Abstract nr. 1

Results of multiantigen DNA-vaccination against neuroblastoma in mouse model

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction
In high-risk neuroblastoma (NB) patients the percentage of relapse reaches 65%, and the survival rate is 50% despite the use of a wide range of therapeutic approaches. DNA vaccination can be used to prevent relapse in patients with neuroblastoma. A vaccines efficacy mainly depends on the antigen (AG) chosen as the target, as well as the delivery method.

Aims
To compare the immunogenicity of DNA vaccines based on three NB-associated AG (tyrosine hydroxylase (TH), Survivin, Phox2b) and to evaluate the effect of vaccine delivery using polyethyleneimine (PEI) and *Salmonella enterica* on tumor progression

Methods/Materials
Sixty-four C57Bl/6 mice were used for immunogenicity study. The mice were injected intramuscularly three times with 10 μg TH/Survivin/Phox2b-PVXCP DNA vaccines (together or separately) in combination with a 20 kDa PEI (DNA-PEI), or an oral gavage of a bacterial suspension of an *Salmonella enterica* attenuated strain (DNA-SE) was performed. A/J mice (n = 58) were used to assess the effect of vaccination on tumor progression when 10^6 NB41A3 cell line were engrafted subcutaneously. The immune response (IR) was evaluated by ELISPOT (production of interferon gamma (IFNγ) and cytotoxicity test (CTT) against NB41A3 murine neuroblastoma cells.

Results
According to the CTT results the delivery of the DNA-PEI conjugate caused a significant increase

in cellular IR for all AGs and their combination, except TH ($p < 0.05$). Oral delivery with *S.enterica* induced a strong IR, which was at least 2 times higher compared to DNA-PEI for all AGs and significantly higher compared to the control. The median percentage of lysed NB41A3 cells in the group of mice vaccinated with *Salmonella* was significantly increased compared to DNA-PEI: 10.2% versus 4.9% for TH; 23.8% versus 11.5% for Survivin; 21.23% versus 7.9% for Phox2b and 34.21% versus 8.16% for TriMixComparison of AGs showed a sequential increase in immunogenicity in the following row: TH → Survivin → Phox2b → TriMix.
IFNγ secretion in response to costimulatory protein PVXCP was recorded in 62.5% of vaccinated mice. The highest AG-specific cytokine production was observed after incubation with TH and Phox2b, where the results were 7.2 and 8.4 times higher (respectively) than in the control group. Vaccination with TriMix-SE showed the best results, because the IFNγ response was obtained for all tested AGs.
The rate of tumor progression was evaluated only for monovaccinations. PEI-DNA showed better results than DNA-SE in all experimental groups. The median tumor volume after vaccination with TH-PEI on the 25th day of monitoring was 918.2 versus 2328 mm^3 in TH-SE. The final tumor volume after vaccination with Survivin-PEI was 2.7 times smaller than in the control (512 versus 1417 mm^3), and after vaccination with Survivin-SE it was 2 times smaller (1302 versus 2664 mm^3). Vaccination with Phox2b-PEI reduced the final tumor volume in 6.3 times (420 mm^3) compared with the control group (2664 mm^3), and Phox2b-SE decreased in 4.7 times (634 mm^3). The low vaccination efficiency with *Salmonella* is probably associated with a toxicity of tumor and bacterial load and potentially can be eliminated by reducing the last one.

Summary/Conclusions

The use of a multi-antigen vaccine is more effective and can help prevent the tumor IR escape by suppressing targeted antigen expression. Delivery of the DNA vaccine with *Salmonella enterica* was accompanied by a stronger IR than the DNA-PEI conjugate, but the latter caused more significant tumor regression.

Type Basic

Presentation Preference Rapid Fire Poster Presentation

Abstract nr. 3

Modification of Home-Made HPLC Method Allowed the Detection of Interference in The Measurement of Catecholamines and Metanephrines

Author - Dr. Tamir, Ronit, Rambam Medical Center, Haifa, Israel (Presenting Author)
Topic Other

Background/Introduction

Quantification of urine catecholamines (norepinephrine, epinephrine and dopamine) and their metabolites (metanephrine and normetanephrine) is commonly requested in the diagnosis and monitoring of neuroblastoma patients. Levels of these analytes are measured by our home-made HPLC method. Interference in the estimation of these analytes could be due to unknown substances in the urine sample that are either similar in chemical structure or have similar chromatographic migration pattern to catecholamines/metanephrines.

Aims

To investigate whether modification of Home-made HPLC method may detect suspected interferences in the measurement of catecholamines and metanephrines.

Methods/Materials

Urine samples were prepared for analysis using a home-made assay involving ion-exchange resins. Catecholamines and metanephrines were extracted with Boric acid and Hydroxide Ammonia, respectively. Catecholamines and fractionated metanephrines were measured by reversed phase, ion pair high pressure liquid chromatography (HPLC) with electrochemical detection. A phosphate buffer including 2% acetonitrile (ACN) was used as a mobile phase.

Results

A urine sample of a 2-year-old child suspected with neuroblastoma was tested for urine catecholamines and metanephrines. The patient's sample chromatogram revealed a minor deviation in the Retention times (RT) of epinephrine and of metanephrine, suggesting interference in the separation of these analytes. Although this interference was slight, it nevertheless produced an artifact measurement of these analytes which were falsely above the normal reference range. Altering the concentration of ACN in the mobile phase from 2% to 2.8%, the peaks of epinephrine and metanephrine were each splitted into two peaks, providing adequate retention times and accurate measurement. Changing the composition of the mobile phase eliminated the interference which falsely increased the estimation of the analytes.

Summary/Conclusions

We can conclude that by changing the composition of the HPLC mobile phase, we can avoid false positive results which may occur from interferences that commonly affect HPLC assays for urine catecholamines and metanephrines.

Type Clinical

Presentation Preference Traditional poster

Abstract nr. 4

Quercetin alleviate cognitive decline in ovariectomised mice by targeting chromatin machinery and relieving transcriptional repression.

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Topic Gene regulatory networks and epigenetics: from basic science to therapy

Background/Introduction

The synergism between estrogen and histone tail acetylation-mediated memory formation is not clearly understood.

Aims

Here, we attempt to study the altered histone acetylation homeostasis linked changes in cognition following ovariectomy and evaluate the protective effect of quercetin.

Methods/Materials

Changes in spatial memory and learning functions assessed by Morris water maze, Novel object recognition test and Elevated plus maze. Differenet neuroplasticity markers, histone modifications and chromatin remodelers (HAT and HDAC) were measured through western blotting and real time PCR.

Results

A significant reduction in estradiol levels with subsequent depletion in spatial memory and learning functions were observed in ovariectomised (OVX) mice. Correspondingly, a significant decline in neuroplasticity markers like Brain derived neurotrophic factor (BDNF), Synaptophysin (SYP), Post synaptic density (PSD)-95 were observed in cortex and hippocampus of OVX animals. Notably, histone acetyl-transferase (HAT)/histone deacetylase (HDAC) balance was significantly disrupted in cortex and hippocampus of OVX mice. Lowered extracellular signal regulated kinases (ERK) and cAMP response element binding protein (CREB) activation observed in OVX brain regions might account for diminished HAT activity. Altered HAT/HDAC homeostasis results in lowered histone (H)3 acetylation in OVX brain that may suppress transcriptional activation of neuroplasticity-related genes. Quercetin supplementation to OVX mice for 4 weeks was able to ameliorate cognitive impairment by restoring HAT/HDAC homeostasis through ERK

activation and reversing alterations in neuroplasticity markers in cortex and hippocampus of OVX mice.

Summary/Conclusions

Taken together, our results suggest that quercetin alleviate ovariectomy-induced cognitive decline by targeting chromatin assembly.

Type Translational

Presentation Preference Traditional poster

Abstract nr. 5

Oncogenic transcription factors are over-represented in mutated active DNA binding sites in neuroblastoma

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Topic Gene regulatory networks and epigenetics: from basic science to therapy

Background/Introduction

The contribution of coding mutations to oncogenesis has been largely clarified whereas little is known about somatic mutations in noncoding DNA and their role in driving tumors remains controversial.

Aims

Here, we used an alternative approach to interpret the functional significance of noncoding somatic mutations in promoting tumorigenesis of neuroblastoma (NB).

Methods/Materials

Noncoding somatic mutations obtained from whole genome sequencing of 151 NBs were integrated with ENCODE v3 data of DNase Hypersensitive Sites and Transcription Factors Binding Sites (TFBSs). We located somatic mutations in regulatory elements specifically active in NB cells (tsa-TFBS), non-specifically active in NB cells (ntsa-TFBS), and non-active (na-TFBS). Within these types of DNA elements, enrichment analysis was used to identify transcription factors (TFs) whose binding sites harboured more or less mutations than the expected by using the ENCODE data as reference. A gene expression signature including the TFs enriched in mutated tsa-TFBS was built using RNAseq data from 498 NB tumors. We then integrated DNA and RNA sequencing data and assessed the effects of mutations in tsa-TFBS on mRNA levels.

Results

The number of somatic mutations in tsa-TFBSs was significantly lower as compared to ntsa-TFBS and na-TFBS ($P<0.05$) and altered, on average, more TFBSs compared to the others ($P<0.0001$). Surprisingly, even if the number of somatic mutations was lower in tsa-TFBS, their median of pathogenic scores, estimated by CADD and FunSeq2, was higher than those in ntsa-TFBS and na-TFBS ($P<0.0001$).

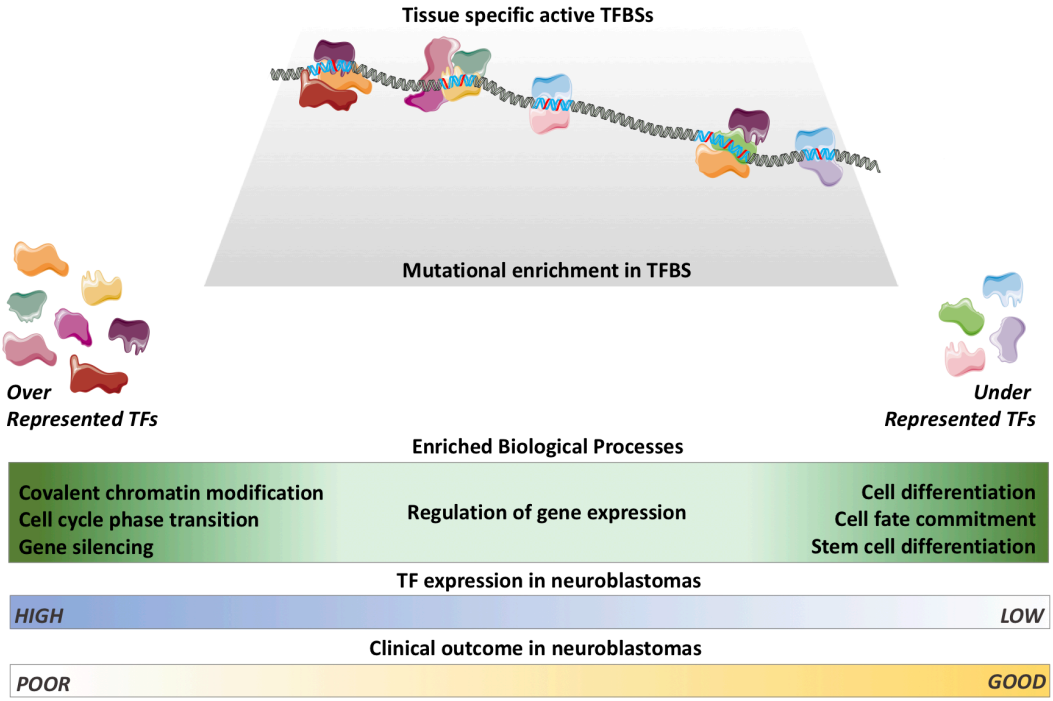
Within the mutated tsa-TFBSs, we found 18 over-represented TFs involved mainly in cell cycle phase transitions, and 15 under-represented TFs primarily regulating cell differentiation.

Among tumors positive for the over-represented TFs-based gene signature, a significantly higher frequency of *MYCN* amplified ($P=2.4x10^{-29}$), INSS stage 4 ($P=1.27x10^{-29}$) and high-risk tumors ($P=1.5x10^{-51}$) was observed. This signature was also able to predicted overall survival and event-free survival ($P=2.0x10^{-16}$).

Moreover, we searched for recurrent mutations in tsa-TFBSs of over-represented TFs. We found a cluster of mutations altering EZH2 binding site and that significantly reduced *MCF2L* ($P=3.97x10^{-18}$) and *ADPRHL1* ($P=3.35x10^{-03}$) expression. Interestingly, the low expression of these genes correlated with unfavourable NB prognostic markers ($P<0.001$) and poor survival ($P<1.00x10^{-07}$). A second cluster of mutations, nearby *CTTNBP2*, altered the binding sites of 6 over-represented TFs (RBBP5, EZH2, SIN3A, POLR2A, TAF1, E2F1). Low mRNA levels of *CTTNBP2* were found in the mutated samples ($P=8.86x10^{-07}$) and correlated with poor prognosis ($P<0.05$). Similarly, a third cluster of mutations, within POLR2A binding site, reduced the expression of *LSP1*, involved in ERK/MAPK pathway ($P=2.48x10^{-03}$), whose low expression correlated with unfavourable NB prognostic markers ($P<0.05$) and poor survival ($P<0.01$).

Summary/Conclusions

We propose a novel approach to study the involvement of regulatory mutations in NB that could be extended to other cancers and provide further evidence that alterations of gene expression may have relevant effects in NB development.



Type Translational

Presentation Preference Oral presentation

Abstract nr. 6

The role of the neurotrophin receptors, TrkA/NTRK1 and TrkB/NTRK2, in modulation of radiation responses in neuroblastoma.

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Radiotherapy is an integral part of treatment in many cancer types including neuroblastoma. Neuroblastoma is the most common extracranial tumor of childhood and shows high diversity in disease outcome. While in some patients tumors differentiate spontaneously, in others the disease spreads aggressively and even after successful initial therapy tumor relapse might occur. High expression of TrkA/NTRK1 has been found to be associated with a good prognosis, while high TrkB/NTRK2 expression on the other hand is associated with an unfavorable outcome. Both, TrkA/NTRK1 and TrkB/NTRK2 are members of the neurotrophin receptor family and are involved in multiple processes during neuronal development including differentiation and apoptosis. In vitro models of neuroblastoma with different TrkA/NTRK1 and TrkB/NTRK2 levels suggested a role for neurotrophin receptors in modulation of chemo- and radiation resistance.

Aims

The aim of the project is to investigate if TrkA/NTRK1 and TrkB/NTRK2 or their downstream targets are involved in radioresistance in neuroblastoma.

Methods/Materials

We previously established inducible TrkA/NTRK1 or TrkB/NTRK2 overexpression in the human neuroblastoma cell line SH-SY5Y. To investigate the effects of TrkA/NTRK1 and TrkB/NTRK2 expression and activation in the context of response to irradiation (IR), we monitored changes in cell-cycle distribution by PI-staining and H3pS10-assay. The effects of TrkA/NTRK1 and TrkB/NTRK2 overexpression and activation in combination with different irradiation intensities were analyzed by MTT and colony formation assay.

Results

We generated human neuroblastoma SH-SY5Y cells with inducible or stable overexpression of TrkA/NTRK1 or TrkB/NTRK2 by using tetracycline-controlled transcriptional activation. Expression of NTRKs was unaffected by irradiation. Phenotypic and molecular characterization of the radiation response indicated a defective G2/M checkpoint in NTRK1-overexpressing cells,

indicated by a higher mitotic index upon ionizing radiation (IR). This effect could be rescued by addition of the NTRK-inhibitor, Loxo-101, suggesting a direct impact of NTRK1 on the response to IR. However, DNA damage-sensing is still intact in SY5Y cells as ATM- or ATR-inhibition is sufficient to abrogate IR induced checkpoint.
Previously, TrkB/NTRK2 expression had been linked with chemo-resistance in NB cells by NTRK2-dependent activation of the PI3K-pathway and by up-regulating MDR (multidrug-resistance) genes (Jaboin et al., Cancer Research, 2002). Our results also point to a TrkB/NTRK2-mediated enhancement in clonogenic survival upon IR. This effect could be confirmed by assessing cell viability in a short term survival assay when TrkB/NTRK2 was either stably of inducibly expressed

Summary/Conclusions

Our experimental data indicate that both, TrkA/NTRK1 and TrkB/NTRK2, are modulators of the radiation response. While increased TrkA/NTRK1 activity in SH-SY5Y cells led to DNA damage independent cell-cycle progression, increased TrkB/NTRK2 signaling was characterized by an increased long term survival upon irradiation. Both findings are in line with clinical data, which correlated high TrkA/NTRK1 expression with a favorable and high TrkB/NTRK2 expression with an unfavorable prognosis.
However, further investigation of the molecular downstream mechanisms of these receptors is important to understand underlying principles and to identify potential therapeutic targets.

Type Basic

Presentation Preference Traditional poster

Abstract nr. 7

Clinical analysis of 458 children with high-risk neuroblastoma in a single center in China

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Topic Other

Background/Introduction

Despite the multi-disciplinary treatment, the prognosis of high-risk neuroblastoma (HR-NB) is still poor. The treatment of HR-NB is still one of the biggest challenges in pediatric oncology.

Aims

To analyze clinical characteristics and prognosis of HR-NB children in the single center in China.

Methods/Materials

The clinical data of children with HR-NB who were diagnosed and treated at the hematology-oncology center of Beijing Children's Hospital (BCH) from February 1st, 2007 to June 30th, 2018 were retrospectively analyzed. The clinical features were summarized and the prognosis was evaluated. The SPSS 19.0 statistical package was used for data analysis.

Results

A total of 458 children with HR-NB were enrolled in this study, including 265 males (57.9%) and 193 females (42.1%), with a median age of 40 months old (4.5-148 months old). Primary tumors were located on retroperitoneal/adrenal glands in 374 cases (81.7%) and on mediastinum in 64 cases (14%). The bone marrow metastases were seen in 338 cases (73.8%), bone metastases were seen in 316 cases (69%) and the distant lymph node metastasis were seen in 272 cases (59.4%). The median follow-up time was 22 months (0.2-138 months), and the median time of event was 14 months (0.2-72 months). 233 patients survived and 196 died. 172 patients (87.8%) died from tumor progression/recurrence, 9 patients (4.6%) died from treatment-related complications, and 15 patients (7.7%) died in the early. The 3-year event-free survival (EFS) of 458 patients was 42.3%±2.6% and the 5-year EFS was 33.8%±2.8%. The 3-year overall survival (OS) was 53.9%±2.7% and the 5-year OS was 43.1%±3.2%.166 cases (36.2%) received autologous peripheral blood stem cell transplantation (APBSCT). There was no significant difference in EFS and OS between the patients who received transplantation and those who did not (3-year OS 56.8%±4.2% vs. 52.5%±3.4%, 5-year OS 39.4%±5.2% vs. 46.8%±3.9%, P=0.123; 3-year EFS 44% ±4.2% vs 41.5%±3.3%, 5-year EFS 28.9%±4.3% vs 37.9%±3.5%, P=0.237). The median time of tumor recurrence/progression was longer in transplantation group than in non-transplantation group (17.5 months vs 11 months, P=0.000). The children with bone metastasis or bone marrow metastasis had higher OS in transplantation group than in non-transplantation group (P=0.004, P=0.002). There were 86 cases (20.6%) with MYCN gene amplification. The 3-year OS of children with MYCN gene amplification was 28%±5.6%, and the 5-year OS was 28%±5.6%, which was significantly lower than that of children without MYCN amplification (3-year OS 62.6%±3.1%, 5-year OS 47.1%±4.2%, P=0.000). Comparison of transplantation group and non-transplantation group in children with MYCN gene amplification was 3-year EFS 26.8%±8% vs 20.9%±6.5% (P=0.021), and 3-year OS 31.4%±8.6% vs 26.1%±7.4% (P=0.07). Univariate analysis showed poor prognosis in children with MYCN gene amplification, bone or bone marrow metastasis, and LDH>1500 U/L (P<0.05).

Summary/Conclusions

The 3-year OS of HR-NB in the single center was 53.9%±2.7%, and 5-year OS was 43.1%±3.2%. APBSCT can improve the prognosis of patients with bone or bone marrow metastasis and can retard recurrence or progression in patients with MYCN amplification. Seeking more effective treatment method during maintenance period is the key to improve the prognosis of children with HR-NB.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 8

Repurposing a psychoactive drug for children with cancer: p27Kip1-dependent inhibition of metastatic neuroblastomas by Prozac.

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

The MYC family of transcription factors are major drivers of human cancer and potential therapeutic targets. However, no clinically viable drugs have been yet developed that are able to directly tackle MYC oncoproteins. In our laboratory, we are exploring alternative approaches aiming to disturb signalling downstream of MYC. MYCN is frequently activated in neuroblastoma, a paediatric solid malignancy that, in its metastatic form, has a very poor prognosis. An important pathway regulated by MYC is the CKS1/SKP2/p27^{kip1} axis. In this study, we have repurposed the anti-psychotic drug Prozac to disrupt CKS1/SKP2/p27^{Kip1} signalling and assess its potential as an anti-neuroblastoma agent *in vitro* and *in vivo*

Aims

Validate Prozac as an anticancer drug for MYC+ CKS1b+ neuroblastomas

Methods/Materials

crispr cas9 DNA editing; MTT and colony assays; cell cycle profile and apoptosis assays using flow cytometry; NSG metastatic mouse models.

Results

Using DNA editing technology, we show that stabilisation of $p27^{Kip1}$ operated by Prozac in MYC-activated cells is essential for the anti-neuroblastoma activity of the drug.
Furthermore, dosing mice with a concentration of Prozac equivalent to that used in long-term clinical trials in children with psychiatric disorders caused a significant reduction of metastatic disease in two models of high-risk neuroblastoma. The anti-proliferative effect of Prozac was strictly dependent on MYCN levels, suggesting that its expression could be used as a bio-marker to guide therapeutic interventions.

Summary/Conclusions

The favourable toxicity profile of Prozac suggests that long term treatments might be implemented in children with MYC/$CKS1^{high}$ neuroblastomas.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 9

MYCN regulates metabolism through vesicular transfer of glycolytic kinases

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Cancer cells expressing MYC proteins modify the tumour microenvironment via the activation or inactivation of growth factors, cytokines and immune checkpoint regulators. The hypothesis that MYC expression could induce non-cell autonomous effects is also supported by evidence of heterogeneous (focal) amplification of *MYCN* in neuroblastoma tumours. Focally amplified neuroblastomas have a negative prognosis, indistinguishable from uniformly amplified cases, suggesting that MYCN could promote a cross talk between parts of the cancer with a different amplification status. We therefore hypothesised that MYCN could promote neuroblastoma growth by regulating the secretion or content of exosomes. Exosomes are small extracellular vesicles of endocytic origin that change the behaviour of recipient cells by the delivery of proteins and nucleic acids. Cancer-produced exosomes play a key role in the metastatic dissemination and/or pro-angiogenic activity of solid tumours. While the pro-tumourigenic or pro-metastatic role of exosomes has been highlighted in different types of adult cancers it is not known whether they play a role in in the context of *MYCN* amplified neuroblastoma.

Aims

we have investigated the hypothesis that *MYCN* amplified tumours could spread oncogenic signals to other body and tissue compartments via regulation of exosomes protein cargo.

Methods/Materials

We have used a switchable *MYCN* expression system and mass-spectrometry analysis to identify MYCN-regulated vescicular proteins; gene overexpression/knockout, sea-horse analysis and microscopy to study their function.

Results

MYCN regulates distinct sets of proteins in the exosomes secreted by neuroblastoma cells. Exosomes produced by MYCN expressing cells or isolated from neuroblastoma patients induced the Warburg effect, proliferation, Histone H3 phosphorylation and c-MYC expression in target cells. Knockdown of Rab27 in $MYCN^{high}$ cells abolished the phenotypic effects observed in recipient cells in co-culture experiments, demonstrating the key role of vescicular trafficking. Mechanistically, we linked the cancer promoting activity of extracellular vesicles to the glycolytic kinase pyruvate kinase M2 (PKM2) that was enriched in exosomes secreted by MYCN expressing neuroblastoma cells. Importantly, the glycolytic enzymes PKM2 and Hexokinase II were detected in the exosomes circulating in the blood stream of neuroblastoma patients, but not in those of non-cancer children.

Summary/Conclusions

We conclude that MYC activated cancers might spread oncogenic signals to remote body locations through extracellular vesicles.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 10

ALYREF/USP3/MYCN signaling axis is a novel molecular vulnerability in high-risk neuroblastoma

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Topic Gene regulatory networks and epigenetics: from basic science to therapy

Background/Introduction

Segmental copy-number gains occur most frequently on Chr17q21-ter and strongly predict adverse outcomes in neuroblastoma (NB). Target genes in this region are poorly characterised and actionable molecular targets have yet to be discovered for these patients.

Aims

Our aim was to identify novel molecular targets for diagnosis and therapy of high-risk NB with 17q21-ter gain.

Methods/Materials

We first used bioinformatic techniques to systematically investigate genes on the 17q21-ter amplicon for independent associations with NB prognosis. For mechanistic studies, we used ChIP sequencing, ubiquitin assay, protein half-life studies, quantitative PCR, western blotting, co-immunoprecipitation, cell viability, cell proliferation and colony formation assay. For in vivo studies, microarray on ganglia from homozygote TH-MYCN transgenic mice (Ooi et al, 2018), and Balbc/nude NB xenograft models were used.

Results

We investigated genes on the 17q21-ter amplicon and identified ALYREF as a key regulator of NB. High ALYREF expression correlated with poor overall survival in a large cohort of primary NB patient tumour samples (n = 498, SEQC dataset). Multivariate analysis showed that high ALYREF expression was independently prognostic compared with other clinical features in NB. MYCN is a major driver of tumourigenesis in NB, and high ALYREF expression correlated with MYCN expression. Microarray analysis carried out on the ganglia tissues of homozygote TH-MYCN transgenic NB mice showed that ALYREF was significantly up-regulated at MYCN-driven tumour initiation. Mechanistic experiments revealed that MYCN, is functionally dependent on ALYREF and the two proteins act in a positive forward feedback expression loop to promote NB progression. ALYREF exerts its effect on NB progression by directly binding to the region between MB Box III and IV of MYCN in the nucleus. Stable genetic suppression of ALYREF expression reduced cell viability and colony formation of MYCN-amplified NB cell lines. Most importantly, ALYREF knock-down in MYCN amplified NB cells resulted in a significant inhibition of tumorigenesis *in vivo*. Genome-wide profile of ALYREF-chromatin interactions using ChIP sequencing suggested that ALYREF promotes tumour formation by transcriptionally up-regulating the ubiquitin-specific protease 3 (USP3). USP3 forms a protein complex with MYCN and protects it from ubiquitin-mediated degradation.

Summary/Conclusions

Our findings suggest a crucial role for ALYREF in high risk NB through effects on MYCN ubiquitination and suggest that inhibition of ALYREF-mediated MYCN turnover is a potential therapy for high-risk NB patients.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 11

Quantitative assessment by liquid biopsy of MYCN and chromosome 11q LOH in neuroblastoma

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Topic Other

Background/Introduction

The “liquid biopsy”, detecting genomic alterations of tumors using cell-free DNA (cfDNA) in plasma, has recently attracted attention as a non-invasive alternative of surgical tissue biopsy. However, most of liquid biopsy is qualitative, detecting only presence targeting specific genomic alterations.

Aims

We here challenged quantitative liquid biopsy to detect *MYCN* amplification and 11q LOH, important prognostic factors of neuroblastoma.

Methods/Materials

Plasma samples at the onset were obtained 17 neuroblastoma patients diagnosed from June 2017 to April 2019. The cfDNA was extracted from 1 ml of plasma and the analyzed results were compared to that of genomic DNA from tumor tissues. The copy number of MYCN and NAGK genes were quantitatively analyzed by droplet digital PCR (ddPCR). All tumor specimens were routinely examined for MYCN amplification using fluorescent in situ hybridization (FISH). LOH at 11q was assessed by detecting allelic imbalances of heterozygous SNPs in the q arm of chromosome 11 using ddPCR. Multiplex ligation probe amplification (MLPA) was also performed to investigate aberrations of 11q in tumor DNA. The proportion of circulating tumor DNA in plasma cfDNA was estimated by examining the variant allele frequency (VAF) of a tumor-specific single nucleotide variant (SNV) identified by whole-exome sequencing for tumor DNA.

Results

The median age of the 17 patients was 29 months old (range, 0-80) [INRGSS L1 (n = 1), L2 (n = 4), M (n = 11) and MS (n = 1)]. The presence (n = 6) and absence (n = 10) of MYCN amplification in tumor specimens using FISH analysis were correctly detected by liquid biopsy in all cases. Results of MYCN copy number analysis using cfDNA were highly concordant to that of tumor specimens, and the correlation coefficient was 0.73 with statistical significance (P = 0.007). 11q LOH was also detected in five cases using MLPA. Analysis of cfDNA using ddPCR detected 11qLOH in all 4 cases with stage M except a case with stage L2 which could detected in cfDNA at the time of recurrence.In terms of VAF of tumor specific SNV, very high correlation of 0.964 (P=0.003) was observed in patients with stage M. Interestingly, the VAF of tumor specific SNV in cfDNA was higher than that in tumor DNA in 2 of 5 patients analyzed.

Summary/Conclusions

MYCN copy number and 11q LOH could be accurately analyzed in plasma cfDNA by ddPCR, especially in stage M. Liquid biopsy is a non-invasive and repeatable method which can detect quantitative genomic alterations in NBs.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 12

Metallothionein-3 is an important factor for development of chemoresistance to CDDP and extravasation and intravasation of neuroblastoma cells

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Topic Other

Background/Introduction

Neuroblastoma (Nbl) is a challenging childhood malignancy, with a very high percentage of patients relapsing following acquisition of drug resistance, thereby necessitating the identification of mechanisms of drug resistance as well as new biological targets. Cisplatin (CDDP) is one of the most commonly used drugs in the treatment of Nbl. Human metallothionein-3 (hMT-3) is presumed to participate in the processes of heavy metal detoxification and protection against oxidative damage of free radicals in the central nervous system. It is worth noting that the concentration of MTs increases in the moment of administration of the platinum-based drugs. Such stimulated MTs can also rapidly bind the administered CDDP, which can result in decrease of drug concentration below the effective level. However, the primary functions of hMT-3 and the mechanism underlying its multiple functions in neuroblastoma have not been elucidated so far.

Aims

We aim to gain further understanding of the mechanisms underlying development of CDDP resistance using a chick chorioallantoic membrane xenograft model (CAM assay).

Methods/Materials

The CAM sustains rapid tumor formation within 5-7 days after neuroblastoma cells grafting. This feature provides a unique experimental model for a rapid study of the intravasation and colonization steps of the metastatic cascade. Furthermore, using quantitative PCR to detect species-specific sequences, such as *Alu*, the chick embryo CAM model can be used to monitor and quantify the presence of the xenografted, ectopic tumor cells in distant tissues.

Results

CAM (UKF-NB-4 cells) treated with 100 µM CDDP during 24 h exhibited inhibition of the weight of primary tumors. The number of UKF-NB-4 cells in CAM distal, liver, lung and brain decreased significantly, compared with the control (no treatment). In contrast, CAM induced with CDDP resistant cells (UKF-NB-4^{CDDP}) did not show significant differences between 0 − 100 µM CDDP. The obtained results indicate that, due to the acquired chemoresistance, UKF-NB-4^{CDDP} cells also exhibit high rate of chemoresistance in CAM xenograft model. Also, we used the CAM assay to examine whether CDDP had any effect on metastatic spreading after hMT-3 overexpression in UKF-NB-4 cells. The tumors were very reliably grown after 6 days. Then, the 100 µM CDDP was added topically on the upper CAM during 24 h. The data showed no significant differences in primary tumor weight in mock *vs*. hMT-3 in UKF-NB-4 cell line upon treatment with 100 µM CDDP, (91.6 mg *vs*. 86.7 mg, respectively). However, hMT-3 overexpression in UKF-NB-4 cells increased the capacity of intravasation, extravasation and metastatic spreading of Nbl cells.

Summary/Conclusions

We identified a significant increase in chemoresistance to CDDP due to hMT-3 overexpression in CDDP sensitive UKF-NB-4 cell line in *in vitro* and *in vivo* model. In addition, hMT-3 markedly stimulated the ability of UKF-NB-4 cells to extravasate and intravasate in CAM. The results provide new insights into better understanding of the role of hMT-3 in progression of Nbl.

Type Basic

Presentation Preference Traditional poster

Abstract nr. 13

MYCN-KDM6B axis regulates an Rb-E2F transcription program that is positively correlated with GSK-J4 sensitivity

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Topic Gene regulatory networks and epigenetics: from basic science to therapy

Background/Introduction

Although outcomes of children with low- or intermediate-risk neuroblastoma are excellent, overall survival rates for those with high-risk neuroblastoma, including N-MYC amplified tumors, remain less than 40%, despite the use of intensive multimodal therapies. Thus, there is a pressing need to identify novel therapeutic targets for MYCN-amplified neuroblastoma. The H3K27me3/me2 demethylase KDM6B is a promising therapeutic target although the role of KDM6B in neuroblastoma is not clear.

Aims

The objective of this study was to understand how KDM6B connects to key oncogenic pathways and identiify the molecular markers predicting sensitivity to the KDM6B inhibitor GSK-J4.

Methods/Materials

KDM6B expression in tumor cohorts and normal neural crest tissues was analyzed using the R2 microarray program. RNA-seq or microarray was performed for gene pathway analysis. The impact of epigenome of MYCN and KDM6B was analyzed using ChIP-seq and ATAC-seq. Chemoinformatics was used to analyze the association of KDM6B inhibition with the CDK4/6 inhibitor palbociclib. Cell lines with overexpression of MYCN or CDK4/6 were generated by transduction of retroviral or lentiviral based vectors. Crystal violet staining was performed to assess the effect on cell viability.

Results

MYCN broadly binds at KDM6B genomic locus, in line with a broad occupancy of active transcription marks, H3K4me1 and H3K27Ac. Genetic or pharmacologic inhibition of KDM6B leads to downregulation of the E2F transcriptome. Chemical genetics data showed that high E2F activity of cancer cells but not KDM6B itself is positively correlated with sensitivity to the KDM6 inhibitor GSK-J4. KDM6B inhibition transcriptomically phenocopies the specific CDK4/6 inhibitor palbociclib and vice versa. Mechanistically, inhibition of KDM6B activity reduced the chromatin accessibility of E2F target genes, accompanying with elevated repressive transcription mark H3K27me3. Overexpression of CDK6 confered neuroblastoma cell resistance to palbociclib but these cells retained sensitivity to the KDM6B inhibitor GSK-J4.

Summary/Conclusions

MYCN-KDM6B interacts with the CDK4/6-Rb-E2F pathway in neuroblastoma cells, revealing a new alternative strategy to target KDM6B for cancer cells resistant to CDK4/6 inhibitors.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 15

Universal targeting of neuroblastoma via adapter chimeric antigen receptor (AdCAR)-modified NK-92 cells

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

Neuroblastoma emerges from immature neural crest cells of the sympathetic nervous system. Representing 7% of all pediatric cancers neuroblastoma is the most common extracranial tumor affecting children and accounts for about 15% of overall childhood cancer mortality. Neuroblastoma is a complex and heterogeneous disease with nearly 50% of patients having a high-risk phenotype characterized by widespread dissemination and poor long-term survival despite intensive multimodal therapy. As such, there is an urgent need for new and effective neuroblastoma treatment options.

Aims

Preclinical evaluation of universal adapter CAR (AdCAR)-engineered NK-92 cells as a treatment option for high risk neuroblastoma.

Methods/Materials

In the context of this project, the FDA approved human NK cell line NK-92 was genetically modified by lentiviral transduction to express a universal adapter chimeric antigen receptor construct. AdCARs are based on the unique properties of a novel single-chain variable fragment (scFv) targeting a “neo”-epitope-like structure derived from the endogenous vitamin biotin, linked to a monoclonal antibody which serves as adapter molecule. Using single cell sorting, AdCAR NK-92 clones with the highest CAR expression were selected and tested in combination with biotinylated therapeutic or clinical phase I/II antibodies targeting a variety of antigens expressed on different neuroblastoma cell lines. Calcein AM-based cytotoxicity assays, real-time impedance measurements and flow cytometry analysis were used to evaluate the specificity and activity of

AdCAR NK-92 cells *in vitro*.

Results

AdCAR-engineered NK-92 cells in contrast to parental unmodified NK-92 cells induced specific and potent neuroblastoma cell lysis in an antibody and antigen dose-dependent manner. The selective cytotoxicity of AdCAR NK-92 cells stringently relied on the presence of biotin-conjugated antibodies, verifying the specificity of the AdCAR system.
Irradiation of AdCAR NK-92 cells, as required in all active clinical trials, had no immediate effect on target cell lysis or chemokine receptor expression, but led to a gradual decline in cytotoxicity and viability over several days post radiation exposure.

Summary/Conclusions

AdCAR NK-92 cell effector functions can be redirected against one or multiple antigens allowing universal and flexible targeting of neuroblastoma cells, thus counteracting antigen loss and immune escape. Moreover, AdCAR NK-92 cells can be manufactured as an "off-the-shelf on demand" standardized cellular product improving the practicality of CAR NK cell therapy combined with the possibility of tailoring an antibody platform for patient-individualized neuroblastoma treatment.

Type Translational

Presentation Preference Traditional poster

Abstract nr. 16

Therapeutic targeting of ubiquitin-specific protease 5 in MYCN-driven neuroblastoma

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Topic Gene regulatory networks and epigenetics: from basic science to therapy

Background/Introduction

Histone deacetylase (HDAC) inhibitors are effective in preclinical models of the childhood cancer, neuroblastoma. HDAC inhibitor, suberanoyl hydroxamic acid (SAHA), has shown clinical success in the treatment of cutaneous T-cell lymphoma but lacks efficacy as a single agent in many solid tumours, including neuroblastoma.

Aims

To identify new compounds that potentiate SAHA activity and characterize their molecular mechanism.
To determine the oncogenic role of USP5 in neuroblastoma.

Methods/Materials

We first performed a cell-based, high-throughput drug screen using a MYC-expressing breast cancer cell line, treated with 1μM of SAHA to identify novel compounds which act synergistically with SAHA. For mechanistic studies, we performed cell viability, colony formation assay, co-immunoprecipitation, ubiquitin assay, and chromatin immunoprecipitation (ChIP) assays. For the *in vivo* studies, we used neuroblastoma-bearing transgenic TH-MYCN homozygous mice and MYCN;GFP zebrafish.

Results

Previously we have shown that a small enhancer molecule, SE486-11, was able to synergistically enhance the cytopathic effects of SAHA in neuroblastoma cells. Treatment with the combination

led to a decrease in cell viability, an increase in MYCN ubiquitination and completely inhibited tumour growth in TH-MYCN homozygous mice.A SILAC (Stable Isotope Labelling by Amino Acids in Cell Culture) analysis of protein from neuroblastoma cells treated with combination therapy, revealed that Ubiquitin-Specific Protease 5 (USP5) expression was significantly reduced by the combination.
More recently, MYCN;EGFP transgenic fish, with established neuroblastomas were treated with the combination which markedly inhibited neuroblastoma tumorigenesis in the zebrafish. A major role of USP5 is regulation of the relative cellular levels of unanchored mono- and polyubiquitins. We found that the combination treatment caused an increase in Lys48-linked polyubiquitination and that overexpression of USP5 reduced polyubiquitin levels following combination treatment. An online data base revealed that high USP5 expression in primary human neuroblastoma samples predicted poor prognosis in MYCN amplified patients. This implicates USP5 as a potential oncogenic driver in neuroblastoma. To determine the oncogenic role of USP5 in MYCN-driven neuroblastoma, USP5 was knock-downed by specific siRNAs in MYCN-amplified neuroblastoma cell lines, causing a significant decrease in MYCN protein expression and its half-life. Chromatin immunoprecipitation assays demonstrated an enrichment of MYCN protein near the USP5 transcription start site. Doxycycline-induced repression of MYCN expression in SHEP-tet21N correlated with decreased USP5 protein and mRNA levels. Co-immunoprecipitation assays showed that USP5 and MYCN formed a protein complex. Genetic suppression of USP5 in MYCN-amplified neuroblastoma cell lines led to a significant reduction in cell viability, cell proliferation and colony formation.

Summary/Conclusions

Taken together, our findings suggest that USP5 and MYCN participate in a forward feedback expression regulatory loop and that inhibition of USP5 function in neuroblastoma cells using small molecule inhibitors could be a novel therapeutic approach for MYCN-amplified tumours.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 17

An oncogenic role for the splicing associated protein, SNRPD3 in MYCN-driven neuroblastoma

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Topic Gene regulatory networks and epigenetics: from basic science to therapy

Background/Introduction

Neuroblastoma is a rare solid tumour in early childhood with less than 50% survival rate for high-risk patients. Amplification of the MYCN oncogene remains the single most important genetic predictor of poor prognosis. Many of the pro-tumorigenic functions of MYCN are attributed to its ability to regulate global gene expression programs. Alternative splicing is another important component of gene expression regulation. Alternative splicing allows for diverse transcriptional variants that can lead to diverse cellular phenotypes and characterising a wide variety of biological functions. Dysregulation of alternative splicing has been found to effect pathways that make up the hallmarks of cancer. We hypothesise that dysregulation of alternative splicing may be associated with an aggressive MYCN-driven neuroblastoma phenotype.

Aims

In this study, we aim to identify the molecular mechanism by which MYCN regulates alternative splicing programs in neuroblastoma.

Methods/Materials

To address the above hypothesis and aims siRNA knockdown of SNRPD3 in BE2C, Kelly and SHEP.tet21N cells were performed to assess cell viability, proliferation and colony formation. Protein and mRNA levels were measured using western blotting and RT-qPCR. RNA-sequencing was performed to assess differentially spliced genes using the leafcutter program.

Results

Microarray analysis on ganglia and tumour tissue collected during tumour initiation in transgenic TH-MYCN$^{+/+}$ and wildtype mice revealed a significant increase in the mRNA expression of SNRPD3, a core component of the spliceosome. Analysis of a 649 neuroblastoma patient cohort

(Kocak dataset) revealed high SNRPD3 expression is correlated with poor prognosis. Using co-immunoprecipitation experiments, we identified SNRPD3 as a novel MYCN binding protein. siRNA knockdown of SNRPD3 reduced cell viability, proliferation and colony number in MYCN-amplified neuroblastoma cell lines. Further investigation using the MYCN-inducible, SHEP.tet21N cells, showed that SNRPD3 knockdown exhibited a more pronounced lethality in the presence of MYCN.

RNA-sequencing was performed in SHEP.tet21N cells with suppression of SNRPD3 by siRNAs to identify aberrantly spliced genes and signalling pathways involved in the regulation of alternative splicing in neuroblastoma. Our analysis revealed that there is an overall increase in the number of genes being spliced when MYCN is expressed. Interestingly, SNRPD3 knockdown in the presence of MYCN resulted in a further 25-fold increase in the number of genes being differentially spliced. This pronounced effect on splicing was MYCN-specific, with SNRPD3 knockdown alone showing little effect on differential splicing. Gene ontology and KEGG pathway analysis of MYCN+ve/SNRPD3-ve dependent splicing identified enrichment in cell cycle pathways.

Summary/Conclusions

Based on these results, we conclude that MYCN-driven transcriptional up-regulation and physical interaction with SNRPD3 contributes to changes in alternative splicing that perpetuate MYCN-driven oncogenesis. Disruption of SNRPD3 in the presence of MYCN was sufficient to induce widespread mis-splicing. That impacts cell cycle machinery and results in cell cycle inhibition/cell death. The selective relationship exhibited between MYCN and SNRPD3 indicates that SNRPD3 could serve as a novel therapeutic target in MYCN-amplified neuroblastoma.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 18

Enhanced targeting of the mitochondrial adenine nucleotide translocase SLC25A5 by modulating p53 dynamics in neuroblastoma

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

Mitochondria-targeted agents are steadily being integrated into cancer therapies as they induce strong apoptotic phenotypes and potentiate treatment responses to cytotoxic chemotherapy in several cancer types. Mitochondria-associated genes (MAGs) encode for mitochondrial proteins and/or mediate mitochondrial signalling pathways. Several MAGs have been strongly associated with increased malignancy and drug resistance in neuroblastoma (NB). Therefore MAGs represent promising therapeutic targets in NB.

Aims

We aimed to identify potential therapeutic targets among MAGs in NB. We then aimed to design rational combination therapies around specific MAG targets.

Methods/Materials

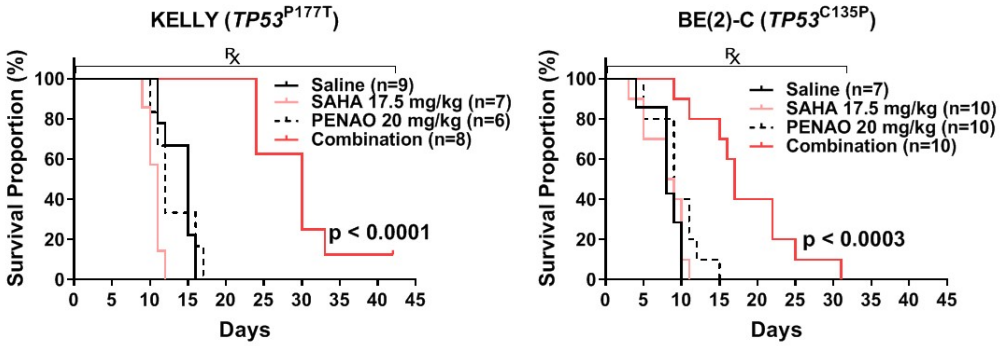
We took an integrative functional genomics approach to prioritise MAGs, where we leveraged published; RNA-seq, ChIP-seq, ATAC-seq and CRISPR interference data derived from NB patients or cell lines. To design rational combination therapies we further utilised drug response data from the Genomics of Drug Sensitivity in Cancer (GDSC) database. To assess the mechanisms of our prioritised MAG and targeted combination therapy we utilised several *in vitro* methodologies including; cell viability, cell cycle, apoptosis, microarray, protein half-life and western blot assays. To assess the *in vivo* efficacy of the targeted combination therapy we utilised NB cell line xenograft and transgenic NB TH-MYCN mouse models.

Results

Our integrative functional genomics approach identified a subset of MAGs whose expression was associated with higher chromatin accessibility at gene promoters, higher gene dependency for NB cell survival and poor NB patient survival. Multivariate analysis of this MAG subset identified mitochondrial adenine nucleotide translocase 2 (SLC25A5) to be the strongest independent predictor of poor NB patient prognosis. We found that siRNA mediated knockdown of SLC25A5 reduced NB cell viability and direct inhibition with a selective SLC25A5 inhibitor, PENAO, markedly reduced cell viability in a panel of NB cell lines. We found that PENAO resistance was primarily driven by p53 mutations, so we then used drug response data to model potential combination therapies to overcome PENAO resistance in p53 mutant NB cell lines. This led to the identification of therapeutic synergy between PENAO and SAHA, a histone deacetylase inhibitor, in both mutant and wild type p53 NB cell lines. We found that PENAO combined with SAHA drastically reduced mutant p53 protein levels in NB cell lines and linked this to mutant p53 protein destabilisation. Combining PENAO and SAHA *in vivo* significantly prolonged the survival of mice inoculated with two mutant p53 NB cell lines and in TH-MYCN transgenic mice compared with single agent therapy or the control.

Summary/Conclusions

PENAO is currently in phase II clinical trials, whilst SAHA is FDA approved for multiple solid tumours. Our promising data on this combination therapy warrants further evaluation for an early phase clinical trial for NB. Furthermore, given that p53 mutations arise more frequently in relapsed NB and confer multi-drug resistance, our proposed combination therapy may offer an effective therapeutic avenue for these patients.



In vivo efficacy of the targeted drug combination in two xenograft models of mutant p53 neuroblastoma

Type Translational

Presentation Preference Oral presentation

Abstract nr. 19

New therapeutic strategies for neuroblastoma: Targeting Gal-3BP with a highly potent Antibody-Drug Conjugate

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

Neuroblastoma is a solid tumor affecting the peripheral nervous system accounting for 15% of all pediatric oncology deaths. Despite multimodal therapeutic treatments, the majority of relapsed,

high risk neuroblastoma patients still succumb to the disease. Antibody Drug Conjugate (ADC)-based therapy has been successfully implemented at preclinical and clinical level, with different types of ADCs showing activity in a variety of malignancies. Galectin-3 binding protein (Gal-3BP) (aka Mac-2 BP or 90K) is a highly glycosylated protein abundantly expressed by the majority of human cancers, including neuroblastoma, while being virtually undetectable in normal adult tissues. In clinical reports, Gal-3BP was associated with low survival rate, development of cancer progression, and enhancement of metastasis. Moreover, Gal-3BP has been found to be overexpressed in the neuroblastoma microenvironment, leading to IL-6 production by monocytes and bone marrow mesenchymal stem cells (BMMSC).

Aims

In this study, we have investigated whether Gal-3BP is a suitable target for non-internalizing ADC-based therapy in neuroblastoma.

Methods/Materials

We have developed a non-internalizing ADC that specifically targets Gal-3BP, by directly coupling the maytansinoid thiol-derivative DM3-SH to unpaired cysteine residues of the engineered anti-Gal-3BP antibody 1959-sss, resulting in site-specific, linker-less thiol drug conjugation, with a drug-antibody ratio of 2. Therapeutic studies were conducted using subcutaneous, metastatic and orthotopic mouse models with conventional human neuroblastoma cell lines. To increase the clinical relevance of the results, we have also used patient-derived tumor (PDX) models expressing high levels of GAL-3BP.

Results

Gal-3 BP-ADC induced complete, durable regression of xenotransplanted tumours formed by conventional cell lines or patient-derived neuroblastomas. Preliminary toxicology studies suggest that therapeutic concentrations of the ADC are well tolerated in rabbits, which is the only species where the 1959 antibody binds endogenous Gal-3BP with an affinity similar to the human antigen. The ADC was highly stable in plasma in vitro and in vivo, and displayed a favorable biodistribution.

Summary/Conclusions

Our results validate Gal-3BP as a suitable cancer target and provides a rationale for further clinical development of this newly engineered ADC for the treatment of Gal-3BP-expressing neuroblastoma.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 20

A single amino acid mutation in the RNF121 protein blocked tumour neuroblastoma development in TH-MYCN mice

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Topic Neuroblastoma as developmental disease

Background/Introduction

Embryonal cells produced in excess to requirements for organogenesis are normally deleted in a programmed manner following trophic factor withdrawal, whereas failure of this process is the first step in embryonal cancer initiation.

Aims

MYCN amplification is a poor prognostic indicator in neuroblastoma, occurring in approximately 20% of patients. Identification of co-factors of MYCN are valuable as novel therapeutic targets in this disease.

Methods/Materials

We used ENU mutagenesis on neuroblastoma-prone *TH-MYCN* transgenic mice in a forward

genetic screen to discover genes whose loss of function inhibited neuroblastoma tumourigenesis.

Results

We generated founder lines with heritable, decreased tumour incidence, and used large-scale mapping, focussed exome and next-generation sequencing of the mutant mouse genomes to identify Ring Finger Protein 121 (RNF121) as a candidate mutant gene linked to loss of tumour formation. The point mutation in RNF121 is located in a transmembrane domain of the protein, and the molecular modelling indicated that it can result in loss of function due to steric clashes with surrounding transmembrane domains. Immunofluorescence demonstrated RNF121 is localized in the cis-Golgi membrane of neuroblastoma cells, which was further supported by cell fractionation experiments. The point mutation in RNF121 disrupts localisation of RNF121 to cis-Golgi. Analysis of patient datasets and prognosis indicated that high RNF121 expression is associated with worse prognosis, particularly in MYCN-amplified patients, and higher RNF121 expression is observed in human MYCN-amplified neuroblastoma cell lines. Suppression of RNF121 by siRNAs in MYCN-amplified cell lines significantly impaired cell viability and colony formation, and induced G2/M arrest, whilst overexpression of RNF121 enhanced cell viability and proliferation. Consistent with an association of RNF121 with MYCN amplification, suppression of RNF121 by siRNAs decreases MYCN protein half-life and stability in MYCN-amplified cell lines.
Furthermore, we have shown that the amplification of RNF121 (18%) and c-MYC (41%) in esophageal tumours samples. Amplification of RNF121 was found in TCGA datasets of Head and Neck Cancer (13%), Ovarian (8%) and MYCN-driven neuroendocrine prostate cancer (NEPC) (20%). To examine the functional role of RNF121 *in vivo*, we have generated RNF121 a conditional knockout mouse model. The RNF121 homozygous knockout mice are embryonic lethal at E12.5. The model is currently being crossed with the *TH-MYCN* mice.

Summary/Conclusions

We found that RNF121 is: a cis-Golgi trans-membrane protein, a prognostic factor in NB, amplified in head and neck cancer and MYCN-driven neuroendocrine prostate cancer, and increase MYCN protein stability. The importance of this completely novel target lies in the fact that the mutant mice were not only cancer-free despite the presence of the MYCN transgene, but healthy, suggesting RNF 121 inhibitors will have a wide therapeutic index.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 21

MODULATION OF THE UNFOLDED PROTEIN RESPONSE IN NEUROBLASTOMA INDUCES APOPTOSIS ESPECIALLY IN MYCN-AMPLIFIED, CHEMORESISTANT CELLS

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

We have previously reported that activation of the calcium-sensing receptor (CaSR) inhibits neuroblastoma (NB) tumor growth by promoting Endoplasmic Reticulum (ER) stress-mediated apoptosis and differentiation. The ER is a major regulator of multiple intracellular processes, such as the proper folding of the newly synthesized proteins. The tumor cell is exposed to several extra and intracellular stresses that can impair this folding efficiency, thus inducing the so called Unfolded Protein Response (UPR). The UPR can promote survival by hindering translation and inducing autophagy, but if the stress becomes unbearable cells undergo apoptosis. UPR is also involved in the acquisition of chemoresistance, the major responsible for the progression or relapse in many cancer patients, including NB, after the first line of treatment.

Aims

To characterize the ER stress/UPR modulation in NB as a putative therapeutic strategy, especially for aggressive, chemoresistant tumors.

Methods/Materials

Six NB cell lines with different *MYCN* and *TP53* status were used to assess toxicity promoted by *in vitro* exposure to several ER stress inducers (bortezomib, thapsigargin and tunicamycin) and one autophagy inhibitor (chloroquine). Synergism between these ER stress inducers and the autophagy inhibitor was also evaluated according to the Chou-Talalay method. Synergism was also evaluated *in vivo* in two xenograft models generated in immunocompromised mice with *MYCN*-Amplified IMR5 and *MYCN*-Non Amplified SH-SY5Y cell lines. Cell lines resistant to chemotherapeutic agents commonly used for NB treatment (cisplatin, doxorubicin and vincristine) were generated. Rescue of this chemoresistance by the modulators of the ER stress response bortezomib and chloroquine was evaluated in the resistant cell lines and their wild type counterparts.

Results

All the cell lines tested showed similar sensitivity to bortezomib, thapsigargin and tunicamycin. Apoptosis occurred due to induction of ER stress and the subsequent UPR as determined by RT-qPCR and western blot. The combination of these stimuli with the inhibition by chloroquine of the pro-survival autophagic branch of UPR resulted in an increased cell death but, interestingly, a synergistic collaboration was only significant in some of the cell lines (IMR5, SK-N-JD, SK-N-LP and LA-N-1). A similar scenario was found when evaluating the rescue of chemoresistance by the ER stress/UPR modulators. *In vivo* studies confirmed these results. Although the *MYCN*-Amplified cell lines were clearly the most responsive to the interactions tested, we also explored other features as putative contributors for this differential response. p53 and mTOR pathways and stemness status exhibited relevance in this phenotype.

Summary/Conclusions

These data would provide proof-of-principle indicating that modulation of the ER stress and the UPR might be a new therapeutic approach for malignant neuroblastomas, particularly the aggressive, *MYCN*-amplified ones with acquired or constitutive chemoresistance.

Type Translational

Presentation Preference Traditional poster

Abstract nr. 22

Role of radiotherapy dose-escalation for high-risk neuroblastoma with post-surgical primary site gross residual disease: a report from the COG ANBL0532 study

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

Despite an intensive multimodal approach, including chemotherapy, multi-agent myeloablative regimens with stem cell transplantation, surgery, radiotherapy, and immunotherapy, outcomes for high-risk neuroblastoma remain inadequate with relapse occurring in approximately 50% of patients. Locoregional relapse continues to be a significant contributor to treatment failure for high-risk neuroblastoma patients even after radiotherapy (RT).

Aims

We aimed to examine the effects of increasing local dose of radiation to the residual primary tumor for patients with less than a gross total resection on cumulative incidence of local progression (CILP), event-free survival (EFS), and overall survival (OS) in the Children’s Oncology Group (COG) ANBL0532 phase 3 study, a question that constituted a Primary Objective of the trial.

Methods/Materials

Newly diagnosed high-risk neuroblastoma patients were enrolled on COG ANBL0532 from November 2007 to February 2012. Patients were randomized or assigned to receive single vs. tandem autologous stem cell transplantation (SCT). Local control of the primary tumor consisted of surgical resection during induction chemotherapy and RT following last SCT. Patients were prescribed RT (21.6 Gy in 1.8 Gy fractions) to the preoperative primary tumor volume. For patients with incomplete surgical resections of the primary tumor (defined as >1 cm^3 residual soft tissue density as assessed by central review of end-induction scans), an additional boost of 14.4 Gy was delivered to the gross residual tumor for a total dose of 36 Gy. OS, EFS, and CILP were compared to COG A3973 historical cohort, in which all patients received 21.6 Gy without a boost. Kaplan-Meier curves of OS and EFS were compared using a log-rank test. CILP curves, with adjustment for competing risks (previous progression outside the primary, or death), were compared using Gray's test.

Results

323 patients received RT on ANBL0532. Of these, 133 patients received boost RT. For all patients receiving RT, five-year CILP, EFS, and OS were 11.2±1.8%, 56.2±3.4%, and 68.4±3.2% for the COG ANBL0532 cohort (n=323) compared to 7.1±1.4% (p=0.0590), 47.0±3.5% (p=0.0090), and 57.4±3.5% (p=0.0088) for COG A3973 cohort (n=330), respectively. Five-year CILP, EFS, and OS for COG A3973 patients who had incomplete resection and received RT (n=47) were 10.6±4.6%, 48.9±10.1%, and 56.9±10.0%, respectively. In comparison, five-year CILP, EFS, and OS for COG ANBL0532 patients receiving boost RT (n=133) were 15.1±3.1% (p=0.4632), 52.1±5.4% (p=0.4053), and 67.0±5.1% (p=0.2863), respectively; and five-year CILP, EFS, and OS for COG ANBL0532 patients randomized or assigned to single SCT and received boost RT (n=74) were 16.3±4.3% (p=0.4126), 50.9±7.0% (p=0.5084), and 68.1±6.7% (p=0.2835), respectively.

Summary/Conclusions

CILP was not improved on COG ANBL0532 despite the implementation of boost RT to gross residual tumor present at end-induction. New strategies are needed to decrease the risk of locoregional failure.

Gainesville,

Children's Oncology Group Statistics and Data Center

Naranjo

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Type Clinical

Presentation Preference Oral presentation

Abstract nr. 23

More is not always better: Results of the randomized open-label GPOH trial NB2004-HR on the induction chemotherapy in high-risk neuroblastoma

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

Induction chemotherapy is a mainstay for treating patients with high-risk neuroblastoma.

Aims

This trial aimed to assess whether the addition of two courses of topotecan, etoposide and carboplatin compared to the GPOH standard induction chemotherapy is able to improve event free survival.

Methods/Materials

An open-label, multi-center, randomized prospective controlled trial was performed at 58´institutions in Germany and Switzerland. Patients with neuroblastoma diagnosed according to

the INSS criteria either with stage 4 and age ≥1 - 21 years or with MYCN amplification and age ≥6 months – 21 years were eligible. The randomization was done at diagnosis by blocks and stratified by stage, LDH elevation, age, and MYCN amplification. The primary endpoint was event free survival (EFS). The patients were randomly assigned to standard induction chemotherapy consisting of six alternating courses N5 (cisplatin, etoposide, vindesine) and courses N6 (vincristine, dacarbazin, ifosfamide, doxorubicine) or to two courses consisting of topotecan, cyclophosphamide, and etoposide followed by standard induction chemotherapy (total eight courses). Induction chemotherapy was followed by high-dose chemotherapy with autologous hematopoietic stem cell transplantation and by nine courses of isotretinoin over 1 year. Radiotherapy to the primary tumor and/or mIBG therapy were applied only to patients with active tumor at the end of induction chemotherapy.

Results

Between October 12, 2004 and December 31, 2016 536 patients were enrolled and eligible for random allocations. Of 422 randomized patients (79%) patients, 211 were assigned to the control and 211 to the experimental arm (intent to treat cohort). Median follow-up time was 3.32 years (IQR 1.65-5.92). At the data lock (September 1, 2018), 138/211 patients of the experimental arm and 145/211 patients of the control arm had events. The 3 year event free survival proportions were 34% (95%-CI 28-40) and 32% (26-38) respectively (P = 0.258, 3-stage adaptive design, total observation period). The proportions for 3 year overall survival (OS) were 54% (95% CI 46-62; experimental arm) and 48% (40-56; control arm; P=0.558). The response to induction chemotherapy after 2 courses and before high-dose chemotherapy was not different between the arms. Patients of the experimental arm needed longer time for the induction chemotherapy (median 195 days, 95% CI 191-201) compared to patients of the control arm 134 days (130-138) (P<0.001). Grade 3 and 4 thrombocytopenia was more frequently seen in the experimental arm (86% vs. 79%, P<0.001) and grade 3 and 4 oral mucositis more in the control arm (6% vs. 3%, P =0.022). The median number of non-fatal grade 3 and 4 toxicities per patient was higher in the experimental group (31; 95% CI 29-33) compared to the control group (22; 95% CI 22-23; P <0.001). In both treatment arms two toxic deaths were caused by induction chemotherapy.

Summary/Conclusions

The patients had no event free survival benefit from two additional chemotherapy courses. The longer chemotherapy period was associated with more grade 3 and 4 toxicities. The addition of chemotherapy courses consisting of topotecan, cyclophosphamide and etoposide to the standard induction chemotherapy cannot be recommended.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 24

Risk classification for patients with first recurrence of stage 4 neuroblastoma

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

Reports on the outcome of patients with first recurrence of stage 4 neuroblastoma frequently lack an adequate risk-matched control group.

Aims

This study aimed to identify relevant prognostic variables and proposes a new risk score.

Methods/Materials

Inclusion criteria were: first recurrence of neuroblastoma stage 4 aged ≥18 months and enrollment in first line trials between 1997 and 2016. Patients were randomized into a training set (n=310) and an independent validation set (n=159). The primary endpoint for this analysis was secondary event-free survival. The main exclusion criteria were death due to toxicity in first line therapy and presence of second malignancy at any time.

The treatment elements of the first line therapy were analyzed as binary variables (n=61). Therapies with curative intent after the first recurrence were included as time-dependent variables (n=8) in order to exclude a time-of-treatment-associated effect. Palliative treatment was calculated as binary variable.

A five-step multiple time-dependent Cox regression analysis was performed on the training set to identify prognostic variables adjusted for the individual frontline treatment the patients received. The selected variables resulted in a prognostic index (PI) which was then used to build a risk score system. The score was validated with the patients included in the validation set.

Results

Of the 469 patients, 372 (79%) were treated with curative intent and 97 (21%) with palliative intent. The median survival time from first to a second recurrence (secEFS) was 5.4 months and to death (secOS) 12.6 months for the total group. Curatively treated patients had a median secEFS of 8.0 and a median secOS of 16.1 months. Aberrations of chromosome 1p and MYCN amplification were prognostic in univariable, but not relevant in multivariable analysis. The parameter estimates of the multivaribly selected non-therapy variables were used to generate the prognostic index (PI). The PI is a measure of the risk for a secondary event independent of the received therapy. The PI values of the selected variables were

+ 0.821 (if >1 recurrence organs, hazard ratio [HR]=2.27)
+ 0.707 (if recurrence time <18 m, HR=2.03)
+ 0.570 (if liver metastasis at diagnosis, HR=1.77)
+ 0.438 (if primary tumor recurrence, HR=1.55)
+ 0.253 (if age <42 m, HR=1.42)

The sum indicates the related risk group: lower PI 0 to ≤0.50, intermediate PI >0.50 to ≤1.20, higher risk group 3 PI >1.20. The risk groups were confirmed in the validation set. The scoring system was also useful for the curatively or palliatively treated subgroups.

Summary/Conclusions

A new risk score system consisting of clinical variables for patients with first recurrence of stage 4 neuroblastoma aged ≥18 months at diagnosis is described.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 26

Discovery of novel thiazoles (TT-42) as potent inhibitor of Phosphatidylinositol 3-kinase (PI3K) against neuroblastoma

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

Neuroblastoma is a cancer often found in the small glands on top of the kidneys (adrenal glands). It can develop in the stomach, chest, neck, pelvis and bones. The existing modalities to treat neuroblastoma are associated with a low survival rate. Phosphatidylinositol 3-kinase (PI3K) is a cellular proto-oncogene and an essential lipid kinase, that plays an important role in the regulation of cell proliferation, survival and motility. Several preclinical studies have shown that this pathway is hyper activated in neuroblastoma.

Aims

The present study showed the development of novel PI3K inhibitors against neuroblastoma.

Methods/Materials

The compounds were designed and developed on the basis of pharmacophoric requirements to interact with the active site of PI3K. The molecule was allowed to dock with 3D crystal structure of PI3K protein to assess the structural requirement for binding. The molecules were tested for their activities against PI3Kα enzyme via Kinase-Glo Luminescent Kinase Assay kit (Promega, Madison, WI, USA).

Results

The molecules were developed in considerable yields. The docking results showed excellent shape complementarity between ligand and the binding pocket. The thiazole moiety occupied the ATP binding pocket, where N1 nitrogen forms a critical hydrogen bond to Val882, and Lys833 in the protein hinge region. The morpholine group fragment lies over Trp812, apparently adding a hydrophilic shield to the hydrophobic heterocycle. The π-stacking interaction was also observed with Asp964. The compound showed IC_{50} of 2.31 μM against PI3Kα.

Summary/Conclusions

In summary, using structure-based design approach, we have successfully designed and

synthesized a novel series of thiazoles as PI3K inhibitor against neuroblastoma.

Type Basic

Presentation Preference Traditional poster

Abstract nr. 27

Roles of Src family kinases and their substrates in the biology of neuroblastoma

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Recent studies suggested the implication of activated anaplastic lymphoma kinase (ALK) in malignant aspects of wide range of neuroblastoma regardless to the presence of genetic alteration of this kinase. During the analysis of phosphotyrosine-containing proteins associated with activated ALK in neuroblastoma cells, we have noticed that several known substrates of Src family kinases (SFKs) such as cortactin and delta-catenin are involved in the complex. It is also reported that inhibition of SFKs cause suppressive effect in the growth of neuroblastoma, while it is not clear how the Src signaling modulates oncogenic potential of neuroblastoma.

Aims

SFKs are known to be activated in various types of cancers and involved especially in the progression of the disease. The objective of this study is to elucidate the role of SFKs and their substrates in the progression of neuroblastoma.

Methods/Materials

Neuroblastoma cell lines with activated ALK such as NB39-nu are treated with several Src inhibitors along with ALK inhibitors and changes in the phosphotyrosine-containing proteins are analyzed by immunoblotting and compared. Dose-dependent effects of these inhibitors on cell proliferation and cell migration were measured by WST-1 assay and Transwell assay, respectively. As for several identified substrates of SFKs affected by Src inhibitors, effects of knockdown by siRNA treatment was analyzed.

Results

SFK inhibitors such as saracatinib, bosutinib and dasatinib generally caused more significant suppressive effects in cell migration than in cell proliferation in NB39-nu cells. Among SFK inhibitors, dasatinib showed outstanding inhibition of cell migration at the concentration as low as

10 nM. At this concentration, dasatinib caused inhibition of the phosphorylation of several substrates of SFKs such as paxillin and p130Cas. On the other hand, siRNA inhibition of these substrates did not show significant effect on the migration of NB39-nu.

Summary/Conclusions

Since the siRNA inhibition of paxillin or p130Cas caused enhanced tyrosine phosphorylation of these substrates possibly by some feedback mechanism, the complete knock out of these proteins might be required to see the involvement of these proteins. Identification of the key substrates of SFKs for migration of neuroblastoma which are sensitive to dasatinib should be performed for further understanding of Src-mediated regulation of neuroblastoma. This approach will lead to the development of novel therapy targeting the Src-mediated signals in advanced stages of neuroblastoma.

Type Basic

Presentation Preference Traditional poster

Abstract nr. 28

Cross-talk between Tumor Associated Macrophages (TAM) and Cancer Associated Fibroblasts (CAF) in Neuroblastoma (NB) Contributes to a Pro-tumorigenic Microenvironment (TME) that Promotes TAM survival and immune escape

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

A major challenge in NB therapy is to understand the mechanisms leading toward therapeutic resistance and immune escape. These processes are deeply influenced by the TME and in particular, as shown previously by our group, the presence of TAM and bone marrow derived mesenchymal stromal cells (MSC) that share phenotypic and functional characteristics of CAF (MSC/CAF).

Aims

To determine the mechanisms by which TAM and MSC/CAF (stromal cells) cooperate to promote resistance and immune escape in NB tumors

Methods/Materials

We used a combination of five antibodies (anti CD163, anti CD31, anti SMA, anti CD105 and anti Phox2B) in multiplex immunohistochemistry on formalin fixed paraffin embedded primary NB tumors to qualitatively and quantitatively examine the presence of TAM, and MSC/CAF and their interaction with tumor cells. We studied the interaction between NB cells (one MYCN-NA and two MYCN-A cell lines) and fresh peripheral blood monocytes from healthy donors and three primary MSC/CAF cell lines (healthy donor, patient bone marrow, and primary NB tumor) in in vitro co-cultures. Analysis of cytokines and chemokines released was done at the RNA level by qRT-PCR

and at the protein level by ELISA on the conditioned media. Phospho-protein analysis was used to identify pathways activated in NB cells.

Results

An analysis of 17 untreated primary NB tumors revealed the presence of TAM and MSC/CAF in 14 tumors but a significant degree of inter-tumoral heterogeneity. The stromal cells differed in their distribution with TAM being present closely associated with NB cells and MSC/CAF being present in the stroma surrounding tumor nodules. In *in vitro* experiments, we observed that MSC/CAF cultured alone or in the presence of NB cells or their conditioned medium prevented apoptosis in monocytes. We then demonstrated that when cultured alone NB cells did not secrete significant amount of cytokines and chemokines. However, when co-cultured with TAM and MSC/CAF several pro-tumorigenic cytokines like TGFβ1, IL-6, IL-8, IL-10, M-CSF, LIF and MCP1 were detected in the culture medium (some >4-fold increase) or by qRT-PCR (>2-fold increase) in the absence of anti-tumorigenic cytokines such as TNFα,IFNγ, IL-11, IL-12 and IL-20. Whereas both TAM and MSC/CAF each stimulated cytokine production, a maximum effect was observed in the presence of both stromal cells cultured together with NB cells. In the case of the MYCN-NA cell line, at the exception of TGFβ1, TAM and MSC/CAF were the source of the cytokines whereas in the case of MYCN-A cell lines, both tumor cells and stromal cells were the source of production. The presence of these cytokines in the medium, induced the activation of several signalling pathways in NB including STAT-3, MAPK1/2 and SMAD that promoted survival and immune escape.

Summary/Conclusions

The data point to the heterogenous nature of the TME landscape in NB and to the cooperation between TAM and MSC/CAF in their contribution to a protumorigenic TME. The analysis of the presence of these cells and of the pathways they activate in NB tumors should provide important information in the design of therapeutic approaches that simultaneously address drug resistance and immune escape.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 29

MYT1 attenuates neuroblastoma cell differentiation by interacting with the LSD1/CoREST complex

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Topic Neuroblastoma as developmental disease

Background/Introduction

Recent studies have shown that myelin transcription factor 1 (MYT1) promotes vertebrate neurogenesis by regulating gene expression. It can act as either a transcriptional repressor or activator depending on the cell context. However, the roles of MYT1 in regulating cell developments in neuroblastoma remains unknown.

Aims

To understand the molecular mechanisms of MYT1 in regulating cell differentiation of neuroblastoma.

Methods/Materials

MYT1 expression across the clinical samples was evaluated by mass-spectrometry based quantitative proteomics. Tissue array and relevant clincal data were used to investigte the potential roles of MYT1 in neuroblastoma. Further biological mechanism of MYT1 in regulating cell differentiation was investigated with RNA-seq and cell experiments

Results

MYT1 was differentially expressed among neuroblastoma patients with different pathological diagnoses. Analysis of clinical data showed that overexpression of MYT1 was associated with a significantly shorter 3-year overall survival rate and poor differentiation in neuroblastoma specimens. MYT1 knockdown inhibited proliferation and promoted the expression of multiple differentiation-associated proteins. Integrated omics data indicated that many genes involved in neuro-differentiation were regulated by MYT1. Interestingly, many of these genes are targets of the LSD1/CoREST complex and we show that MYT1 physically interacts with this complex. Depletion of LSD1 or inhibition of LSD1 by ORY-1001 decreased the expression of MYT1, providing an alternative approach to target MYT1. Taken together, our results indicate that MYT1

significantly attenuates cell differentiation by interacting with the LSD1/CoREST complex. MYT1 is, therefore, a promising therapeutic target for enhancing the neurite-inducing effect of retinoic acid and for inhibiting the growth of neuroblastoma.

Summary/Conclusions

Our results identify an association between MYT1 expression and the differentiation status of neuroblastoma cells and provide evidence that MYT1 knockdown in combination with inhibition of either retinoic acid or LSD1 can significantly enhance neuroblastoma differentiation. Mechanistically, MYT1 may stabilize REST expression, inhibit differentiation-related gene expression by forming a complex with LSD1 and coREST, and reduce the expression of RARs. Thus, LSD1 inhibitor may alternative provide potential therapeutic benefit for high-risk neuroblastoma patients with high levels of MYT1 expression.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 30

High-risk neuroblastoma: mIBG Curie scores at the time of diagnosis and the end of induction, their prognostic value in comparison with data of MMD and MRD at the same time.

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Topic Other

Background/Introduction

Despite a multi-modality approach combining chemotherapy, surgical resection, radiotherapy, autologous stem cell transplant and biotherapy, survival for high risk neuroblastoma remains poor, with 5-year EFS 30-49% The use of the anti-GD2 chimeric antibody post-transplant time was improvements in EFS .For patients who develop relapsed disease, specifically those who relapse 6–18 months from initial diagnosis, 5-year OS is < 20% The identification of prognostic markers of response and survival early in a patient’s treatment may have significant impact on therapy and outcome.

Aims

Assessment of prognostic value for patients with high-risk neuroblastoma of the mIBG data in comparison with data of MMD and MRD at the time of diagnosis and the end of induction.

Methods/Materials

44 patients (pts) who underwent I 123 MIBG scintigraphy from April 2011 till December 2017.Sex: 24 - boys, 20- girls. Age median 2.84 years (from 0.76 years to 7.71 years). The distribution according to the stages is the following: stage 2 -1 pts, stage 3 – 3 pts, stage 4 - 40 pts. All pts received treatment according to NB2004 protocol. Determination of MMD and MRD by PCR, the relative expression level of TH and PHOX2B genes in the bone marrow was evaluated.
To assess the significance of differences, the $\chi 2$ test, the Whitney U-test were used. EFS rate for pts was estimated using the Kaplan-Meier method and survival function was compared using the long-rank test.

Results

It was revealed that the patient's with the presence of mIBG avid disease at the time of diagnosis according to I^{123} mIBG scintigraphy of more than 5 CS (Curie scores) significantly worsens the results of therapy (5-year EFS 74 ± 13%, vs. 32 ± 14%). In addition, when evaluating the response to therapy after the end of induction treatment, it was found that the preservation of the the presence of mIBG avid disease according to I^{123} MIBG scintigraphy more than 3 CS significantly worsens the results of the therapy (4-year EFS 54 ± 9%, vs. 13 ± 12%).
The data of MIBG scintigraphy are comparable with the data on MMD and MRD in bone marrow. Thus, differences in the medians of relative expression of TH and PHOX2B genes at the time of diagnosis of the disease at 5 points (CS) was 0.003 versus 0.0776 ($p = 0.012$) and 0.0078 against 0.0503 ($p = 0.059$), respectively. At the end of the induction, the differences in the medians of the relative expression of TH and PHOX2B genes in bone marrow of the disease at 3 points (CS) was 0 against 0.0062 ($p = 0.0056$) and 0 against 0.0001 ($p = 0.0075$).

Summary/Conclusions

The cut-off data for the relative expression level of PHOX2B gene at the end of induction therapy coincides with the median if there are less than 3 (SC) points. For the relative expression level of TH gene in the bone marrow at the time of induction, the cut-off data are within the same order (0.002 and 0.006), respectively. Taking into account the above, we consider it reasonable to use both indicators simultaneously to highlight the ultra-high risk group of patients with neuroblastoma. This group is characterized by an unfavorable prognosis in terms of EFS, the correction of the therapy for this group of patients is definitely necessary.

Type Clinical

Presentation Preference Traditional poster

Abstract nr. 31

Preliminary results of Phase I study of T-cell Engaging Anti-GD2 Bispecific Antibody Hu3F8-BsAb (NCT03860207): T-cell activation and Clinical Toxicities.

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

T-cells have potent anti-cancer properties but their utility in pediatric malignancies is limited because of low tumor mutational burden, sparse tumor infiltration, chemotherapy-related immunosuppression and an immunosuppressive tumor microenvironment. In leukemia, chimeric antigen receptors and T-cell engaging bispecific antibodies (BsAb) have successfully redirected polyclonal T cells for anti-cancer activity. Built on the IgG(L)-scFv format, the first fully humanized BsAb targeting the carbohydrate antigen GD2, hu3f8bsAb showed high potency in vitro and in vivo against GD2(+) xenografts without neurotoxicity (Cancer Immunol Res 3:266, 2015). A phase I/II study was initiated in 2019 to test the toxicity and efficacy of hu3F8-BsAb.

Aims

The primary objectives of the phase I part of this study are to evaluate the toxicity of hu3F8-BsAb in patients with GD2-expressing tumors and to establish a recommended phase II dose. Secondary objectives include investigating pharmacokinetics, immune modulation, immunogenicity (by human anti-bispecific antibody response [HABA]) and anti-tumor activity.

Methods/Materials

Salient eligibility criteria include presence of measurable or evaluable high-risk disease resistant to standard therapy, absolute lymphocyte count >500/ul, lack of human anti-human antibody if previously exposed to monoclonal antibody. After premedication with acetaminophen, anti-histamines and dexamethasone hu3F8-BsAb is administered on days 1 and 7 with a dose-limiting toxicity observation period of 28 days. The first dose is administered in-patient with monitoring for at least 48 hours.Subsequent doses may be administered out-patient. Cohorts of one patient per dose level were planned with 3-10-fold dose-escalation between cohorts until a grade 2 related toxicity was encountered. Further dose-escalation follows a continuous reassessment method. Toxicity is assessed using CTCAE version 4.03. HABA and pharmacokinetics are measured using

ELISA. Immune effects are evaluated by immunophenotyping of lymphocyte subsets and by serum cytokine levels.

Results

Six patients (5 with neuroblastoma and one with osteosarcoma) have thus far received 10 cycles of hu3F8-BsAb. Dose escalation commenced at 0.009 mcg/kg per cycle. At dose level 4 (2.6mcg/kg/cycle), grade 2 cytokine release syndrome (CRS) characterized by hypotension, hypoxia, myalgia, lymphopenia and thrombocytopenia was encountered. A subsequent patient was enrolled at that dose level and after no >grade 2 toxicities were encountered, dose was escalated to dose level 5 (4.5mcg/kg/cycle) with a fixed first dose of 1.3mcg/kg dose and a second dose of 3.2mcg/kg. Grade 3 CRS that had resolved to grade 1 within 48 hours was observed in this latter patient. Management of CRS included rescue doses of dexamethasone, tocilizumab, fluid boluses and supplemental oxygen. Onset of CRS occurred 2-4 hours after the end of hu3F8-BsAb infusion and resolved within 24-48 hours. CRS was most marked after the first dose, with mild to no symptoms with subsequent doses. Neither other related >grade 2 toxicities (including neurotoxicity) nor HABA were seen even after 4 cycles. Dose-limiting toxicity has not yet been encountered. CRS was characterized by log-fold, though transient elevations of IL-6, IL-10, IL-13, IL-2, IL-8, IFN-γ and TNF-α.

Summary/Conclusions

Hu3F8-BsAb activates T-cells with associated cytokine release syndrome. Dose-escalation is ongoing, and phase II will commence after recommended dose is established.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 32

MicroRNA-based therapeutics for the treatment of high-risk neuroblastoma

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Topic Gene regulatory networks and epigenetics: from basic science to therapy

Background/Introduction

Neuroblastoma is a deadly childhood cancer arising in the developing sympathetic nervous system. High-risk patients are currently treated with intensive multi-layered chemotherapy which is not always curative and leaves surviving patients with life-long side effects, underscoring an urgent need for safer treatments. miRNA-based drugs represent an exciting new class of cancer therapeutic. However, our understanding of miRNA function in neuroblastoma is fragmented, restricting our ability to capitalise on their therapeutic potential.

Aims

We aim to screen and validate miRNAs that sensitise neuroblastoma cells to chemotherapy *in vitro,* and test candidate therapeutic miRNAs in pre-clinical models of high-risk neuroblastoma.

Methods/Materials

An extensive functional genomic screen of >1200 miRNA mimics was conducted in neuroblastoma and normal-like cell lines to discover miRNAs with therapeutic promise. This was performed in combination with low doses (IC30) of doxorubicin and vincristine chemotherapies. Selected candidate miRNAs are currently being tested in the pre-clinical COG-N-519 patient-derived xenograft (PDX) model, originating from a patient with high-risk disease after several lines

of treatment. Star-POEGMA nanoparticles are used to deliver candidate miRNAs either as a single agent, or in combination with topotecan chemotherapy. Ongoing work includes mechanistic experiments to assess miRNA uptake and their impact on gene expression, and therapeutic experiments to monitor tumour growth and survival.

Results

Three miRNAs (miR-99b-5p, miR-380-3p and miR-485-3p) had potent synthetic lethal interaction with doxorubicin *in vitro*, specifically in high-risk cell lines (Kelly and SK-N-DZ), but not low-risk neuroblastoma (SHEP) or normal (IMR90 and MCF-10A) cell lines. Analysis of a clinical cohort revealed that these miRNAs are possible tumour suppressors as they undergo recurrent copy number loss, and low expression predicts poor outcome.

Encouragingly, miR-99b-5p could be effectively delivered (40-fold overexpression) to PDX tumours. RNA-Seq analysis revealed downregulation of key neuroblastoma dependency genes *MYCN*, *PHOX2B*, *HAND1/2*, *GATA3, ISL1* and *EZH2* in miR-99b-5p-treated tumours. Importantly, many of these genes encode core regulatory lineage-defining transcription factors in neuroblastoma (1,2), and are considered to be 'undruggable'. Additionally, miRNA treatment resulted in upregulation of neuronal differentiation genes, suggesting that miR-99b-5p can either differentiate the tumour cells, or selectively kill undifferentiated tumour cells. Future single cell RNA-Seq experiments will be used to address these possibilities.

Summary/Conclusions

We have identified novel chemosensitising miRNAs specific to high-risk neuroblastoma that can be administered *in vivo* to drive favourable gene expression changes. If effective in longer term preclinical studies, we predict that restoring the function of tumour suppressive miRNAs represents an attractive new therapeutic strategy for the treatment of neuroblastoma patients.

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MicroRNA-based therapeutics for the treatment of high-risk neuroblastoma

Type Translational

Presentation Preference Oral presentation

Abstract nr. 33

Association of heterogeneous MYCN amplification with clinical features, biological characteristics, and outcomes in Neuroblastoma: A Report from the Children's Oncology Group

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

MYCN amplification (MNA) is associated with poor outcomes in children with neuroblastoma. In most cases, *MYCN* amplification is seen homogeneously throughout tumor cells, typically with high-level amplification (>4-fold ratio of *MYCN* signals to reference probe signals). With the advent of fluorescent in situ hybridization (FISH) methods to assess *MYCN* status, it has become clear that MNA may be heterogeneous, defined as amplification in a minority (≤20%) of tumor cells in a sample.

Aims

1. To describe the clinical characteristics and biologic features of patients with heterogeneous *MYCN*amplification. 2. To compare clinical outcomes of patients with heterogeneous MNA to patients with either homogeneous MNA or *MYCN* wild-type tumors.

Methods/Materials

In this retrospective cohort study from the Children's Oncology Group Neuroblastoma Biology Study (ANBL00B1), we categorized neuroblastoma patients as having tumors with *MYCN* wild-type, homogeneous MNA or heterogeneous MNA. We used chi-squared or Fisher's exact tests to compare features between groups. We used log-rank tests and Cox models to compare event-free survival (EFS) and overall survival (OS) between groups.

Results

MYCN status was ascertained in diagnostic tumor samples from 5,975 patients, including 4,937 (82.6%) with *MYCN* wild-type tumors, 981 (16.4%) with homogeneous MNA, and 57 (1%) with heterogeneous MNA. Clinical and biological features differed significantly between patients whose tumors had heterogeneous and homogeneous MNA, including stage, risk group, thoracic primary site, LDH, ploidy, 1p LOH, histology, MKI, grade, and *MYCN* copy number. Despite these differences, there was no statistically significant difference in EFS between patients with heterogeneous MNA compared to patients with homogeneous MNA [5-year EFS 57.5% (95% confidence interval (CI) 39.7-75.3%) vs. 50.7% (95% CI 46.3-55.2%), respectively; p=0.3537].

However, comparing outcomes for patients with heterogeneous MNA vs. wild-type *MYCN*, EFS was significantly higher for patients with wild-type *MYCN* [5-year EFS 78.4% (95% CI 76.6-80.1%; p<0.0001). Further, controlling for other key prognostic factors (age and INSS stage), Cox proportional hazards model for EFS comparing heterogeneous MNA to homogeneous MNA with *MYCN* status as the variable of interest found no difference in EFS (hazard ratio of 0.96; p=0.8453). In a separate model using heterogeneous MNA vs. wild-type *MYCN* and controlling for age and stage, EFS for patients with heterogeneous MNA remained significantly inferior to patients with wild-type *MYCN*(hazard ratio of 1.61; p=0.0284).

Summary/Conclusions

Neuroblastoma with heterogeneous MNA demonstrates significantly different biological and clinical patterns compared to homogeneous MNA. Prognosis is similar between these two groups. These results support current practice that treats patients with heterogeneous MNA similarly to patients with homogeneous MNA.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 34

Single-cell comparison of neuroblastoma stages with mouse embryonic neural crest and human adrenal gland

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

Intra-tumor heterogeneity in neuroblastoma (NB) and the presence of resistant clones represent a challenge for therapy. To understand the heterogeneity and “differentiation-like” status of cancer cells, we need to determine the origin and differentiation paths of cancer-originating cells. We explored intra-tumor heterogeneity using single-nuclei sequencing (Smart-seq2) and examined clinical heterogeneity by sequencing tumors from different stages of favorable or unfavorable outcome. A total of 3,853 tumor cells were sequenced, with 1,412 million reads mapped to annotated genes. Tumors clustered differently depending on stage and outcome. By using a cell type enrichment approach, we identified cell clusters that can be broadly classified as 1) neural, 2) undifferentiated, 3) immune, 4) mesenchymal stroma (MSC), and 5) endothelial. We compared cell populations of the tumors with mouse neural-crest populations from different embryonic ages at E9.5, E10.5, E12.5 and E13.5. We identified gene signatures shared between the sympatho-adrenal lineage of mouse E12.5 and E13.5 and the neural populations of NB. We further compared tumor populations with human normal adrenal gland populations, and determined commonalities in gene expression between cell clusters.

Aims

Methods/Materials

Results

Summary/Conclusions

Type Basic

Presentation Preference Oral presentation

Abstract nr. 35

Stage 4S Neuroblastomas: Molecular, Histological and Immunohistochemical Characteristics and Presence of 2 Distinct Patterns of MYCN Protein Expression: A Report from the Children's Oncology Group.

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

Stage 4S neuroblastoma (4SNB: <1yr at diagnosis, INSS stage 1 or 2 primary, metastasis limited to liver, skin and/or bone marrow) is often associated with spontaneous regression and a good clinical outcome. *MYCN* oncogene amplification and augmented expression of MYC-family (MYCN/MYC) protein indicate aggressive clinical behavior in NBs.

Aims

Molecular, histological and immunohistochemical characteristics of 4SNB tumors were analyzed along with clinical outcomes of the patients.

Methods/Materials

A total of 185 4SNBs filed at the COG NB Pathology Reference Laboratory were available in this study. Their *MYCN* oncogene status [Non-amplified(NA) vs. Amplified(A)] by FISH, MYC-family protein expression [no-overexpression(-)/(+/-) vs. overexpression(+)] by immunohistochemistry(IHC), and histopathology by INPC [Favorable Histology(FH) vs.

Unfavorable Histology(UH)] with a special attention to the nucleolar hypertrophy [NH, supporting increased protein expression, (-) vs. (+)] were correlated with patient survival.

Results

As shown in the Table, *MYCN* oncogene status (165NA;20A) distinguished favorable and unfavorable clinical outcomes of the patients. The vast majority of tumors showed *MYCN* NA, MYC-family protein(-)/(+/-), FH, NH(-), and excellent survivals (conventional NA tumors). Among *MYCN*-NA tumors, however, 11 demonstrated MYCN protein(+) with uniform and moderate intensity: they were FH(10/11), NH(-) and one showed MYC protein(+). Also found were 5 MYC protein(+) and MYCN(-)/(+/-) tumors: they were FH without NH(4/5). Among the *MYCN*-A tumors, 18 had MYCN protein(+) with heterogeneous and strong intensity regardless of INPC [UH(9, conventional A tumors)/FH(9)] and 15 of them had NH(+). Two tumors had MYCN protein(-)/(+/-) despite *MYCN*-A; both were FH and NH(-).

Summary/Conclusions

In most of the 4S NBs (89%), all or majority of the factors tested in this study were favorable, indicating an excellent prognosis of the patients. However, rare tumors (11%) were *MYCN*-A, and had a poor prognosis when MYCN protein was (+). Two patterns of MYCN protein(+) were distinguished: (1) In *MYCN*-A tumors, the expression was associated predominantly with NH, showed a heterogenous and strong pattern, and indicated a poor prognosis and (2) Protein expression in *MYCN*-NA tumors was mostly not associated with NH, showed a uniform and moderate pattern, and indicated an excellent prognosis. The definition of 4SNB should be refined to adhere to the biological characteristics of tumors, especially to the *MYCN* status, expression pattern of the MYCN protein and the nucleolar morphology.

MYCN oncogene	MYCN protein	MYC protein	INPC	NH	3-year EFS (SE)%	3-yar OS (SE)%
Non-Amplified (165)						
Conventional (147)	(-)/(+/-)	(-)/(+/-)	FH	(-)	88.5 (3.1)	94.1 (2.3)
Others (10)	(+)*	(-)/(+/-)	FH(9);UH(1)	(-)	100 (0)	100 (0)
(1)	(+)*	(+)	FH	(-)	100 (0)	100 (0)
(5)	(-)/(+/-)	(+)	FH	4(-);1(+)	100 (0)	100 (0)
(2)	(-)/(+/-)	(-)/(+/-)	UH	(-)	100 (0)	100 (0)
Amplified (20)						
Conventional (9)	(+)**	(-)/(+/-)	UH	2(-);7(+)	44.4 (23.4)	44.4 (23.4)
Others (9)	(+)**	(-)/(+/-)	FH	1(-);8(+)	68.6 (19.2)	68.6 (19.2)
(2)	(-)/(+/-)	(-)/(+/-)	FH	(-)	N/A	N/A

MYCN expression by IHC: *Moderately and uniformly positive; **Strongly and heterogeneously positive.

Type Translational

Presentation Preference Traditional poster

Abstract nr. 36

Investigation of a novel approach in the detection of circulating tumor cells in pediatric neuroblastoma patients

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Topic Other

Background/Introduction

Neuroblastoma (NB) is the most common extracranial solid tumor in children. The prognosis of NB varies greatly. Children with high-risk NB often have poor prognosis. Although a variety of aggressive therapies have been taken, this situation is still not optimistic.

Aims

The aims of this study are 1) to establish a new model to capture circulating tumor cells (CTCs) in peripheral blood of NB patients; 2) to examine the efficacy of the utilization of CTCs in the monitoring of patient outcomes and to identify early relapses.

Methods/Materials

A newly designed microfluidic chip was developed for the isolation of CTCs in the peripheral blood of NB patients based on the size differences between tumor cells and normal peripheral blood cells. All red blood cells and over 99.99% of white blood cells were successfully excluded from the blood samples. The remaining cells were applied for immunofluorescence staining with markers to identify NB cells. $CD45^{-}GD2^{+}PHOX2B^{+}$ cells were classified as NB CTCs.

Results

The average capture rate of CTCs was evaluated by spiking SH-SY5Y NB cells into healthy blood samples. We examined the capture rate by spiking 6 to 87 cells per 3 ml of blood of each of the 7 donors. The capture rate varied between 83.3% and 90.8%. In sum, 93 patient blood samples were tested and 4 healthy blood samples were used as controls. CTCs were detected in none of the healthy samples. NB patients were divided into low-risk (stage 1 and 2 NB) and high-risk (stage 3 and 4) groups based on INSS classification. The average count of CTCs per 3ml of blood

from the 49 low-risk patients was 0.61 ± 0.29 (mean ± SEM). In comparison, the average count of CTCs per 3ml of blood from the 46 high-risk patients was 12.30 ± 4.55 (mean ± SEM). There was a significant increase of CTC count between the high-risk group and the low-risk group (**P* < 0.01, Mann-Whitney *U* test). We tested 7 samples that were collected from newly diagnosed NB patients before the initiation of any treatment. CTCs were positively detected in all of these 7 samples, with an average count of 6.29 ± 2.78 per 3ml of blood (mean ± SEM). Furthermore, we tested 9 patients for CTCs counts both before and after chemotherapy. There were significant reductions of CTCs counts of all of these patients after chemotherapy (10.22 ± 3.27 vs. 1.11 ± 0.66, ***P* < 0.01, Mann-Whitney *U* test).

Summary/Conclusions

A newly designed microfluidic chip was presented for enriching, purifying, and counting circulating tumor cells (CTCs) in peripheral blood of NB patients with high sensitivity and specificity. CTCs detection in NB patients showed promising applications in diagnosis, evaluation of treatment, and dynamic monitoring of NB disease.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 37

Surveillance for Early Detection of Neuroblastoma Central Nervous System Relapse

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

Neuroblastoma relapsing in the central nervous system (CNS), though uncommon, is historically incurable. However, a recent multimodality approach that includes intra-ventricular radioimmunotherapy has significantly improved long term survival (J. Neurooncology 97:409, 2010). Timely detection of CNS relapse may reduce or prevent morbidity and mortality. In co-operative groups, surveillance for patients on or after treatment for high-risk neuroblastoma usually includes whole-body MIBG scans but does not require dedicated anatomical imaging of the brain. At Memorial Sloan Kettering Cancer Center (MSK), head MRI at 3-6 monthly intervals is standard-of-care.

Aims

The objective of this retrospective study was to determine the optimal imaging modality for the detection of CNS relapse in patients with neuroblastoma.

Methods/Materials

After MSK IRB approval, records of all patients with CNS neuroblastoma seen at MSK from 2004-2019 were evaluated. In most cases, relapse was diagnosed at other institutions before referral to MSK. Analyzed data included symptoms and radiological findings on brain CT/MRI and MIBG scans at CNS relapse. Data on available scans from neuroblastoma diagnosis until CNS relapse were also analyzed.

Results

153 patients, all with MIBG-avid disease at initial diagnosis were identified; 5 had CNS disease at diagnosis. For the remaining 148 patients, median time to CNS relapse from diagnosis was 18 (range 0-104) months. 102 (67%) patients experienced CNS relapse after achieving complete remission (CR) at a median of 9.8 (range 0.8-72) months later, while 43 patients had relapse without achieving CR. Of those relapsing post-CR, CNS was the initial site of relapse in 93 (90%).

97/153 (66%) patients had neurological symptoms at time of CNS disease; the rest (34%) were asymptomatic, relapse being detected on surveillance. Multiple (>1) parenchymal lesions were noted on imaging in 58 (38%), diffuse leptomeningeal disease without parenchymal involvement in 8 (5%) and solitary lesions in 87 (57%), located to cerebellum and cerebrum in 14 (16%) and 73 (84%), respectively. Median diameter of the largest lesion on imaging was 2.7 (range <0.5-6.8) cm. Anatomical imaging was performed with MRI (82; 54%), CT (14; 9%) or both (57;37%). Of those undergoing both scans, CNS relapse was missed on CT in 3/57 (5%) patients. 115 patients had MIBG scans prior to resection of CNS relapse. All sites of disease noted on MRI/CT were also positive by MIBG in 40 (35%) patients. However, MIBG scan was either totally or partly negative in 56 (49%) and 19 (15%) patients, respectively. Even for lesions ≥2cm in diameter, MIBG was negative in 24/61 (39%). Of the 76 (50%) patient undergoing prior MR/CT of the brain (0.3-34 months before relapse), CNS disease was not detected in any; only 51/153 (33%) had MRI/CT surveillance within 6 months prior to relapse.

Summary/Conclusions

MIBG scan has poor sensitivity for the detection of CNS neuroblastoma, regardless of size or location. Although CT or MRI are both effective in detecting CNS relapse, the former can miss some lesions. Given that most relapses occur within 2 years of diagnosis, we recommend brain MRI for surveillance of high-risk neuroblastoma at regular intervals for ≥3 years after initial diagnosis.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 38

Therapeutic targeting of KSP in high-risk neuroblastoma

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

High-risk neuroblastoma patients have a very poor prognosis and relapse is common despite heavy treatment. Neuroblastomas exhibit relatively few recurrent and potentially druggable targets. In addition, high-risk neuroblastomas show significant inter- and intratumoral heterogeneity, suggesting that any highly specific targeting may need to be complemented with a broader treatment strategy, for example cell-cycle inhibition, to completely eradicate tumors and minimize the risk of acquired resistance.

Aims

To perform a high-throughput drug screen to identify and test novel treatments against high-risk neuroblastoma.

Methods/Materials

We used PDX-derived tumor organoids representing multiple high-risk MYCN-amplified neuroblastomas and performed a high-throughput drug screen consisting of approved and/or emerging oncology drugs, thereby facilitating rapid translation of candidates to the clinics. The drug screen was performed at different oxygen tensions and drugs were tested against control cells to stringently select drugs with high predicted activity but low potential for off-target effects. Selected drugs were further tested by cell viability and cell death assays, cell cycle analysis, RNA sequencing, and PDX-testing *in vivo*.

Results

Several novel anti-neuroblastoma drugs, approved for other conditions, were identified; we selected kinesin spindle protein (KSP) inhibition to overcome potential tumor heterogeneity effects. Bioinformatic analyses of large data sets revealed that high expression of the KSP encoding gene *KIF11* is associated with poor outcome in neuroblastoma and across multiple tumor types, *KIF11* gene dependency is highest in neuroblastoma. KSP inhibition showed efficacy in multiple high-risk neuroblastoma organoid models. Mechanistically, KSP inhibition resulted in formation of abnormal monoastral spindles, mitotic arrest, upregulation of mitosis-associated genes, and apoptosis. *In vivo*, KSP inhibition caused complete regression of neuroblastoma PDXs and increased survival of orthotopic high-risk neuroblastoma PDXs.

Summary/Conclusions

Our results suggest that KSP inhibition could be a promising treatment strategy in children with high-risk neuroblastoma.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 39

LIN28B increases neural crest cell migration and leads the transformation of trunk sympathoadrenal precursors

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Topic Neuroblastoma as developmental disease

Background/Introduction

The RNA-binding protein LIN28B regulates developmental timing and stem cell identity by suppressing the *Let-7* family of microRNAs. The post-embryonic reactivation of LIN28B is a hallmark of poor prognosis and metastasis in different types of cancer, including neuroblastoma, a pediatric solid tumor deriving from neural crest cells (NCC). However, the extent to which LIN28B activation participates in the modulation of NCC behavior in neuroblastoma remains unclear.

Aims

The aim of this study was to assess the molecular mechanisms by which LIN28B influences the viability of the NCC toward a malignant and metastatic phenotype.

Methods/Materials

The effects of transient LIN28B overexpression on NCC migration and differentiation were analyzed in the two vertebrate models *Danio rerio* and *Xenopus leavis.* The phenotype and the expression of related genes were analyzed by time-lapse microscopy, in situ hybridization, immunolabelling, and western blot analyses. The doxycycline-inducible neuroblastoma SH-SY5Y ^{LIN28B} cell line was obtained by lentiviral infection. The cell migratory and invasive capabilities were detected through transwell migration assay, matrigel invasion assay, scratch test and *in vivo* xenotransplantation model. Flow cytometric analyses, western blot, and real-time PCR were used to characterize the LIN28B-related molecular phenotypes.

Results

In this study, we provided evidence that the overexpression of LIN28B inhibits the sympathoadrenergic cell differentiation and increases the speed of NCC migration *in vivo*. Moreover, we show that LIN28B sustains the invasive motility of neuroblastoma cells *in vitro* and in zebrafish xenotransplanted embryos, where tumor cells show increased spread throughout the animal body. Our data support a growing body of evidence that LIN28B may re-establish neuroblastoma heterogeneity through the epithelial-to-mesenchymal transition (EMT), leading to tumor progression and metastatic tumor cell invasion. The EMT/PI3K/AKT regulatory axis is triggered by the up-regulation of the two integrins, ITGA5 and ITGA6, upon LIN28B overexpression, sustaining the metastatic phenotypes of this disease.

Summary/Conclusions

Taken together, our results establish the relevance of LIN28B in the regulation of NCC migration during embryonic development. Our data suggest a mechanism by which a long-term overexpression of LIN28B in neuroblastoma cells supports the integrin-mediated cell spreading and migration by facilitating the formation of focal contacts.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 40

Case report of a metastatic VIP-secreting neuroblastoma in remission without postoperative chemotherapy

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

Introduction Neuroblastoma represents around 10% of malignant diseases in children, with 120-150 cases per year in France, with a peak of incidence at the age of 18 months. The mode of discovery is variable and often not very specific. In the metastatic stage, neuroblastomas lead in the majority of cases to heavy management by surgery, followed by high-dose chemotherapy,radiotherapy and immunotherapy.

Aims

Methods/Materials

Results

The case We report here the case of a metastatic VIP-secreting neuroblastoma in remission without postoperative chemotherapy. This is a 15-month-old child who presented a chronic diarrhoea, for several months. Clinically he presented abdominal distension without mass, hepatomegaly and hypertension. Biologically there was an hypokalemia. Additional examinations revealed a calcified left adrenal mass and secondary pulmonary lesions. MIBG scintigraphy did not reveal any fixation. The dosage of urinary cathecholamines was high and the VIP 10 times normal. Biopsy of the mass confirmed the diagnosis of differentiated neuroblastoma without MYCN amplification. The CGH analysis found: +3(p26.3 - p22.2), +14q11.2, +15(q22.2-q25.3), 15(q26.1 –qter)-. The child was included in the HLBL SIOP protocol, RAPID Cojec arm, treated

with chemotherapy. Chemo-resistance of the tumour was noted, with no decrease in volume, neither resolution of diarrhoea or decreasing of metastatic lung lesions. Surgery was then performed by total left adrenalectomy, lumbo-aortic curage and resection of 2 pulmonary nodules. Anapatological analysis of these nodules confirmed the neuroblastic origin. Following surgery, the digestive symptoms disappeared and close surveillance was decided. Postoperative treatment with high dose chemotherapy has not been initiated. Clinical, biological and radio-logical monitoring notes no tumour and metastatic evolution. There is a continued quasi-normalisation of the VIP rate at 29 months of diagnosis.

Summary/Conclusions

Conclusion In conclusion, this case is original by the unusual therapeutic development, because a clinical-biological remission was obtained without post-operative chemotherapy, despite the metastatic nature of the tumour. Metastases are most often bone, hepatic, lymph node or sub-hepatic, here it is a case with pulmonary metastases. The absence of fixation with MIBG can make us ask the question of a particular component of this tumour. In addition, genetic analysis in “classic” neuroblastoma can find chromosomal deletions, typically 1p, 3p, 4p, 6q, 11q or gains such as 1q, 2p or 17q, in our case the atypical CGH profile has given us leads to an in-depth molecular study, studies are underway.

Perspective Metastatic neuroblastoma cases with Vaso-Intestinal-peptide secretion appears as a special entity among neuroblastic tumors, with a possible favourable prognosis, that might be related to the spontaneous maturation of the tumor and the metastases. A retrospective collection of such cases could help to confirm these data at the clinical, biological and therapeutic level.

Advances in Neuroblastoma Research (ANR) Meeting
ANR 2020
Amsterdam
11-14
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Type Clinical

Presentation Preference Traditional poster

Abstract nr. 41

CD44-high neural crest stem-like cells are associated with tumour aggressiveness and poor survival in neuroblastoma tumours

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Topic Neuroblastoma as developmental disease

Background/Introduction

Neuroblastoma is a paediatric tumour originated from sympathoadrenal precursors and characterized by its heterogeneity and poor outcome in advanced stages. Intra-tumoral cellular heterogeneity has emerged as an important feature in neuroblastoma, with a potential major impact on tumour aggressiveness and response to therapy. CD44 is an adhesion protein involved in tumour progression, metastasis and stemness in different cancers; however, there have been controversies about the significance of CD44 expression in neuroblastoma and its relationship with tumour progression.

Aims

We aim to explore the function of CD44 in neuroblastoma and its role as a potential marker for neural crest stem-like cells within neuroblastoma tumours

Methods/Materials

We have performed transcriptomic analysis on patient tumour samples studying the outcome of patients with high CD44 expression. Adhesion, invasion and proliferation assays were performed in sorted CD44high neuroblastoma cells. Tumoursphere cultures have been used to enrich in undifferentiated stem-like cells and to asses self-renewal and differentiation potential. We have finally performed in vivo tumorigenic assays on cell line-derived or Patient-derived xenografts.

Results

We show that high CD44 expression is associated with low survival in human neuroblastoma, independently of MYCN amplification. CD44 is expressed in a cell population with neural crest

stem-like features, and with the capacity to generate multipotent, undifferentiated tumourspheres in culture. These cells are more invasive and proliferative in vitro. CD44 positive cells obtained from tumours are more tumorigenic and metastatic, giving rise to aggressive neuroblastic tumours at high frequency upon transplantation.

Summary/Conclusions

We describe an unexpected intra-tumoral heterogeneity within cellular entities expressing CD44 in neuroblastoma, and propose that CD44 has a role in neural crest stem-like undifferentiated cells, which can contribute to tumorigenesis and malignancy in this type of cancer.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 42

Chick embryo model of neuroblastoma metastasis identifies novel genes associated with poor patient outcome

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Neuroblastoma is the second most common cancer in childhood but still has one of the lowest survival rates of all childhood malignancies. More than 60% of neuroblastoma cases are invasive and despite treatment advances, metastasis is still one major obstacle to overcome. We have established the chick embryo as a powerful and convenient preclinical *in vivo* model to study neuroblastoma aggressiveness and demonstrated that preconditioning neuroblastoma cells in hypoxia triggers their metastasis, and the regulation of genes supportive of tumour dissemination.

Aims

Our aim was to elucidate new molecular markers of neuroblastoma metastasis and to identify drugs that can revert this phenotype.

Methods/Materials

Using our established chick embryo model for neuroblatoma tumour growth and controlled metastasis, together with microarray analysis we measured the transcriptomic profile of non-metastatic and metastatic (hypoxic preconditioned) neuroblastoma primary tumours formed by SK-N-AS cells.

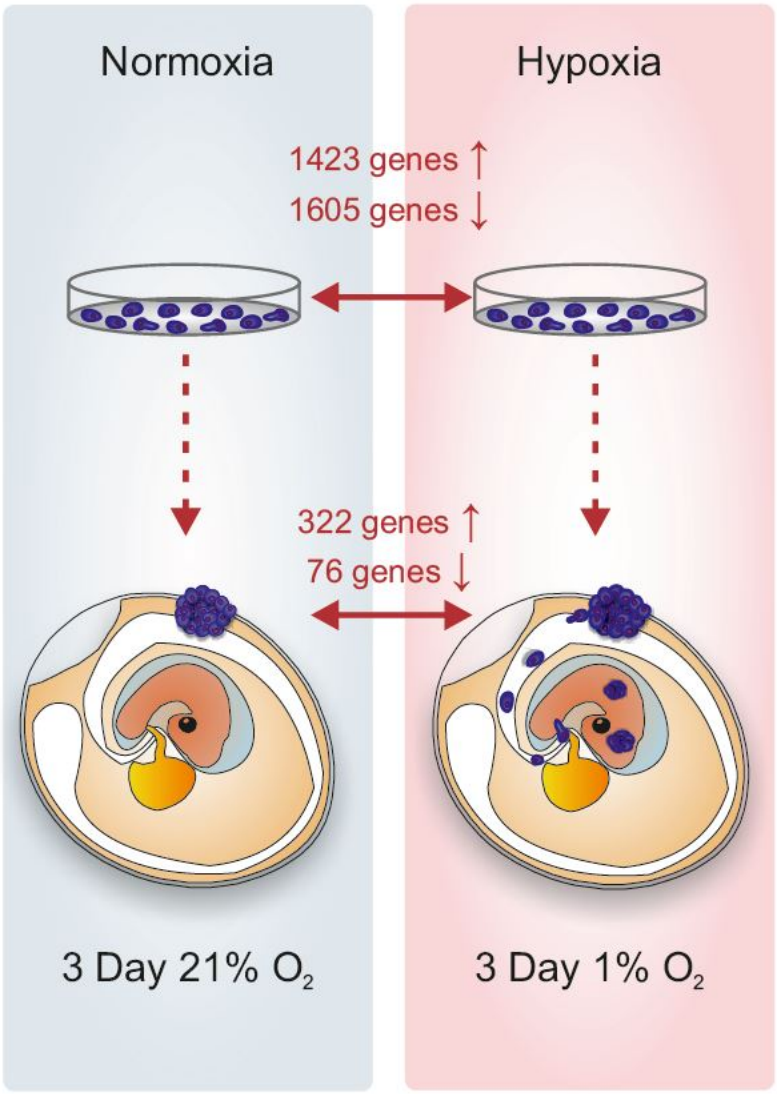
Results

We identified the significant ($p<0.01$) regulation of 398 genes in neuroblastoma tumours formed by hypoxic preconditioned cells. Out of these, only 93 genes were in common with the hypoxic gene signature observed in cells *in vitro*. To test the prognostic power of the genes identified by the hypoxic preconditioning experiment we performed a survival analysis using two independent human neuroblastoma gene expression studies (TARGET and SEQC). We discovered that 59 out of the 398 genes were putative predictors of survival. Upon series of selection criteria to search for

genes with prognostic power, two genes matched the strictest filtering: ZDHHC23 and UCP3. We confirmed their regulation at protein level in tumours formed by hypoxic cells, making them potential new biomarkers for use in a diagnostic worksflow. In addition, through computational analysis, we identified a number of drugs that are potentially capable of reverting the hypoxic gene signature.

Summary/Conclusions

We identified novel biomarkers that are indicative of poor prognosis in NB patients and could aid disease stratification especially in the non-MYCN amplified patient cohort. Our approach also identified specific drugs indicative of reversing the metastatic phenotype. Together this work paves the way for new neuroblastoma metastatic molecular markers and future therapeutic strategies for high-risk metastatic neuroblastoma patients.



Gene expression analysis of metastatic and non-metastatic neuroblastoma tumours grown in the chick embryo.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 43

Depletion of CD11b myeloid cells augments anti-neuroblastoma immune response induced by the anti-GD2 antibody dinutuximab beta

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

We recently showed CD11b+ myeloid cell-mediated upregulation of the PD-1/PD-L1 checkpoint on neuroblastoma (NB) cells treated with the chimeric anti-GD2 antibody dinutuximab beta and a blockade of this checkpoint increased anti-tumor efficacy of a GD2-directed treatment.

Aims

Here, we addressed whether depletion of CD11b+ immune suppressive cells augments immunotherapeutic effects of dinutuximab beta against NB.

Methods/Materials

Expression of CD11b+ myeloid cell-associated and modulating genes (*M-CSF, M-CSFR, GM-CSF, CCL2, TGF-β1, IL-1β, IL-4, IL-6, IL-6R, CXCL8, IL-10, VEGF-A, Arg1, IDO, NOS2* and *IFN-γ*) was analyzed using RT-PCR. Flow cytometry and immunohistochemistry were used to assess tumor infiltrating leukocytes. Anti-tumor effects of a GD2-directed treatment with dinutuximab beta (15 mg/kg, days 4-8 after tumor cell inoculation) in combination with depletion of CD11b+ myeloid cells by anti-CD11b Ab (25 mg/kg, days 4, 7, 11, 14 and 18) or 5-Fluorouracil (5-FU; 50 mg/kg, days 4, 11 and 18) were evaluated in a murine syngeneic NB model.

Results

53% of all leukocytes found in tumor tissue were CD11b+. Analysis of mRNA expression in NB cells used for tumor induction showed high expression of *M-CSF, TGF-β1, IL-6, and VEGF-A* genes that were further induced in primary tumor tissue. Although analysis of tumors revealed strong mRNA expression of all genes analyzed, the strongest induction was observed for *M-CSFR, CCL2, IL-1β, IL-4, IL-6R, CXCL8, Arg1 and NOS2*. Compared to controls, application of

anti-CD11b antibodies resulted in reduction of both tumor infiltrating CD11b+ cells and mRNA expression of the genes analyzed. Additionally, CD11b blockade showed delayed tumor growth and prolonged overall survival that could be further improved by either dinutuximab beta or 5-FU treatment. Importantly, the combinatorial immunotherapy with dinutuximab beta and 5-FU showed the strongest anti-tumor effects and superior survival rates.

Summary/Conclusions

Depletion of immune suppressive myeloid cells augments anti-neuroblastoma efficacy of a dinutuximab beta-based immunotherapy representing a new effective treatment strategy against GD2-positive cancers.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 44

Clinical significance of PHOX2B gene expression for predicting metastasis and prognostic evaluation in newly diagnosed neuroblastoma patients

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Paired-like homeobox 2B (PHOX2B) is specifically expressed in nervous system including neuroblastoma cells, but little is known about the clinical significance of the expression of *PHOX2B* in bone marrow (BM) and peripheral blood (PB) samples of neuroblastoma patients.

Aims

The aim of this study was to test the clinical significance of PHOX2B gene expression in newly diagnosed neuroblastoma patients.

Methods/Materials

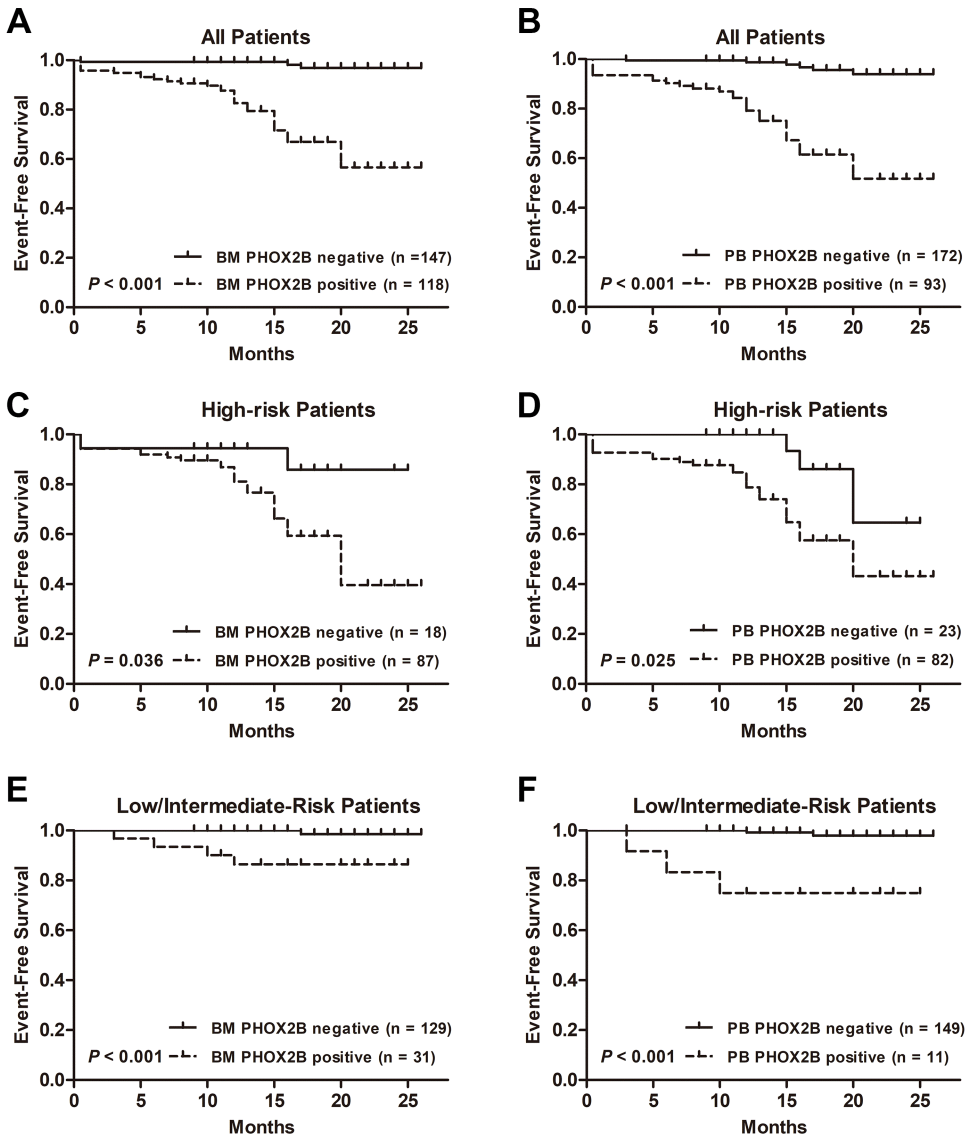
The expression of *PHOX2B* in samples of BM and PB was tested by quantitative reverse transcriptase polymerase chain reaction (RT-PCR). The predicting metastasis significance of *PHOX2B* was established through receiver operating characteristic (ROC) analysis. The prognostic significance of BM and PB involvement at diagnosis was assessed by event free survival (EFS).

Results

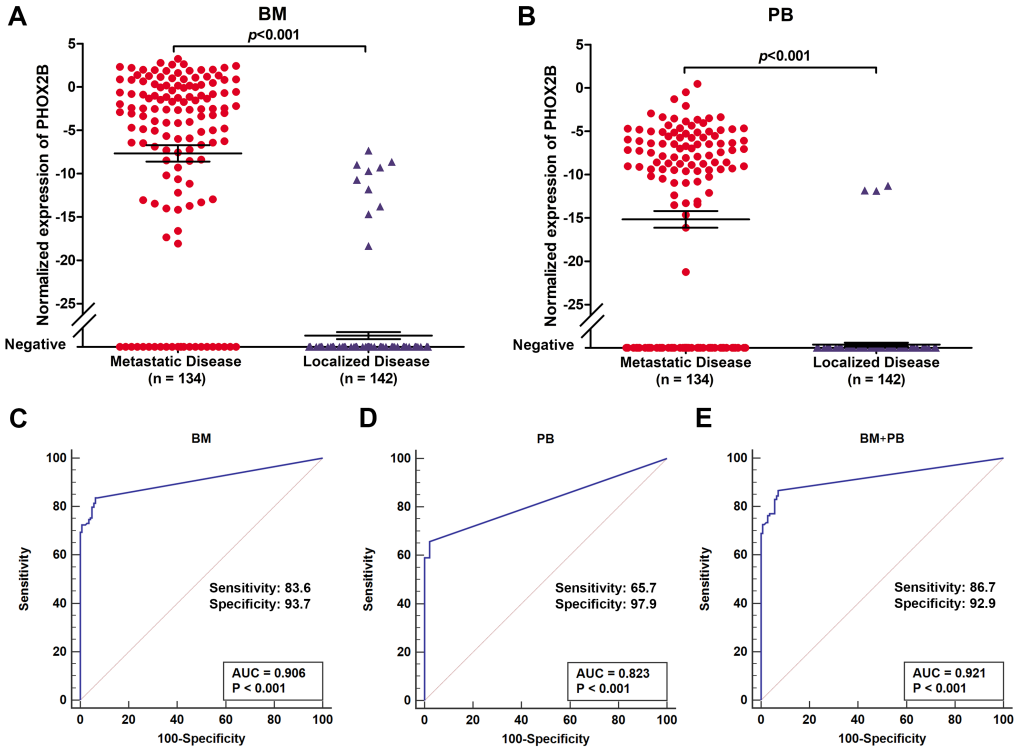
We identified the combined expression of *PHOX2B* in both BM and PB provided a high diagnostic accuracy for metastasis of neuroblastoma (AUC = 0.920) with the sensitivity and specificity of 86.7% and 92.9%, respectively. At last, 265 children were enrolled for prognostic analysis. The median follow-up time was 16 months. Positive expressions of *PHOX2B* in BM and PB at diagnosis were associated with worse EFS in patients with neuroblastoma ($P < 0.05$). What's worse, 19.4% (31/160) and 6.9% (11/160) patients with positive expression of *PHOX2B* in BM and PB at diagnosis in low/intermediate-risk group with favorable prognosis also had worse EFS ($P < 0.05$).

Summary/Conclusions

The expressions of *PHOX2B* in BM and PB were high in patients with unfavorable clinically characteristics. *PHOX2B* could be an appropriate biomarker for predicting metastasis in neuroblastoma patients. The expressions of *PHOX2B* in BM and PB at diagnosis had adverse prognostic significance in neuroblastoma patients as well as in patients with low-/intermediate-risk group.



The expression of PHOX2B in BM and PB samples for predicting metastasis of neuroblastoma



Event-free survival of positive and negative expression of PHOX2B in BM and PB of neuroblastoma patients

Type Clinical

Presentation Preference Traditional poster

Abstract nr. 45

The Emergence of a Novel Scientific Society: Dynamics of Collaboration between Scientists at the Neuroblastoma Research Congresses between 1975 and 2016

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Topic Other

Background/Introduction

This study investigates how collaborations among scientists in the field of neuroblastoma research developed throughout 41 years. Based on abstracts submitted to the ANR conference series we constructed a network of collaboration by inferring co-author relationships between scientists appearing together on an abstract.

Aims

Our aim was to quantitatively describe the evolution of scientific collaboration, to analyze how network position relates to academic success, and to identify structural tendencies in the formation and maintenance of co-author ties.

Methods/Materials

The data set was collected from the published abstracts of the Advances of Neuroblastoma (ANR) congresses from 1975-2016. Based on the co-authors' collaboration network, degree centrality (local connections), closeness (global connections), and betweenness centrality (brokerage) were calculated and related to the neuroblastoma-specific Hirsch index (h-index, Web of Science). In addition, stochastic actor-oriented models (SAOMs) were used to uncover how collaboration in neuroblastoma research changed its structure as the society matured. SAOMs are multivariate network models capable of controlling for individual attributes in modeling the development of networks and rely on a simulation of decisions to start, end or maintain ties.

Results

The data set comprised 18 conferences with 8459 authors affiliated at 553 institutions in 53 countries. 74% of authors and 39% of institutions contributed at one congress only. 3993 abstracts were presented in total. Centralization in the distribution of co-author ties increased steadily throughout time (skewness of the degree centrality distribution rose from 0.99 (1975) to 3.69

(2016)). This finding suggests that a small group of researchers attracted many co-authors especially at the later ANR conferences. A high number of presentations and of co-authors (measured by fractionalized counting (weight) and degree centrality) were strongly associated with a high h-index as measure of scientific productivity (R^2 = 0.51 and 0.47, resp.). In addition, the application of SAOMs showed that the number of co-authors a scholar had was already influential for the accumulation of additional co-author ties in an early stage of the field's development. This Matthew effect was statistically significant from the conference held from 1996 onward.
As more authors and institutions entered the field, we observed that scholars who often occurred on abstracts in last author positions attracted more co-authors than researchers who occupied mostly first or middle positions. This parameter for a status effect was observed first in 2008 (P < .00001). Moreover, we found that collaborations were more strongly shaped by status signals among authors within the same institution compared to authors who were not affiliated at the same institution (interaction between same institution and status effect, P=0.006). In summary, our results indicate the emergence of a global, densely connected core of authors who were highly productive and managed to attract many co-authors primarily from their home institutions.

Summary/Conclusions

The 41 year long history of ANR congresses (1975-2016) allowed us to study the emergence of a scientific society. Our data set quantified the growth of neuroblastoma research and indicated that different social processes were at work in earlier compared to later periods of the society.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 46

Enhancement of anti-tumor immunity by T cells engineered to restore HLA expression in neuroblastoma

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

T cells can target cancer cells by recognizing tumor-specific peptides presented by HLA proteins. HLA downregulation is one of the most common neuroblastoma (NB) immune escape mechanisms, and it is associated with an adverse prognosis. Interferon-gamma (IFNγ) has been shown to rescue HLA expression on NB cells; however, no strategies exist for the targeted delivery of IFNγ to upregulate HLA on tumor cells.

Aims

To present a novel approach to induce HLA expression in NB cells using targeted cellular therapy.

Methods/Materials

We genetically engineered GD2-specific T cells expressing synthetic-Notch (synNotch) receptors. In our receptors, an internal Notch domain is cleaved upon binding of the target antigen GD2, which releases a transcription factor driving expression of IFNγ (IFNγ-synNotch). We analyzed co-cultures of IFNγ-synNotch T cells and human NB cell lines by flow cytometry, enzyme-linked immunosorbent assay, enzyme-linked immunospot assay, and luminescence-based cytotoxicity assays.

Results

Treatment with recombinant IFNγ led to a strong induction of HLA expression in 6/6 NB cell lines. Next, we generated 13 synNotch receptors targeting NB-specific antigen GD2, and one CD19-specific synNotch receptor. One of the 13 GD2-targeting synNotch receptors showed strong surface expression and no basal IFNγ expression. T cells expressing this construct (GD2-IFNγ-synNotch T cells) produced IFNγ in the presence of GD2-positive NB cells and not GD2-negative cells. In vitro, GD2-IFNγ-synNotch T cells efficiently upregulated HLA on NB cell lines SK-N-DZ and NB-1643. HLA upregulation was maintained for seven days after removing GD2-IFNγ-

synNotch T cells from the co-culture. In contrast, treatment with GD2-IFNγ-synNotch T cells did not increase PD-L1 on NB cells or PD-1 on T cells. In vivo, NOD.Cg-PrkdcscidIl2rgtm1Wjl/SzJ (NSG) mice were engrafted with the human NB cell line Kelly. Once tumors reached a diameter of 5 mm, GD2-IFNγ-synNotch T cells were injected intratumorally. Three days after treatment, mice were euthanized, and their tumors were harvested and analyzed by immunohistochemistry for HLA induction. Mice treated with GD2-IFNγ-synNotch T cells, but not CD19-IFNγ-synNotch T cells, showed HLA upregulation in their tumors. Finally, GD2-IFNγ-synNotch T cells significantly enhanced the killing of HLA-A*02+ NB cell lines by T cell receptor (TCR) transgenic T cells targeting the tumor antigens NY-ESO-1 and PRAME.

Summary/Conclusions

Induction of HLA on tumor cells using engineered IFNγ-synNotch T cells is feasible and increases T cell-mediated tumor cell killing. Treatment with GD2-IFNγ-synNotch T cells alone or in combination with TCR-transgenic T cells represents a promising approach to improve anti-tumor immunity in solid tumors.

Type Translational

Presentation Preference Traditional poster

Abstract nr. 47

Extrachromosomal circular DNA drives oncogenic genome remodeling in neuroblastoma

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Extrachromosomal circularization of DNA is an important genomic feature in cancer. Neuroblastoma is one of the first tumor entities in which extrachromosomal oncogene circularization in the form of *MYCN* proto-oncogene double minute chromosomes was detected. Since the first descriptions in 1965, the structure, composition and genome-wide frequency of extrachromosomal circular DNA have not been extensively profiled.

Aims

We hypothesized that extrachromosomal circular DNA could represent a genome-wide, driving mutagenic process in neuroblastoma with functional consequences beyond oncogene amplification. We set out to systematically describe the spectrum and impact of extrachromosomal circular DNA in neuroblastoma using different genomic and transcriptomic approaches.

Methods/Materials

We combined methods for circular DNA enrichment (Circle-seq) with short- and long-read genomic (DNA sequencing) and transcriptomic approaches (RNA sequencing) to describe the landscape of extrachromosomal circular DNA in a cohort of 93 primary neuroblastomas. Since DNA circularity can be computationally inferred, we applied an algorithm utilizing paired-end read orientation to detect circularity. Additionally, five established algorithms for the detection of structural variants were applied to whole genome sequencing data. This enabled to distinguish intra- from extrachromosomal structural variants and uncover the contribution of extrachromosomal circular DNA to genome remodeling in neuroblastoma.

Results

Our analysis identifies and characterizes a wide catalog of somatically acquired and previously undescribed extrachromosomal circular DNAs. Moreover, using assembly-based reconstruction we find that extrachromosomal circular DNAs are an unanticipated major source of somatic rearrangements, contributing to oncogenic remodeling through chimeric circularization and reintegration of circular DNA into the linear genome. Cancer-causing lesions can emerge out of circle-derived rearrangements leading to aberrant fusion transcript expression, proto-oncogene amplification and enhancer hijacking. Importantly, such lesions are associated with adverse clinical outcome.

Summary/Conclusions

Our work provides a comprehensive map of extrachromosomal DNA circularization in neuroblastoma, revealing this mutagenic process to be more frequent than previously anticipated. We demonstrate that the majority of genomic rearrangements in neuroblastoma involve extrachromosomal circular DNA, challenging our current understanding about cancer genome remodeling. Thus, extrachromosomal circular DNAs represent a multi-hit mutagenic process, with important functional and clinical implications for the origins of genomic remodeling in cancer.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 48

Whole genome sequencing of recurrent neuroblastoma reveals somatic mutations that affect key players in cancer progression and telomere maintenance

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Despite recent improvements in multimodal treatment, survival for children diagnosed with high-risk neuroblastoma (NB) remains below 50% at 5 years from treatment. Although an initial response to treatment is seen in a majority of patients, a significant portion will subsequently relapse with lesions resistant to standard therapy, and for NB patients with relapsed or refractory metastatic disease, survival is less than 10% despite recent advances in treatment regimen.

Aims

The aim of this study is to investigate and compare molecular and genetic aberrations in relapsed NB tumors with tumor material from time of diagnosis in order to find mechanisms associated to refractory disease.

Methods/Materials

In order to study genomic alterations seen in association with tumor relapse and therapy resistance, whole genome sequencing was performed on diagnostic and relapsed/refractory lesions together with constitutional DNA from seven neuroblastoma patients. Sequencing was performed using Illumina instrumentation for an average coverage of at least 60X and 30X for tumor samples and normal tissue respectively. Mapping and variant calling was done using TNscope from Sentieon, copy number calling through the Canvas tool while structural variants were called using the software Manta.

Results

Sequencing of the patients with relapse show somatic alterations in a diverse set of genes that includes genes involved in RAS-MAPK signaling, that promote cell cycle progression or function in telomere maintenance. Among recurrent alterations *CCND1*-gain, *TERT* rearrangements and point mutations in *POLR2A*, *CDK5RAP* and *MUC16* was seen in at least two individuals. In our cohort we also find examples of converging genomic alterations in primary-relapse tumor pair indicating dependencies related to specific genetic lesions. Furthermore, we also detect rare genetic germline variants in DNA repair genes, e.g. *BARD1*, *BRCA2*, *CHEK2* and *WRN*, which might cooperate with somatically acquired variants in this patient group with highly aggressive recurrent neuroblastoma. The number of somatic mutations at time of diagnosis respective time of relapse/progression shows correlation with the patients´ age at time of sampling although one patient appears to deviate with a pronounced increase in mutation rate as compared to the other samples.

Summary/Conclusions

In this study of recurrent NB we identify alterations that affects genes promoting cell cycle progression, activating ALK-RAS-MAPK signaling or that is important for telomere maintenance and immortalization. Our findings in combination with previous studies of relapsed NB provide a rational for that comprehensive genomic characterization of recurrent neuroblastoma is required in order to optimize treatment and prolonged survival. This could be used as an incitement for novel combination therapies with compounds abrogating MAPK signaling, cell cycle progression and telomere activity.

Type Basic

Presentation Preference Traditional poster

Abstract nr. 49

Enhancer hijacking determines intra- and extrachromosomal circular MYCN amplicon architecture in neuroblastoma

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Topic Gene regulatory networks and epigenetics: from basic science to therapy

Background/Introduction

MYCN amplification drives one in six cases of neuroblastoma. The additional *MYCN* copies are found extrachromosomally as circular double minute chromosomes and intrachromosomally as homogeneously staining regions. Amplicons often comprise several parts of the genome and are internally rearranged. In how far this structural complexity plays a functional role in *MYCN*-amplified neuroblastoma is unknown.

Aims

Here, we characterized *MYCN*-driving enhancers in the context of intra- and extrachromosomal *MYCN* amplification. We hypothesized that *MYCN* amplicon contents and structure are largely shaped by the selective pressure to retain specific non-coding elements.

Methods/Materials

We integrated H3K27ac and transcription factor ChIP-seq data for 25 neuroblastoma cell lines with copy number data for 240 *MYCN*-amplified primary neuroblastomas to inspect for significantly co-amplified coding and non-coding regions. The exact structure of the *MYCN* amplicon was investigated in four neuroblastoma cell lines using Illumina whole-genome sequencing followed by structural variant analysis and Nanopore long-read sequencing followed by *de novo* assembly. In the same cell lines, we characterized the chromatin landscape (ATAC-seq, H3K27ac/H3K4me1/CTCF ChIP-seq) and spatial chromatin conformation (4C-seq, Hi-C) of the

MYCN amplicon.

Results

We identified five putative enhancers (e1-e5) that were specific for *MYCN* expression in non-MYCN-amplified neuroblastoma cell lines based on H3K27ac histone marks. ChIP-seq analysis showed that four of these enhancers were likely driven by noradrenergic neuroblastoma core regulatory circuit factors (PHOX2B, HAND2, GATA3).

Copy-number analysis for 240 *MYCN*-amplified primary neuroblastomas revealed a strikingly asymmetric distribution of *MYCN* amplicon boundaries which can be explained by a selective pressure to co-amplify the core regulatory circuit-driven enhancer e4 in 90% of cases, much more often than expected by chance (empirical *P*=0.0003). In two *MYCN*-amplified cell lines, e4 presents as a functional enhancer as determined by ATAC-seq, ChIP-seq and 4C-seq analysis. 10% of neuroblastomas did not co-amplify the key local enhancer e4. Intriguingly, these samples were more likely to harbor complex amplicons consisting of more than one fragment (66.7% vs. 35.7%, *P*=0.003). This could be indicative of a selective pressure to incorporate distal regulatory elements compensating for e4 loss in these cases.

Therefore, we inspected two cell lines that did not co-amplify e4. Nanopore long read-based *de novo* assembly of the amplicon and short-read based structural variant analysis independently demonstrated that the amplicon is complex and consists of several distal parts of chromosome 2. In both cases, core regulatory circuit-driven super-enhancers from distal parts of the genome were juxtaposed next to *MYCN* by structural rearrangements on the amplicon.

Mapping Hi-C reads to the reconstructed amplicon sequence demonstrated the existence regulatory loops and insulation on the amplicon, irrespective of intrachromosomal or extrachromosomal location. Neo-TAD formation in both cases enabled *MYCN* regulation by the juxtaposed super-enhancers.

Summary/Conclusions

We demonstrated different reconstruction methods for complex parts of cancer genomes and their integration with epigenomic data. This showed that patterns of co-amplification on the *MYCN* amplicon are in large parts shaped by a selective force to retain core regulatory circuit driven enhancers on the amplicon.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 50

In vitro 3D bioprinted neuroblastoma: A tunable platform to assess extracellular matrix impact on growth and phenotypic cell behavior

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

The composition of extracellular matrix (ECM) and its structural and mechanical properties can control cancer cell behavior, developing a tumor microenvironment that promotes cell migration and invasion. We have previously described in human biopsies that ECM stiffness is related to poor outcome in neuroblastoma (NB) patients. 3D bioprinted in vitro models can imitate in a simplified and controlled way the ECM stiffness, which makes them a useful tool for the study of ECM-dependent tumor cell behavior.

Aims

This work aims to study the effect of ECM stiffness over the time on several parameters which characterize cell tumor behavior using different bioprinted 3D NB models.

Methods/Materials

Hydrogels were made of 5% of methacrylated gelatin and different percentages of methacrylated alginate (from 0% to 2%). Cell populations were incorporated during hydrogel fabrication and were together bioprinted applying air pressure extrusion system with 3DDiscovery BioSafety printer. Four types of matrices were generated regarding cell composition, differentiated by the incorporation of SK-N-BE(2) or SH-SY5Y neuroblastic cells and the presence (in a proportion of 10%) or absence of SW10 Schwann cells. The 3D bioprinted hydrogels were cultured from 2 to 6 weeks. Models were paraffin-embedded, sectioned, and stained according to the parameter to be

analyzed. Hydrogel porosity, cell cluster density, cell cluster occupation, cell nucleus size and staining intensity, cell proliferation (anti-Ki67 antibody), mRNA activity (anti-PTBP1 antibody) and vitronectin expression (anti-VN antibody) were quantitatively analyzed in digitalized hydrogel stained sections applying Pannoramic Viewer software modules. Mitosis Kariorrhexis Index (MKI), stem cell markers (anti-SOX2 and anti-OCT4 antibodies), antiapoptosis (anti-BCL2 antibody) and apoptosis (anti-BAX antibody) were qualitatively analyzed by the pathologist and two researchers. All analyses were based on the presence, distribution and intensity of the staining and differences were related to the different time and stiffness conditions.

Results

SK-N-BE(2) cells grew in all hydrogel conditions while SH-SY5Y presented some difficulties. The patterns of growth and behavior presented differences with time and stiffness in both cell lines with or without SW10 cells. Some parameters remained with less variability (cell cluster occupation, mRNA activity, MKI, cell nucleus size and staining intensity), but others, and depending on the analyzed model, inverted their tendency with SW10 cells incorporation, leading to a less aggressive cell behavior.

Summary/Conclusions

Tunable in vitro 3D bioprinted cell culture scaffolds can replicate the NB pathophysiology, which allows to achieve deeper knowledge of cell behavior related to the tumor microenvironment.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 51

Short-term Efficacy Of Autologous Stem Cell Transplantation With Busulfan And Melphalan Regimen In The Treatment Of High-risk Neuroblastoma

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Topic Neuroblastoma as developmental disease

Background/Introduction

To investigate the clinical outcome of children with high-risk neuroblastoma and the prognostic function of Busulfan and melphalan as high-dose chemotherapy for high-risk neuroblastoma.

Aims

Methods/Materials

The clinical and follow-up data of patients who suffered from high-risk neuroblastoma admitted from January 2014 to December 2018 were retrospectively reviewed. The survival rate was calculated and Cox proportional hazard model was used to determine the clinical and pathological factors ,especially the factors of Autologous peripheral blood stem cell transplantation (APBST) on overall survival(OS) of high-risk neuroblastoma .

Results

68 case was included ,among them 38 case received Busulfan and melphalan as high-dose chemotherapy and
APBST. The Kaplan-Meier curve revealed regular therapy significantly improve the prognosis of high-risk neuroblastomaThe 3-year survival rate was 65.6%. Busulfan and melphalan as high-dose chemotherapy for high-risk neuroblastoma improved event-free survival. 3-year event-free survival 67.8%vs 39.8%P<0.05. 3-year overall survival 82.1%vs 42.1%P<0.05.

Summary/Conclusions

Busulfan and melphalan improved event-free survival in children with high-risk neuroblastoma and caused fewer severe adverse events. Busulfan and melphalan should thus be considered standard high-dose chemotherapy and ongoing randomized studies will continue to aim to

optimize treatment for high-risk.

Type Clinical

Presentation Preference Traditional poster

Abstract nr. 52

Biological and clinical significances of DNA methylation profiles in aggressive neuroblastomas and their application to treatments targeting tumor metabolism

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

It is widely accepted that since somatically acquired gene mutations are relatively less frequent, other mechanisms such as epigenetic alterations and/or metabolomic changes would play important roles in tumorigenesis as well as progression of neuroblastoma. Previously, DNA methylation profiling has been reported to have a relation with MYCN amplifications, but epigenetic mechanisms involved in neuroblastoma biology and clinical behavior have yet to be fully elucidated. Except for association with MYCN amplification and oxidative and glycolytic metabolisms, the knowledge of metabolomic alterations is very limited in neuroblastoma.

Aims

We aimed to elucidate DNA methylation characteristics and metabolomic alterations, which involve in aggressive phenotype of neuroblastoma and get better insight into molecular basis of neuroblastoma. We also tried to identify novel therapeutic targets for intractable neuroblastoma.

Methods/Materials

We conducted DNA methylation array analysis, RNA sequencing and target capture sequencing on 34 advanced neuroblastoma specimens. Publicly available data including TARGET were used for validation. For functional evaluation, siRNA-mediated gene silencing analysis and metabolomic screening in neuroblastoma-derived cell lines were also performed.

Results

Based on the DNA methylation profiles, we obtained two distinct methylation clusters among the cases with 11q loss of heterozygosity (11qLOH), cluster A and cluster B. These clusters were well correlate with genetic alterations and clinical outcomes. Cluster A was characterized by frequent 4p deletion and ATRX alterations, while these alterations were less common in cluster B. Cluster B was associated with significantly poor prognosis. Because metabolomic alterations were thought to be correlated with aggressive phenotype and drug resistance in many cancers, we explored metabolomic targets in cluster B showing dismal prognosis. In comparison to cluster A, much higher expression of serine biosynthesis related gene, PHGDH, was detected in cluster B. Intriguingly, this upregulation was also found in all cases with MYCN amplifications. Our methylation clustering and gene expression pattern of PHGDH were confirmed in available open data with a large number of cases. Inhibition of PHGDH with siRNA or inhibitors resulted in significant decrease in cell proliferation of neuroblastoma cells. On the other hand, we also observed a similar expression pattern of ASS1, an arginine metabolism regulator, to PHGDH, and both inhibition of PHGDH and arginine metabolism in ASS1-defective neuroblastoma cells with low PHGDH expression showed synergistic cytotoxic effect. In RNA sequencing, SLC7A11, a membrane transplantation of amino acids and NQO1, a polyamine metabolism related gene, were upregulated by treatment of PHGDH inhibitor alone or combination of PHGDH and arginine metabolism inhibitors, suggesting that these genes would be also promising targets. Furthermore, metabolite set enrichment analysis showed that inhibition of PHGDH upregulated several pathways including Warburg effect, and inhibition of arginine metabolism upregulated glycine and serine metabolism. Integrated network analysis visualized the complex effects of inhibition of arginine metabolism towards genes and metabolites which affect serine and glycine metabolism.

Summary/Conclusions

Our results demonstrate a unique methylation subgroup with characteristic genetic features and very dismal prognosis and provide important clues for development of serine/arginine metabolisms targeting therapies in aggressive neuroblastomas.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 53

Casein Kinase II (CK2) as novel therapeutic target in Neuroblastoma

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Casein kinase II (CK2) is a constitutively active, ubiquitously present serine/threonine kinase that plays a critical role in multiple normal cellular processes. CK2 overexpression has been demonstrated in many types of cancers including neuroblastoma. CK2 promotes tumorigenesis and tumor cell survival via several pathways including phosphorylation of transcription factors such as P53, PTEN and Ikaros. CX-4945 (Silmitasertib), is an orally bioavailable, ATP-competitive CK2 inhibitor that show anti-tumor activity in several cancers including leukemia and neuroblastoma. CX-4945 is currently being tested in Phase I/II clinical trial setting for adult solid tumors and pediatric brain tumors.

Aims

Validate CK2 as therapeutic target in neuroblastoma and establish the efficacy of CK2 inhibitor CX-4945 in neuroblastoma preclinical models.

Methods/Materials

Oncomine NBL database was used to analyze correlation between CSNK2A1 gene expression and clinical outcome. Neuroblastoma cell lines SH-SY-5Y, SK-N-RA and SMS-KCNR were used for in vitro experiments. CK2 retroviral transduction of NBL cell lines was used to overexpress CK2. CK2 ShRNA was used for molecular inhibition and CX-4945 for pharmacological inhibition of CK2. RNA sequencing of CK2 altered neuroblastoma cells were done and gene expression changes and pathway analysis were done using Ingenuity pathway analysis software. Athymic nude mice were subcutaneously injected with NBL cells to establish preclinical models. CX- 4945 was purchased from vendor was used at dose of 150mg/kg oral gavage once daily for 14 days. Serial tumor measurement was obtained to compare tumor growth inhibition.

Results

We analyzed several publically available neuroblastoma gene expression data sets which showed that CSNK2A1 expression is positively correlated with older age, increased mortality, high INSS stage and MYCN amplification. In addition, CSNK2A1 mRNA expression is significantly positively correlated to MYCN amplification status, high stage disease, tumor recurrence. In tumors without MYCN amplification, high CSNK2A1 expression is still prognostic for poor outcome in Kaplan-Meier analysis.

Overexpression of CK2 in Neuroblastoma cell lines (SK-N-RA, SH-SY-5Y, SMS-KCNR) via lentiviral transduction results in increase cell proliferation and decreased apoptosis (in vitro) and rapid tumor growth when subcutaneously implanted in athymic mice. Gene expression analysis of CK2 overexpressed NBL cell lines using RNA sequencing showed upregulation of several cancer related genes. NBL cell lines with molecular silencing of CK2 (CK2ShRNA) show decreased tumor growth. CX4945 showed cytotoxic activity in all NBL cell lines with IC50 ranging from 10-12uM. CX4945 is very well tolerated as single agent in mouse models as well as in humans (published data from Phase I study in adults). CX4945 showed significant tumor inhibition (50-60%) in mouse models of NBL cell lines (SH-SY-5Y and SMS-KCNR) at 150mg/kg/day PO given daily for 14 days. CX4945 also delayed tumor progression (tumor endpoint volume >2000mm^3) in treated mice.

Summary/Conclusions

CK2 overexpression in Neuroblastoma cases correlates with poor outcome and decreased survival independent of MYCN status. Inhibition of CK2 is potential therapeutic strategy in neuroblastoma. CX-4945 shows potent antitumor effect in NBL murine models. Further studies to establish mechanism of action of CK2 inhibitors in NBL are underway.

Type Translational

Presentation Preference Rapid Fire Poster Presentation

Abstract nr. 54

Functional analysis of natural antibody and NK cells against neuroblastoma

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Topic Other

Background/Introduction

The mechanism underlying the spontaneous regression of neuroblastoma is unclear. Although it was hypothesized that this regression occurs via an immunological mechanism, there is no clinical evidence, and no animal models have been developed to investigate the involvement of immune systems, especially natural antibodies and Natural Killer (NK) cells, against neuroblastoma.

Aims

We hypothesized that immunological systems correlate to spontaneous regression of neuroblastoma, and investigated immunological function of natural antibody and NK cells against neuroblastoma.

Methods/Materials

Mouse model: We performed an immunological analysis of homo- and heterozygous TH-MYCN transgenic mice in which MHC-Class1 is null expression. The tumor cells obtained from TH-MYCN mice were incubated with plasma obtained from TH-MYCN mice and stained by fluorescence-labeled anti-mouse IgG/IgM, then the fluorescence was detected by flow cytometry. To analyze the function of anti-tumor antibody, complement-dependent cytotoxicity (CDC) assay and antibody-dependent cellular cytotoxicity (ADCC) assay were conducted. Depletion experiments *in vivo* (NK, CD4, CD8 and B) were performed by administration of anti-asialo GM1 antibody, anti-CD4 antibody (clone GK1.5), anti-CD8 antibody (clone 53-6.72) and anti-CD20 antibody (clone 5D2), respectively.
Human model: Highly activated NK cells (GAIA-102) were generated as previously described

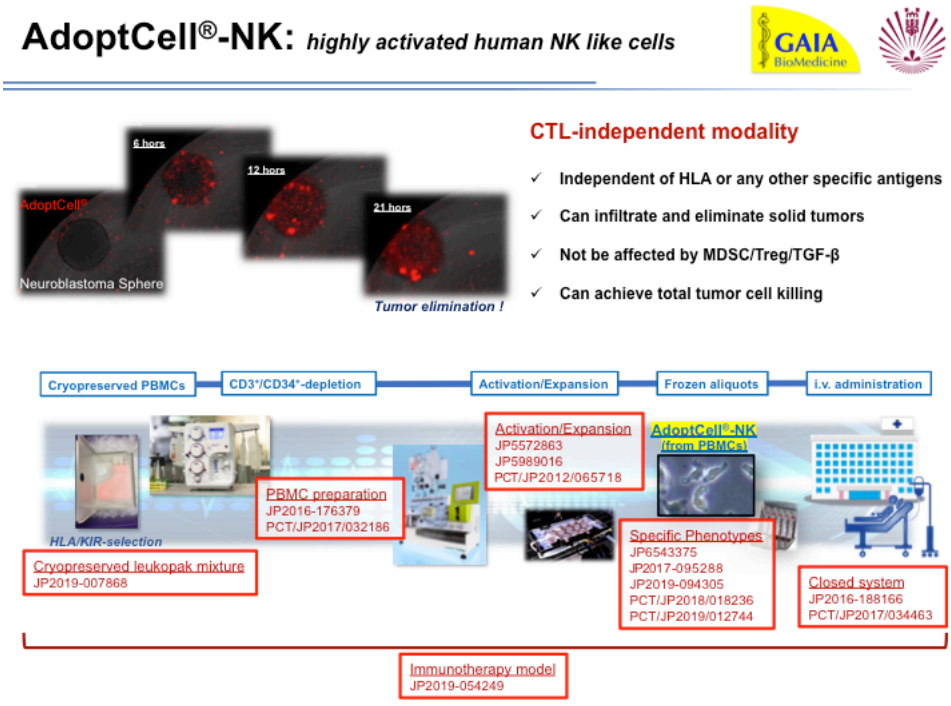
(Saito S, et al. *Hum Gene Ther* 2013, AdoptCell-NK Kit: Cat. no. 16030400). Briefly, CD3-depleted PBMCs were cultured under the condition of adhesion culture for 14 days. A spheroid of IMR32 (neuroblastoma cell line) was co-cultured with fluorescence-labeled GAIA-102, and invasion of the effector cells into spheroid was observed. Spheroids were then manually disrupted, and the number of live tumor cells were counted by flow cytometry.

Results

Mouse model: Mice with no or small (<5 mm) tumors showed higher antibody titers in plasma than mice with large (>5 mm) tumors. A significant negative correlation was observed between the tumor diameter and the titer of antitumor antibody. This antibody had CDC but not ADCC against neuroblastoma cells. Moreover, B-cell depletion had no effect on the tumor incidence *in vivo*. On the other hand, NK-cell depletion showed tendency of increase of tumor incidence. ***Human model:*** In 3D cytotoxicity assay, GAIA-102 efficiently killed IMR32 spheroid as compared to primary NK cells, anti-GD2 antibody or combination of both. Live imaging demonstrated GAIA-102 actively migrate, infiltrate and alter spheroid morphology subsequently mediate tumor cell killing in IMR32 spheroid. In contrast, primary NK cells could not invade into the spheroid.

Summary/Conclusions

We revealed that TH-MYCN transgenic mice have a natural antibody against neuroblastoma that correlate with tumor size. However, this antibody does not correlate with the spontaneous regression of neuroblastoma. Thus, the function of the natural antibody is limited. From our depletion study of NK cells and *in vitro* ADCC assay, it appears that NK cells may be involved in the spontaneous regression. In human model, GAIA-102 exhibits high cytotoxicity and invasion activity against neuroblastoma spheroid. GAIA-102 offers a highly promising approach for the treatment of solid, bulky lesion of advanced neuroblastoma.



Type Basic

Presentation Preference Oral presentation

Abstract nr. 55

Investigating chemoresistance mechanisms in neuroblastoma using a longitudinal cisplatin resistance cell mode

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

A significant challenge to anti-cancer therapy is the heterogeneity within tumours driven by genetically, transcriptionally and phenotypically divergent cancer cell populations. This intra-tumoural heterogeneity equips cancers with innate and/or acquired mechanisms of drug resistance, often among minor subclones. Recent studies have uncovered significant aspects of neuroblastoma heterogeneity that impact their response to drug treatment. Neuroblastoma consists of adrenergic and mesenchymal cells, the latter displaying higher resistance to chemotherapy. It has also been shown that neuroblastoma cells can acquire drug resistance through transcriptional changes leading to dysregulation of chromatin structure and downstream gene expression. These findings highlight the importance of understanding cell plasticity and the substantial variation in the types of molecular alterations, at a single cell resolution, that occur in response to anti-cancer therapy.

Aims

We aimed to assess the transcriptional heterogeneity within and between parental IMR-32 neuroblastoma cell line and its drug-resistant counterparts to identify transcriptional profiles that may explain mechanisms of cisplatin resistance.

Methods/Materials

To investigate heterogeneity and drivers of chemoresistance in neuroblastoma, we established a longitudinal drug resistance cell model using the IMR-32 cell line. Treatment-naive IMR-32 cells were pulsed with increasing concentrations of cisplatin, over 13 months. Three increasingly resistant cell lines were established at separate time points during this period. We used the 10X

Genomics Chromium^{TM} platform to profile the transcriptomes of 39,000 single cells across these four IMR-32 cell lines. We next examined gene expression signatures of individual cells to distinguish unique transcriptional states, cell clusters and reconstruct transcriptional trajectories. We then functionally validated candidate genes involved in cisplatin resistance using immunoflourescence, western blotting and cell viability assays.

Results

Through our single cell analysis, we identified three transcriptionally distinct clusters, each with varying abundances of cells from the four cell lines that provide evidence for depletion or enrichment of cell populations with increasing cisplatin resistance. Hence, we inferred that varying transcriptomically-defined phenotypes are involved in governing drug sensitivity or resistance. We further identified a transcriptionally dormant cluster composed predominantly of cells from the resistant cell lines suggesting that transcriptional inactivation could be a potential mechanism for drug resistance. Through trajectory analysis we observed strong transitions in the expression of cytoskeletal genes with increasing cisplatin exposure, especially of the microtubule destabiliser, stathmin-1 (STMN1). Immunocytochemistry of these targets revealed altered microtubule network structures in the resistant cells. We found that shRNA mediated knockdown of STMN1 sensitised cells to cisplatin treatment in a neuroblastoma cell line, SK-N-BE2.

Summary/Conclusions

Our work suggests that upon cisplatin exposure, specific transcriptionally-defined phenotypes of individual cells influence sensitivity or resistance in IMR-32 cells and that the parental cell line contains a transcriptionally dormant subpopulation which is enriched upon cisplatin treatment. Taken together we find that cisplatin treated neuroblastoma cells become resistant using complex and multifaceted mechanisms involving transcriptomic silencing and cytoskeletal adaptation.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 56

MYCN Amplification Predicts Sensitivity to PC-002 in Neuroblastoma

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

Oncogene MYCN amplification is the most important negative prognostic factor in neuroblastoma (NB). Survival for high-risk patients who are resistant to conventional chemotherapy remains below 40%. Therefore novel therapeutic strategies are urgently needed. PC-002 is a novel small-molecule agent with broad antitumor activity in vitro and in vivo. In this study we investigated the efficacy and the mechanism of PC-002 as an antitumor drug in MYCN-driven neuroblastoma.

Aims

Methods/Materials

Results

We performed EdU incorporation assay to assess cell proliferation 72h after PC-002 treatment. Four cell lines with MYCN amplification (MNA) were highly sensitive to PC-002 with IC50 ranging from 0.62nM to 11.7nM, whereas two cell lines without MYCN amplification (non-MNA) showed much higher IC50 values (59.06nM and >220nM). Apoptosis was triggered in MNA cell line with PC-002 treatment, while non-MNA cells did not show a specific response. PC-002 also caused cell

regrowth inhibition and DNA damage in MNA cells. We further demonstrated that PC-002 significantly downregulated MYCN protein through reducing protein half-life without causing transcriptional repression in MNA cells. USP7 (ubiquitin-specific protease) interacts with MYCN and induces deubiquitination and stabilization of MYCN. PC-002 inhibited USP7 debuiquitinase activity *in vitro* and disrupted MYCN-USP7 binding. In contrast, extremely low MYCN protein expression was detected in non-MNA cells, and MYCN protein level was not significantly affected by PC-002 treatment. The *in vivo* anticancer activity of PC-002 was assessed in neuroblastoma xenograft models. In MNA NB xenograft models, PC-002 significantly inhibited the tumor growth. In contrast, no tumor growth inhibition effect was observed in non-MNA models.

Summary/Conclusions

Taken together, our data suggested that PC-002 selectively inhibited tumor growth in MNA neuroblastoma via MYCN protein degradation. Therefore, future clinical evaluation of PC-002 in MNA neuroblastoma is warranted.

Type Translational

Presentation Preference Traditional poster

Abstract nr. 57

A phase IIa trial of molecular radiotherapy with 177-Lutetium DOTATATE in children with primary refractory or relapsed high-risk neuroblastoma

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

Current treatments for high-risk neuroblastoma are unsatisfactory, as more than half of patients will either respond poorly to induction chemotherapy, or relapse during or after therapy. Innovative treatments are required. Molecular radiotherapy with 177-Lutetium DOTATATE (LuDO) which targets somatostatin receptors significantly prolongs survival in adults with metastatic neuroendocrine cancers. Many neuroblastomas also express somatostatin receptors, and LuDO has shown promise in small pilot studies.

Aims

The objective of this Phase IIa, open label, single centre, single arm, two stage clinical trial was to evaluate the safety and activity of LuDO molecular radiotherapy in neuroblastoma.

Methods/Materials

Children with relapsed or refractory metastatic high-risk neuroblastoma were treated with up to four courses of LuDO. The sample size calculation was based on a Simon Two Stage Minimax design. The administered activity was 75 (first course) to 100 (subsequent courses) MBq kg^{-1} per course, spaced at 8 to 12 week intervals, planned not to exceed a cumulative renal radiation dose of 23 Gy. Outcomes were assessed by the International Neuroblastoma Response Criteria

(primary outcome), progression-free survival (PFS) and overall survival (OS).

Results

Forty three patients were screened for the trial, and 21 were recruited between September 2013 and July 2017. Ten were male, and 11 female. The median age was 5.4 years (range 1.7-16.9 years). Only one patient had MYCN amplified disease. Eight received the planned four courses, two received two courses and ten received only one course. There was dose limiting hematologic toxicity in one case, but no other significant hematologic or renal toxicity. The median cumulative renal radiation dose in the six patients who received four courses of treatment and for whom full dosimetric data were recorded was 16.5 (range 9.5 – 21.5) Gy. None of 14 evaluable patients had an objective response at one month after completion of treatment (Wilson 95% CI: is 0.0, 0.22). The trial did not therefore proceed to the second stage. Three patients had a minor reduction in the semi-quantitative 123-I mIBG scan score after two courses. Eighteen patients have died from their disease, the median follow up for survivors is 17.3 months (13.1 to 37.4). The median PFS was 2.96 months (95% CI: 1.71, 7.66), and the median OS was 13.0 months (95% CI: 3.0, 21.5).

Summary/Conclusions

In the absence of any objective responses, the use of LuDO as a single agent at the dose schedule used in this study is not recommended for the treatment of neuroblastoma. There are several reasons why this treatment schedule may not have resulted in objective responses, for example suboptimal dosing and too long between courses. As other studies do show responses, the treatment should not be regarded as being of no value. Further trials designed to overcome this schedule’s limitations are planned with international collaboration.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 58

COX2 expression is increased in relapsed Neuroblastoma samples with 11q deletion and non-amplified MYCN

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Topic Gene regulatory networks and epigenetics: from basic science to therapy

Background/Introduction

Despite still challenging, important prognostic genetic markers have been associated with the low survival rates in high-risk neuroblastoma. Additionally to the genetic background, the tumor microenvironment inflammatory state plays a role in the modulation of tumor progression. The presence of pro-inflammatory markers was related to the metastatic phenotypes, and more recently, it was suggested that losses in the 11q chromosome could define a subset of therapy-resistant neuroblastoma. At cellular and molecular level, this subset was characterized to be inflammatory driven and with high activity of the COX/mPGES-1/PGE2 pathway.

Aims

This study aimed to evaluate the expression of COX-2 in a set of tumor samples collected in diagnosis and post chemotherapic treatment.

Methods/Materials

Neuroblastic specimens were obtained retrospectively for the years 2001–2014 from the archives of the Division of Anatomical Pathology, Pequeno Príncipe Pediatric Hospital (Brazil) as formalin-fixed paraffin-embedded (FFPE) blocks. A subgroup of 82 specimens were collected at diagnosis prior to any cancer treatment (Pre-CT) and 20 were collected after chemotherapy (Post-CT), out of which 11 cases were paired with pre and post-CT samples. Immunohistochemical expression of COX-2 was evaluated through quantitative analyses of cytoplasmic staining. Copy number alterations (CNA) were analyzed by array-CGH in paired samples of patients (Pre-CT and post-CT). *MYCN* oncogene content was assayed by fluorescence in situ hybridization (FISH). The genes from 11q23 deletion chromosomal locus were extracted using RefSeq gene model as

provided by the a-CGH analysis. Next, the genes were subjected to protein-protein interaction analysis using String database.

Results

COX-2 expression was positive in all NB tumorigenic subgroups. Spearman multivariate analysis showed an anti-correlation between COX-2 expression and *MYCN* amplification (ρ =-0.693, p<0.05). A significant increase in COX-2 expression was verified in post-CT samples when compared with pre-CT unpaired samples (n = 77, p< 0.05) in non-amplified *MYCN*. A-CGH results showed no significant differences in the median of copy number alterations in Pre-CT (15.36 ± 3.2) and Post-CT (12.18 ± 2.4) samples. COX-2 expression in pre-CT samples correlates with 11q23 loss (ρ = 0.631, p = 0.045), 7q gain (ρ = 0.853; p = 0.002), and 7p gain (ρ = 0.798; p = 0.006). Other significant correlations were found for 11q loss with 1q gain (ρ = 0.694; p = 0.038), 7p gain (ρ=0.908; p<0.001), 7q gain (ρ=0.771; p = 0.005), and 9q gain (ρ=0.654; p=0.029). Analysis of the genes lost in the 11q23 chromosomal locus demonstrated a strong and positive involvement of MMPs-related genes that interact or are involved in the collagen formation, including MMP7, MMP10, MMP13, MMP1 and MMP3.

Summary/Conclusions

COX-2 expression is increased in post-treated samples in NB tumors without *MYCN* amplification. It also correlates with chromosome 11q deletions, with strong and positive involvement of MMPs-related genes involved in the extracellular matrix remodeling.

Type Basic

Presentation Preference Traditional poster

Abstract nr. 59

Results of the NB-2015 protocol for treating neuroblastoma in China: experience from multiple centers

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Topic Other

Background/Introduction

Risk-based treatment approaches for neuroblastoma (NB) have been continuously developed for decades. The survival rates of children with NB have been improved through multidisciplinary treatment. However, the diagnosis and treatment strategies are varied across different regions and institutions in China. The collaborative efforts of the Chinese Children's Cancer Group (CCCG) have led to advances in our understanding of NB biology, a standardized classification, and stratified strategies, resulting in improved outcomes in China.

Aims

We aimed to assess the NB-2015 protocol formulated by the CCCG in 2015 and evaluate the extent of the impact of autologous peripheral blood stem cell transplantation (APBSCT).

Methods/Materials

This study enrolled patients with NB between 2015 and 2018 from seven medical centers in China. The clinical characteristics of the patients were noted, and the therapeutic effect was evaluated as the impact on survival rate.

Results

The data of one hundred sixty-one patients with previously untreated NB were reviewed. The 3-year overall survival (OS) and event-free survival (EFS) rates of the low-, intermediate-, and high-risk groups were 100%, 95.5% and 97.2% and 91.6%, 65.6%, and 51.1%, respectively. In total, 3 out of 7 (42.8%) patients with stage 2B disease, none of whom had *MYCN* amplifications, relapsed. A total of 47 of the 78 patients in the high-risk group underwent external-beam radiation to the primary tumor bed (dose ranging from 18 to 36 Gy). The therapeutic effect was significantly different between patients who received radiation therapy and those who did not (3-year OS P =0.041). Within the high-risk group, the 3-year OS rates of the patients with and without APBSCT were 79.0% and 58.3%, respectively (P=0.014). The 3-year EFS rates of the patients with and without APBSCT were 72.6% and 37.1%, respectively (P=0.008).

Summary/Conclusions

This study showed that using the NB-2015 protocol achieved satisfactory results without the occurrence of fatal complications. For patients in the stage 2B group, chemotherapy should be intensified. Multidisciplinary treatment was essential; APBSCT can effectively improve the survival rate of patients with NB, especially high-risk patients. A longer follow-up period is needed to survey the survival rate and incidence of complications for Chinese NB patients who received chemotherapy, radiotherapy and APBSCT.

Type Clinical

Presentation Preference Traditional poster

Abstract nr. 60

Clinical significance of detection of small lesions based on GD2 immunocytology in high-risk neuroblastoma

Author - doctor Li, Jie, Tianjin Medical University Cancer Institute and Hospital, Tianjin, China (Presenting Author)
Topic Neuroblastoma as developmental disease

Background/Introduction

Neuroblastoma is the most common extracranial solid tumor in children, and the most common malignancy in infants. Outcomes vary significantly among patients with this disease, as subsets of very young children fare well with little or no treatment, whereas older children with metastatic disease have an event-free survival (EFS) of approximately 50% despite aggressive, multimodality therapy that results in significant long-term side-effects.

Aims

To explore the predictive effect of GD2-based immunocytology on the prognosis of children with NB, and to compare the sensitivity of bone marrow smear and GD2 staining in the detection of NB small lesions. Meanwhile, to analyze the effect of stem cell transplantation on prognosis of children with high-risk NB.

Methods/Materials

The clinical and pathological data of 56 patients with high-risk neuroblastoma from Tianjin Medical University Cancer Hospital from February 2011 to September 2018 were retrospectively analyzed. The clinical significance of GD2-based immunocytological detection of small lesions in children with high-risk NB is discussed from the following aspects:
Comparative study on sensitivity of detection of small lesions: sensitivity differences of NB small lesions detected by bone marrow smear method and GD2-based immunocytography at the time of diagnosis and the end of chemotherapy were analyzed.
Analysis of pathological factors of GD2 positive in bone marrow at diagnosis: Pathological factors such as gender, age, tumor site, abnormal amplification of MYCN gene and initial NSE level were further studied. In addition, total survival (OS) and disease-free survival (DFS) of children with initial GD2 positive and negative in bone marrow were compared and analyzed.
Correlation between the status of GD2 in bone marrow at each treatment stage and the recurrence: statistical methods were used to analyze the potential relationship between GD2 status of bone marrow and recurrence and prognosis in children with NB at each treatment stage.
Efficacy analysis of stem cell transplantation: To analyze the effect of stem cell transplantation on the prognosis of high-risk NB children, and to group the children with GD2 positive and negative at

the end of chemotherapy, then to explore the efficacy of stem cell transplantation within the groups, respectively.

Results

Comparative study on sensitivity of detection of small lesions: The differences in sensitivity of bone marrow smear and GD2 staining were statistically significant at the time of diagnosis and at the end of chemotherapy in children with high-risk NB, and the sensitivity of detection of microscopic lesions using GD2 staining was better than that of traditional bone marrow smear.
Analysis of pathological factors of GD2 positive in bone marrow at diagnosis: There was no significant difference in gender, age, tumor site, abnormal amplification of MYCN gene and initial NSE level between children with GD2 positive and negative in bone marrow at the time of diagnosis ($P > 0.05$). The difference of disease-free survival and total survival compared between the two groups was also not statistically significant ($P > 0.05$).
Correlation between the status of GD2 in bone marrow at each treatment stage and the recurrence: At the time of diagnosis, and after 2 courses chemotherapy, GD2 positive had no effect on the recurrence and overall survival of high-risk NB children. However, GD2 is still positive after 4 courses chemotherapy and at the end of chemotherapy, which is a risk factor for recurrence and prognosis.
Efficacy analysis of stem cell transplantation: The difference was statistically significant in the effect of stem cell transplantation on the prognosis of children with high-risk NB, and the recurrence rate of children with high risk NB showed a trend of decrease after stem cell transplantation.

Summary/Conclusions

The detection of microscopic lesions based on GD2 immunocytology was more sensitive than bone marrow smear. At the time of diagnosis and after 2 courses chemotherapy, the status of GD2 was not related to the prognosis of children with high-risk NB, but the GD2 staining was still positive after 4 courses chemotherapy, indicating poor prognosis and prone to recurrence. hematopoietic stem cell transplantation can reduce the recurrence rate and the effect is more obvious for children with GD2 positive at the end of chemotherapy.

Type Clinical

Presentation Preference Traditional poster

Abstract nr. 61

Targeting the c-MYC oncogenic signal through novel PA2G4 inhibitors

Author - Dr Massudi, Hassina, Childrens Cancer Institute, Kensington, Australia (Presenting Author)

Topic Neuroblastoma as developmental disease

Background/Introduction

MYC (MYCN and c-MYC) oncoprotein expression is pivotal in over 70% of human cancers and is associated with poor prognosis and disease aggression, making it an attractive target for pharmacological inhibition. MYCN drives 20% of the high-risk paediatric neuroblastoma (NB) patients, whereas, c-MYC has recently emerged as a potent driver gene in a subset of 10% of high-risk NB. We identified a novel oncogenic co-factor, proliferation-associated protein 2G4 (PA2G4), which bound MYCN to increase its stability and thus promoting cancer cell growth. Most importantly, the PA2G4-MYCN protein-protein interaction could be disrupted by a novel small molecule, WS6, which markedly reduced tumorigenesis in several *in vivo* NB models.

Aims

To evaluate whether c-MYC bound PA2G4 and increased its stability/oncogenic function.
To assess the efficacy of WS6 and analogues to target the putative PA2G4-c-MYC interaction in NB and other c-MYC driven malignancies.
To determine the mechanism of a putative PA2G4-c-MYC interaction.

Methods/Materials

Publicly available R2 gene expression database (n = 649, Kocak dataset) was used for gene expression analysis. Mechanism of PA2G4/c-MYC interaction was investigated using plasmid DNA overexpression, siRNA mediated knockdown, cycloheximide chase assay, western blots and colony formation assays. Cell viability and drug treatment assays were conducted to measure drug IC-50 and therapeutic window. Molecular modelling, surface plasmon resonance, differential scanning fluorimetry, and Lapinski's drug like properties assessments were conducted to rank WS6 analogues for potency and efficacy.

Results

For the first time, we showed that PA2G4 and c-MYC mutually stimulate high levels of each protein. High PA2G4 expression was a prognostic marker in c-MYC high NB patient tumor subset. High expression of PA2G4 and c-MYC predicted poor overall survival in MYCN non-amplified, high c-MYC NB patients. In addition, PA2G4 knockdown significantly reduced c-MYC protein

stability and half-life. WS6 treatment showed selective toxicity and sensitivity for high c-MYC expressing NB cells as well as other c-MYC driven cancer cells (B cell leukemia/lymphoma, medulloblastoma). Most importantly, we have identified selective and potent analogues of WS6, which have significant cytotoxicity to NB cells with high PA2G4 expression and less toxicity for normal cells. WS6 analogue treatments reduce the protein levels of PA2G4/c-MYC and significantly reduced colony formation.

Summary/Conclusions

These data strongly support PA2G4's role as an oncogenic cofactor in c-MYC driven cancers. WS6 and its potent selective analogues target the c-MYC oncogenic signal, providing critical data to guide future clinical trials.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 62

A novel therapeutic strategy against MYCN-amplified neuroblastoma by chemical-induced copy number reduction combined with PARP inhibitors

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

MYCN amplification is a predictor for clinical outcome of patients with neuroblastoma and correlates with tumor aggressiveness. Inhibition of MYCN is a promising therapeutic strategy for MYCN-driven neuroblastoma although direct targeting of MYCN has been challenging. We have recently reported a DNA-alkylating pyrrole-imidazole polyamide, MYCN-A3 (CCC-002), that sequence-specifically binds to the *MYCN* gene and demonstrates anti-tumor activity (Yoda, et al., Cancer Res., 2019). CCC-002 induced site-specific DNA damage, copy number reduction of amplified MYCN and cell death in a *similar* manner to CRISPR/Cas9.

Aims

In this study, we examined the efficacy of PARP inhibitors, olaparib and rucaparib, as a potential add-on therapy to CCC-002.

Methods/Materials

CCC-002 was designed to bind to the minor groove of genomic DNA in the coding region of *MYCN* and synthesized for site-specific alkylation of *MYCN*.

Results

Olaparib and rucaparib treatment sensitized *MYCN*-amplified CHP-134 cells to CCC-002 but not in *MYCN*-non-amplified SK-N-AS cells. We also used IMR5-75-shMYCN cells expressing tetracycline-inducible shRNA targeting *MYCN*. Knockdown of MYCN expression showed no synergistic effect with olaparib, suggesting that the above combinatorial effect of CCC-002 and PARP inhibitors may occur by DNA damage accumulation rather than the suppression of MYCN

expression. Indeed, western blot and immunofluorescence analyses confirmed the elevated level of gamma-H2AX and phosphorylated RPA32 after the combination treatment with CCC-002 and olaparib.

Summary/Conclusions

Site-specific DNA damage induced by CCC-002 leads to suppression of the *MYCN* gene and potent anti-tumor activity against *MYCN*-amplified neuroblastoma. The present data suggest that DNA repair inhibition may provide an effective therapeutic strategy to accumulate DNA damage by CCC-002 in *MYCN*-amplified neuroblastoma cells.

Type Translational

Presentation Preference Traditional poster

Abstract nr. 63

KETAMINE BASED MANAGEMENT OF NAXITAMAB (Hu3F8) INDUCED PAIN IN THE OUTPATIENT SETTING AT HSJD.

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

Immunotherapy with anti-GD2 MoAbs has improved outcomes (EFS and OS) in patients with high-risk NB. Generalized neuropathic pain is a dose-limiting toxicity of 3F8 anti-GD2 MoAbs. The pain associated with anti-GD2 therapy is similar to other neuropathic pain syndromes and is relatively opioid-resistant; nevertheless, opioids are routinely utilized for pain control during anti-GD2 infusions. Neuropathic pain is mediated mainly through the N-methyl-D-aspartate (NMDA) receptor and Ketamine is an NMDA-R antagonist. It releases nitric oxide and binds to mu receptors to increase the effectiveness of opioid-induced signaling. These multifactorial actions help in producing effective analgesia.

Aims

We investigated the management of Naxitamab (hu3F8) induced acute neuropathic pain with ketamine.

Methods/Materials

The same outpatient clinic personnel treated all patients and a physician was involved in all bedside decisions including when to give rescue doses of analgesics to control pain. Standard supportive therapy included Gabapentin (10mg/kg q8h po) from day -5; before the infusion paracetamol (15 mg/kg po), cetirizine (2.5-10 mg po), ranitidine (2mg/kg po), ondansetron (5

mg/m2 po) and Morphine Chloride 25-100 mcg/Kg iv. Patients were selected to ketamine management if standard support therapy was ineffective to control reactions during infusion. They received treatment in the procedure room under the supervision of anesthesiologist. Same premedication but instead of Morphine before hu3F8 infusion received Midazolam (0.05 mg/Kg iv bolus), Lidocaine (2 mg/Kg iv bolus), Ketamine (2 mg/Kg iv bolus), and Atropine (0.005 mg/Kg iv if normal heart rate). Middle way through hu3F8 infusion (minute 15) a second dose of Lidocaine (2 mg/Kg iv bolus) and Ketamine (2 mg/Kg iv bolus; up to a total of 4 mg/kg) is provided.

Results

Since June 2017, 115 patients received a total of 659 Naxitamab cycles (range 1-20). 21 (18%) pts were selected for ketamine protocol: four because of complex allergic reactions potentially enhanced by the use of opioids; six because of apnea; four because of refractory hypotension; and seven because of unmanageable pain. 19 of 21 patients successfully completed planned treatment with Ketamine and were all managed outpatient. The recovery time and resume of normal daily activities is faster (3-5 days) compared to standard management (10-15 days). Two very young patients developed apnea in the first few minutes of infusion also with ketamine based protocol and were taken off study.

Summary/Conclusions

Ketamine management of neuropathic pain related to Naxitamab is safe and effective in the outpatient setting.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 64

NAXITAMAB (Hu3F8) plus GM-CSF FOR HIGH-RISK NEUROBLASTOMA IN COMPLETE REMISSION PATIENTS: RESULTS OF A PATIENT RESTRICTED USE PROGRAM (YMABS) AT HSJD.

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

Immunotherapy with chimeric anti-GD2 MoAbs (m3F8; Dinutuximab/beta) has improved outcomes (EFS and OS) in patients with high-risk NB in complete remission (CR) after induction therapy and autologous bone marrow transplantation (ABMT). Anti-GD2 antibodies differ in their in vitro and in vivo properties.

Aims

We investigated the humanized anti-GD2 MoAb Naxitamab (previously termed Hu3F8) in combination with GM-CSF (granulocyte macrophage colony stimulating factor) in patients achieving CR after standard induction therapy.

Methods/Materials

Patients were treated on a patient restricted program based on an ongoing phase 2 trial of the same agents (NCT 03363373). Eligibility criteria included non-evidence of disease by bone marrow studies (4 aspirates), MIBG-SPECT/FDG-PET and whole body MRI. Prior anti-GD2 MoAbs was permitted. Each cycle comprised sc GM-CSF at 250 µg/m2/d on days −4 to 0 (Wednesday through Sunday), followed by 500 µg/m2/d on days 1 to 5 (Monday through Friday); and hu3F8 iv for 30 minutes on days 1, 3, and 5 (3 doses/cycle) at 3 mg/kg/day (total 9 mg/kg or 270 mg/m2 per cycle). Treatment consisted of 5 cycles, all outpatient. Toxicity was measured by CTCAE v4.0.

Results

Since June 2017, 49 patients (34 in first CR and 15 in 2nd CR) received a total of 180 cycles. Six patients had prior isolated CNS relapse; 8 had systemic relapse; and one had both. 11 (22%) patients had MYCN amplified NB. 7 (20%) of 34 in first CR and 9 (60%) of 15 in 2^{nd} CR had prior ABMT. Sixteen (32%) had MRD based on Phox2B qRT-PCR. Toxicities included pain and hypertension expected with Naxitamab. No other >grade 2 related toxicities occurred. 36 completed all planned cycles. 4 and 9 patients could not complete 5 cycles because of toxicity and PD, respectively. With a median F/up of 13.5 months, 7 (20%) of 34 pts in 1^{st} CR and 6 (40%) of 15 pts in 2^{nd} CR have relapsed. Three (6%) patients have died of disease progression. 2-year EFS and OS for all patients is 65.3%, 95% CI = (49.4%; 86.3%) and 87.2%, 95% CI = (73.1%; 100%), respectively. 2-year EFS and OS for 1^{st} CR patients with no prior ABMT (n=27) is 62.2%, 95% CI = (38.4%; 100%) and 80.2%, 95% CI = (55.7%; 100%).

Summary/Conclusions

Naxitamab-based immunotherapy is effective in consolidating both first and second remission in high-risk neuroblastoma.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 65

SOX11 as guardian of epigenetic plasticity in neuroblastoma

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Topic Gene regulatory networks and epigenetics: from basic science to therapy

Background/Introduction

Normal human development is controlled through the complex interplay of multiple transcription factors and the reshaping of epigenetic landscapes. Tumor cells can co-opt normal developmental pathways for functions that are linked to tumor progression and may become addicted to survival mechanisms controlled by developmental master regulators. Neuroblastoma (NB), the most common extra-cranial tumor in childhood, arises from the sympatho-adrenergic lineage during normal embryonic development. The genomic landscape of NB is characterized by a low mutational burden and highly recurrent copy number changes. Consequently, these copy number alterations affecting lineage-dependency factors in the sympatho-adrenal lineage might be strong candidates for NB cell addiction. The study of such oncogenic lineage-dependency factors is considered of strong translational value as they could represent highly selective drug targets compared to conventional cytotoxic therapy.

Aims

We aimed to scrutinize DNA copy number profiles for gains or amplifications of putative lineage-dependency transcription factors involved in the epigenetic landscape and development of NB.

Methods/Materials

Using high resolution copy number analysis of NB tumors we sought for candidate lineage-dependency transcription factors. *In vitro* functional assays were performed upon *SOX11* knockdown and overexpression. Tumor acceleration was evaluated upon *SOX11* overexpression in a *MYCN*-driven zebrafish model. ChIP-seq, ATAC-seq and RNA-sequencing was performed to evaluate the transcriptome and epigenetic landscape, which consequently resulted in the execution of drugging assays for strong candidate targets.

Results

We identified recurrent focal gains and amplifications encompassing the *SOX11* locus and selected this transcription factor as a strong candidate based on (1) its expression in the normal sympatho-adrenal lineage and adrenergic NBs and (2) its control by multiple adrenergic specific distal (super-) enhancers, amongst others. Both *in vitro* SOX11 knockdown in NB cells and data from the Cancer Dependency Map are indicative for strong dependency of NB cell lines to elevated *SOX11* expression levels. Furthermore, SOX11 overexpression in a *MYCN*-driven NB zebrafish model showed accelerated tumor formation. ChIP-sequencing revealed SOX11 controlled transcription of multiple components of major epigenetic modulating protein complexes implicated in chromatin remodeling and enhancer activation (SWI/SNF), chromatin modification (PRC1 and PRC2), DNA methylation as well as several pioneer transcription factors including MYB. Furthermore, ATAC-sequencing indicated SOX11 controlled chromatin opening, predominantly affecting distal enhancers marked by SMARCC1 and MYB binding motifs. In

addition to the strong co-regulation of *MYB* and *SOX11*, *SOX11* high expressing NB cells showed increased sensitivity for peptidomimetic blockade of MYB.

Summary/Conclusions

Our data suggest a key role for SOX11 in the activation of multiple epigenetic modulators in NB leading to an impact on maintenance of adrenergic NB chromatin accessibility and cell identity and contributing to the proliferative *MYCN* driven NB phenotype.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 66

Immunosuppression mechanisms in 11q-deleted neuroblastomas support increased anti-GD2 response when combined with dual immunotherapy

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

Post-consolidation treatment with anti-GD2 immunotherapy has become the standard of care for patients with high-risk neuroblastoma (NB). However, the relapse rate of patients exceeds 50% with fatal outcome. Dissecting molecular hallmarks within high-risk NB subgroups will identify actionable targets for new treatment options.

Aims

To evaluate the response of 11q-deleted NB subgroup to standard anti-GD2 immunotherapy and define potential new targets for tailored therapies.

Methods/Materials

Response to maintenance therapy was evaluated in 84 high-risk (M stage) NB patients. Primary endpoints were 3-year event-free survival (EFS) and 3-year overall survival (OS). Patients received either standard anti-GD2 immunotherapy (n=51) or conventional retinoic acid-based therapy (n=33). Differences in EFS and OS between treatment regimens were compared with the log-rank test. Immune and genetic profiles of high-risk NB were analyzed to examine their relationship with immunotherapy response. CIBERSORT was used to quantify immune cell infiltration. Segmental chromosome aberrations (SCAs) were analyzed by SNP arrays (CytoScan HD, Affymetrix). Gene Set Enrichment Analysis (GSEA) with gene-targeted 11q-miRNAs was performed to explore the effect of 11q miRNAs haploinsufficiency. Differences in immune cell infiltration, gene expression profiles, and SCA profiles between subgroups were tested using one-way analysis of variance (one-way ANOVA).

Results

Anti-GD2 immunotherapy improved outcome in the overall high-risk NB cohort with regard to EFS (52.9 vs 30.3, p=0.013) and OS (74.2 vs. 51.5, p=0.023) at 3 years. Similarly, 11q-deleted subgroup showed improvement in EFS (54.2% vs. 10%, p=0.006) and OS (74.2% vs. 50%, p=0.169), although survival rates are still sub-optimal. Noteworthy, several approaches reveal that 11q-deleted NB exhibit various immune inhibitory mechanisms: higher SCAs, dysregulation of immunosuppressive miRNAs, increased regulatory T cells infiltration (Tregs, p=0.035), and higher expression of several immune checkpoint biomarkers: programmed death-ligand 1 (PD-L1, p=0.029), interleukin-10 (IL-10, p=2.47e-03), transforming growth factor-beta 1 (TGF-β1, p=0.016) and indoleamine 2,3 dioxygenase 1 (IDO1, p=0.021).

Summary/Conclusions

NB patients with 11q-deletion display similar outcome as those with 11q-undeleted NB to anti-GD2 immunotherapy. However, 11q-deleted tumors display a distinct immune suppression profile, supporting the combination treatment of anti-GD2 with immune checkpoint inhibitors to circumvent immune checkpoint blockade. This combination may be a more efficient therapeutic strategy for this tumor subtype.

Type Translational

Presentation Preference Traditional poster

Abstract nr. 67

The incidence of neuroblastoma cases before and after screening in Japan

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Topic Other

Background/Introduction

Mass screening for neuroblastoma in infants in Japan was carried out from1985 to 2003. No accurate epidemiological studies have been performed on changes in neuroblastoma morbidity after screening cessation.

Aims

The purpose of this study was to clarify the prevalence of neuroblastoma before and after pausing infantile neuroblastoma screening.

Methods/Materials

We used 10,868,860 births in 1990-1998 as a cohort during screening conduct, and 5,446,889 births in 2004-2008 as a cohort after suspension. The neuroblastoma registration database was created by registration of the Japanese Society of Pediatric Surgery and the Japanese Neuroblastoma Study Group. Patients' information was collected after deleting personal information, and the study was approved by the ethics review board at the representative research institution.

Results

According to the database, the number of neuroblastoma cases up to 6 years of age was 2,008 among those born between 1990 and 1998 (A) and 449 among those born between 2004 and 2008 (B). A total of 115 and 5 cases of INSS stage 1-3 neuroblastoma per 1 million births detected between 6-11 months age in (A) and (B), respectively (rate ratio [RR] 0.04, 95% confidence interval [CI] 0.03-0.06). A total of 8 and 4 cases of stage 4 neuroblastoma per 1 million births detecteed between 6-11 months age in (A) and (B), respectively (RR 0.46, 95% CI 0.29-0.75). In (A), there were many localized cases of stage 1-3 neuroblastoma. This is presumed to be due to the over-diagnosis by screening. A total of 12 and 24 cases of stage 4 neuroblastoma per 1 million births occurred between 24-72 months of age in (A) and (B), respectively (RR 2.04, 95% CI 1.60-2.61). A total of 3.4 and 1.1 cases of *MYCN*-amplified neuroblastoma per 1 million births detected between 6-11 months age in (A) and (B), respectively. However, 10 and 20 cases of *MYCN*-amplified neuroblastoma per 1 million births detected between 0-72 months age in (A) and (B), respectively (RR 2, 95% CI 1.54-2.59). Furthermore, 3.0 and 13.0 cases of *MYCN*-amplified neuroblastoma per 1 million births occurred between 24-72 months age in (A) and (B), respectively. The number of *MYCN* amplification cases increased after screening was stopped.

Summary/Conclusions

Screening of neuroblastoma resulted in the over-diagnosis of localized cases in infancy. In contrast, the number of high-risk cases with stage 4 and *MYCN* amplification older than 24 months of age increased after screening cessation. These cases may have been excluded earlier by screening for neuroblastoma in infants.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 68

XIAP inhibition through ARTS mimetics as an anticancer therapeutic in Neuroblastoma

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

XIAP (X-linked Inhibitor of Apoptosis), the most prominent member of mammalian inhibitors of apoptosis proteins (IAPs), is a critical regulator of cell death and survival that suppresses apoptosis and enhances survival signaling. Despite being targeted in multiple adult cancers, XIAP inhibition is an underexplored therapeutic strategy in pediatric solid tumors. Neuroblastoma is one of the several neural crest-derived cancers that share a common developmental biology requiring XIAP. XIAP inhibition by XAF1 has previously been demonstrated to play a critical role in mediating KIF1Bβ-driven tumor suppression in neuroblastoma. KIF1Bβ-XAF1-XIAP pathway is required for normal sympathetic nervous system development. Removal of XIAP is required for targeted sympathetic neuronal progenitors to gain apoptotic competence prior to developmental culling. In high-risk, poor prognosis neuroblastoma patients, KIF1Bβ is often deleted or inactivated. This leads to low XAF1 and correspondingly, high XIAP expression. Therefore, we hypothesized that high-risk, poor prognosis neuroblastoma or other neural crest-derived cancers may be addicted to XIAP for aggressive growth and survival and that patients may benefit from a treatment approach targeting XIAP.

Aims

To evaluate the utility, efficacy and mechanism of XIAP inhibition as an anticancer strategy for neuroblastoma stratified according to XIAP.

Methods/Materials

Small molecule compounds targeting pan-IAPs (SMAC mimetics) and XIAP alone (ARTS mimetics) were screened and tested on a panel of commercial and patient-derived neuroblastoma cell lines. Dose response curves established from cell viability assay were used to determine and rank the efficacy of each compound. The expression of IAPs proteins and cell death were assessed via Western Blot, Crystal Violet and biochemical apoptotic assays.

Results

Among the compounds tested, ARTS mimetic A4 demonstrated the best efficacy across the neuroblastoma panel. It is most effective against high XIAP-expressing cells (BE(2)-C and KELLY), which are considered to be aggressive and resistant to conventional chemotherapeutics. ARTS (Sept4_i2) induces apoptosis through direct binding and specifically degrading XIAP through the ubiquitin proteasome system. ARTS mimetics, are distinct from all other IAP antagonist as they can both bind and degrade XIAP. Moreover, its efficacy appears to be stratified according to XIAP and MYCN expression status (a hallmark of high-risk, poor prognosis neuroblastomas). Encouragingly, A4 was best tolerated by immortalized non-cancer cell lines representing liver and bone marrow, two common metastatic sites for neuroblastoma. Preliminary pharmacokinetic testing of A4 showed good stability and availability in plasma. Furthermore, A4 acts synergistically when used in combination with cytotoxic agents such as vincristine and topotecan. Silencing of XIAP induces apoptosis in neuroblastoma cells while forced expression of XIAP protects cells from drug-induced cell death, indicating XIAP inhibition is necessary and sufficient to kill neuroblastoma cells.

Summary/Conclusions

ARTS mimetic A4 with XIAP-specific inhibition, showed the highest efficacy against neuroblastoma cells lines and exhibited the lowest toxicity to non-cancer cells, while pan-IAP inhibitors showed the converse with the lowest anti-tumor efficacy and the highest toxicity. Taken together, inhibiting XIAP in a subset of aggressive neuroblastoma cells characterized by high XIAP may be a potential breakthrough treatment or sensitizer for these cells that are currently resistant to existing chemotherapeutics.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 69

Naxitamab-based Chemoimmunotherapy for Resistant High-Risk Neuroblastoma: Preliminary results of HITS treatment through compassionate use at HSJD.

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

Chemoresistant and relapsed disease are major obstacles to curing high-risk neuroblastoma (HR-NB). Anti-GD2 monoclonal antibody (MoAb) is effective in preventing relapse after remission but responses in relapsed or progressive disease (PD) are rare.

Aims

We investigated the combination of humanized anti-GD2 MoAb naxitamab, (previously termed Hu3F8), irinotecan, temozolomide and sargramostim (GM-CSF): a HITS patient restricted use against resistant HR-NB following the phase II study (NCT03189706).

Methods/Materials

Salient eligibility criteria included evaluable or measurable chemoresistant disease. Prior anti-GD2 MoAb and/or irinotecan/temozolomide (I/T) therapy was permitted. Each cycle comprised of irinotecan 50 mg/m^2/day intravenously (IV) plus temozolomide 150 mg/m^2/day IV or orally (days 1-5); naxitamab 2.25 mg/kg/day IV over 30 minutes, days 2, 4, 8 and 10 (total 9 mg/kg or 270 mg/m2 per cycle), and GM-CSF 250 mg/m^2/day subcutaneously, days 6-10. Toxicity was

measured by CTCAE v4.0 and responses by modified International Neuroblastoma Response Criteria.

Results

Forty-four (36 evaluable and 8 non-evaluable) heavily prior-treated patients (median age at enrollment: 6 years; median number of prior relapses: 2) have received 210 (median 6; range 1-14) cycles to date. 16 patients had HR-NB refractory to induction chemotherapy while 28 had prior relapse. Toxicities included myelosuppression and diarrhea expected with I/T, and pain and hypertension expected with naxitamab. No other >grade 2 related toxicities occurred; treatment was outpatient. Early responses, assessed after 2 cycles, were documented in 17 (47%) of 36 evaluable patients and were complete (n=11), and partial (n=6); 10 patients had stable disease. Nine (25%) patients progressed before or at cycle 2 evaluation. 1-year progression-free survival is 37.0%, 95% CI (21.8%, 62.9%), median time to progression 9.2 months. 1-year Overall Survival is 55%, CI (39.4%, 76.9%).

Summary/Conclusions

High-dose naxitamab-based chemoimmunotherapy is safe and effective against chemoresistant HR-NB.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 70

Academic, Phase I/II Trial on T Cells Expressing a Third-Generation GD2 Chimeric Antigen Receptor and Inducible Caspase-9 Safety Switch for Treatment of Relapsed/Refractory High-Risk Neuroblastoma

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

Treatment of high-risk neuroblastoma (HR-NB) remains a major challenge in the field of pediatric oncology, with an unsatisfactory long-term survival of 50% or even less. Immunotherapy using T-cells genetically modified to express a chimeric antigen receptor (CAR) targeting disialoganglioside-2 (GD2) represents an attractive option.

Aims

At Ospedale Pediatrico Bambino Gesù (OPBG) in Rome, we opened an academic, phase I/II clinical trial on the use of autologous T cells expressing a 3rd generation (CD28-4.1bb) GD2-CAR and the suicide gene inducible caspase-9 (iC9-GD2-CAR), for treatment of pediatric patients with relapsed/refractory (r/r) HR-NB.

Methods/Materials

We developed a clinical-grade retroviral construct, including CD28 and 4.1bb costimulatory domains and the iC9. After obtaining informed consent, the study consecutively enrolled children

and young adults (aged 1 – 18 years) with r/r HR-NB, using a dose escalation/de-escalation design for the phase I portion. All patients received a fludarabine/cyclophosphamide-based lymphodepletion.

Results

Sixteen children were enrolled between February 2018 and September 2019. All patients but one had failed at least 2 lines of therapy. The designed cell dose was successfully produced for all patients and we did not experience any production failure. Three dose levels (DL) were tested (DL3, 3; DL4, 6; DL5, 10×10^6 CAR T cells per kg) and no dose limiting toxicities (DLTs) were recorded, defining the recommended dose as 10×10^6 CAR T cells per kg. Cytokine release syndrome (CRS) occurred in 12/16 patients (75%) and reached grade 4 in 1 patient only, requiring the administration of steroids and the activation of the suicide gene. Upon dimerization of iC9, circulating iC9-GD2-CAR T cells dropped from 77 to 1 cells/ul, leading to a rapid resolution of CRS. Fourteen patients developed grade 3-4 neutropenia, lasting >4 weeks in 4 children (28.6%). Mild peripheral neuropathy occurred in 3/15 (20%) patients. iC9-GD2-CAR T cells expanded *in vivo* and were detectable in peripheral blood by flow-cytometry in 15/16 patients, up to 5 months after infusion, reaching a median peak value of 47,37%$\pm$26,9% of circulating CD3+ cells (103$\pm$87 CAR T cells/ul)(Fig.1). Expansion of CAR-T cells inversely correlated with the presence of myeloid-derived suppressor cells in peripheral blood. Transduced cells were also detected in bone marrow and cerebrospinal fluid. Thirteen children responded to the treatment (overall response rate: 81,3%), with 5 patients obtaining complete response (CR) and 1 treated with non-evidence of disease (NED) maintaining this state. Amongst responders, 5 patients maintain CR or stable disease, while 8 patients ultimately relapsed/progressed. Lastly, the protocol was amended to allow multiple infusions in case of response to the first administration and 9 patients received additional infusions. Reinfusions were overall well tolerated, with 1 patient developing grade 4 CRS; iC9-GD2-CAR T cells expanded after reinfusion and were detectable in peripheral blood in 7/9 patients

Summary/Conclusions

Use of iC9-GD2-CAR T cell in an academic setting is feasible and safe in treating highly resistant/relapsed HR-NB. Peculiar, sometimes severe, toxicities can develop and the activation of the suicide gene iC9 is able to control the side effects. Importantly, iC9-GD2-CAR T cells are able to determine a significant anti-tumor effect.

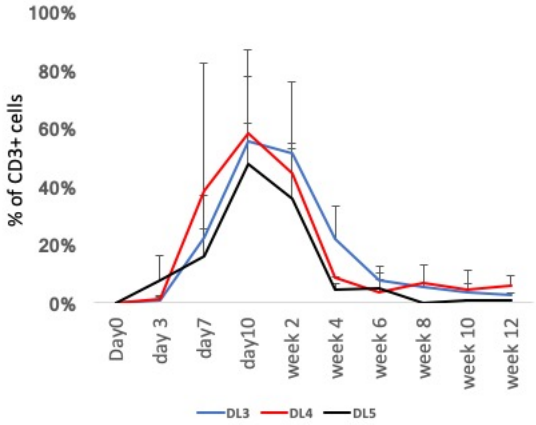

Figure 1. iC9-GD2-CAR T cells expansion in the peripheral blood

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 71

45A ncRNA EXPRESSION IMPAIRS MICROTUBULES DYNAMICS AFFECTING DRUG RESPONSE IN NEUROBLASTOMA

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

45A non-coding (nc) RNA overexpression induces deep modifications of neuroblastoma (NB) cell cytoskeleton leading to a cascade of reactions which interferes with proliferation control, cell migration, tumorigenic potential and cell adhesion properties. In detail, 45A-overexpressing cells show a non-specific metastatic engraftment whereas the silencing of its expression drives to the production of liver-specific metastasis. The reasons behind this modification remains still unclear.

Aims

We demonstrate the tuned regulation of several proteins involved in microtubules dynamics with relevant effects on cancer development driven by 45A ncRNA expression. Indeed, the proper function of these proteins is fundamental for the cytoskeleton organization and its impairment leads to the particularly dangerous condition known as chromosomal instability (CIN).

Methods/Materials

We investigated by Real-time qPCR, western blot and immunocytochemical analysis the different expression and/or localization of GTSE1 (G2 And S-Phase Expressed 1), MCAK (Mitotic Centromere-Associated Kinesin), Aurora B, p53 and the altered organisation of tubulin in different neuroblastoma cell (SKNBE2) models stably overexpressing or downregulating 45A ncRNA.

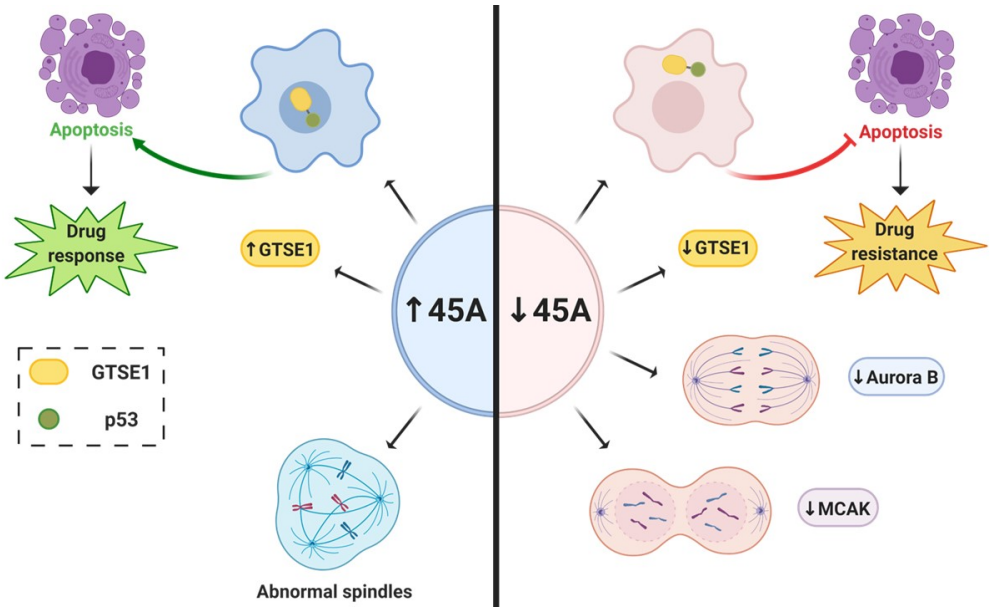
Results

We demonstrate that 45A ncRNA not only directly regulates the expression of aforementioned proteins but can even affect the subcellular localization of GTSE1: in 45A-overexpressing cells the protein is accumulated in nuclei, while 45A-downregulation leads to a significant GTSE1 cytoplasm relocation (nuclear GTSE1 being 86% vs 14% respectively, $p<0.01$). The simultaneous cytoplasmic sequestration of p53, driven by GTSE1, affects apoptosis leading to the observed resistance of 45A-downregulating cells to spindle poisons (e.g. paclitaxel, vincristine, vinblastine). Furthermore, 45A-overexpression leads to an increased number of abnormal spindles, thus

promoting CIN and possibly explaining the increased tumorigenic potential exhibited by 45A-overexpressing cells.

Summary/Conclusions

These data highlight the role of 45A ncRNA in the regulation of the expression of several proteins involved in microtubules dynamics, causing variations in drug response and suggesting its possible relevance in NB prognosis and/or therapy.



Graphical abstract

Type Basic

Presentation Preference Oral presentation

Abstract nr. 72

A novel neuroblastoma-based model to study the effects of suppressive monocytes on CART cell therapy

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Neuroblastoma is a paediatric solid tumour which presents with poor survival rates among patients within the high-risk group. Despite advances in cancer therapy, clinical outcomes remain poor, and novel approaches are needed. Chimeric antigen receptor (CAR) T cells have been demonstrated to be an effective cell-based treatment in blood cancers, where genetically engineered T cells are re-directed to detect highly expressed surface antigens of the tumour cells. However, the immunosuppressive microenvironment that is formed by solid tumours and the surrounding tumour-conditioned immune cells heavily affects the efficacy of CART cell therapy due to inhibition of proliferation and effector functions. Myeloid derived suppressor cells (MDSC) are a key group among tumour-induced immune cells and have been shown to correlate with cancer stage, disease progression, and resistance to standard therapies.

Aims

To successfully overcome local inhibition of CART cells, the effects of the microenvironment of solid tumours need to be studied more closely. Even though they are a key player, the detailed interactions with MDSC are not yet fully understood. The aim of this work is to provide a neuroblastoma-based model to study and overcome interactions between CART cells and suppressive myeloid cells.

Methods/Materials

In an in vitro polarisation assay, healthy monocytes were grown in neuroblastoma-conditioned media to mimic the conditions of the tumour environment. The generated neuroblastoma-conditioned monocytes (NbM) were characterised for changes in their phenotype and their suppressive effect on T cells in co-cultures, using flow cytometry analysis and ELISA.

Results

Phenotype analysis of NbM showed an enrichment of a subtype reflecting mononuclear MDSC (M-MDSC; CD14+ HLA-DR^{low}), as well as early-stage MDSC (eMDSC; CD14- CD15- HLA- DR ^{low}). In co-cultures, polarised monocytes significantly inhibit T cell proliferation and activation (interferon gamma release). Sunitinib malate, a receptor tyrosine kinase inhibitor, can partially recover T cell functions through inhibition of NbM. Scaling to 96 well format shows the level of reproducibility necessary for high-throughput drug library screens for inhibitors of NbM suppression function using interferon gamma secretion as a read out.

Summary/Conclusions

Here we present a neuroblastoma-based model to screen for strategies to overcome myeloid cell-mediated T cell suppression in the tumour environment. The generated NbM reflect phenotype and suppressive effect of M-MDSC, which can be found in the neuroblastoma microenvironment. A 96 well plate inhibition of suppression assay is now being used to identify retargetable agents. Thus, the established model can lay the basis for improving CART cell immunotherapy by reducing MDSC-based suppression.

Type Translational

Presentation Preference Traditional poster

Abstract nr. 73

The effect of PLK4 inhibitor Centrinone combined with Isotretinoin on neuroblastoma

Author - Doctor Liu, Yun, Tianjin Medical University Cancer Institute and Hospital, TianJin, China (Presenting Author)

Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Neuroblastoma is a malignancy of the sympathetic nervous system that almost occurs in early childhood. It has unique clinical biological heterogeneity. Neuroblastoma patients with spontaneous regression or differentiation have good prognosis. However, the prognosis of patients who have an extensive metastatic disease is still unsatisfactory and prone to recurrence even after intensive multimodal treatment. The existence of minimal residual disease is one of the reasons for the recurrence, and isotretinoin (13-cis retinoic acid) is used for the elimination of minimal residual disease. But for patients receiving isotretinoin treatment, the 5-year overall survival rate is still only approximately 60%. In addition, NB has strong intratumoral heterogeneity. All of these provide challenges for the treatment of NB. PLK4 plays an important role in the regulation of centrosome replication, and its abnormal expression is associated with poor prognosis in breast cancer, liver cancer, ovarian cancer, NB and so on. Based on this phenomenon, researchers have developed a variety of inhibitors against PLK4 which as a target of clinical treatment, such as CFI-400945, YLT-11, Centrinone etc. Centrinone can inhibit centriole replication and delay cell mitosis. The purpose of this study was to investigate the effects of the inhibitor Centrinone and its combination with all-trans retinoic acid on centromere replication, DNA damage and cell function in NB cells.

Aims

However, despite its widespread use in neuroblastoma for the past decade, there remain a number of drawbacks to its clinical use. PLK4 (Polo-like kinase 4) plays an important role in centriole duplication. The deregulation of PLK4 can lead to mitotic defect and contribute to tumorigenesis. Centrinone is an inhibitor against PLK4, that can inhibit centriole duplication and delay cell mitosis. The purpose of this study was to investigate the effects of the Centrinone and its combination with Isotretinoin on centriole duplication, DNA damage and apopotosis in NB cells.

Methods/Materials

Method:

1.To analyze the expression of PLK4 in neuroblastoma and its prognostic relationship with NB
Adopting immunohistochemistry and western blot to verify the expression of PLK4 in NB tissues

and cells, then the TCGA database was used to analyze the relationship between the expression level of PLK4 and the prognosis of NB patients.
2. To verify the effect of inhibitor Centrinone on the expression of PLK4 and P-PLK4(S305) in NB cells by western blot test.
3. To verify the effect of Centrinone on the function of NB cells
(1) To detect the IC50 of NB cell line after adding Centrinone by MTT assay.
(2) To verify the effects of Centrinone on proliferation, differentiation and apoptosis of NB cell lines by plate cloning and Western blot.
4. To verify the effect of Centrinone on centriole duplication and DNA damage repair by western blot.
5. To induce differentiation of NB cell lines by using all-trans retinoic acid (ATRA) ,then to detect the expression of Rac1, RhoA, S-100, NSE and other differentiation-related indicators, and after the differentiation model of NB cells was established, test the expression of PLK4, P-PLK4 (S305) and γ-tublin.
6. The centrinone was combined with ATRA and added into NB cells. To verify the effects of the combination on proliferation, migration, differentiation and apoptosis of NB cells by western blot , and test the expressions of γ-tublin, 53BP1 and BRCA1.

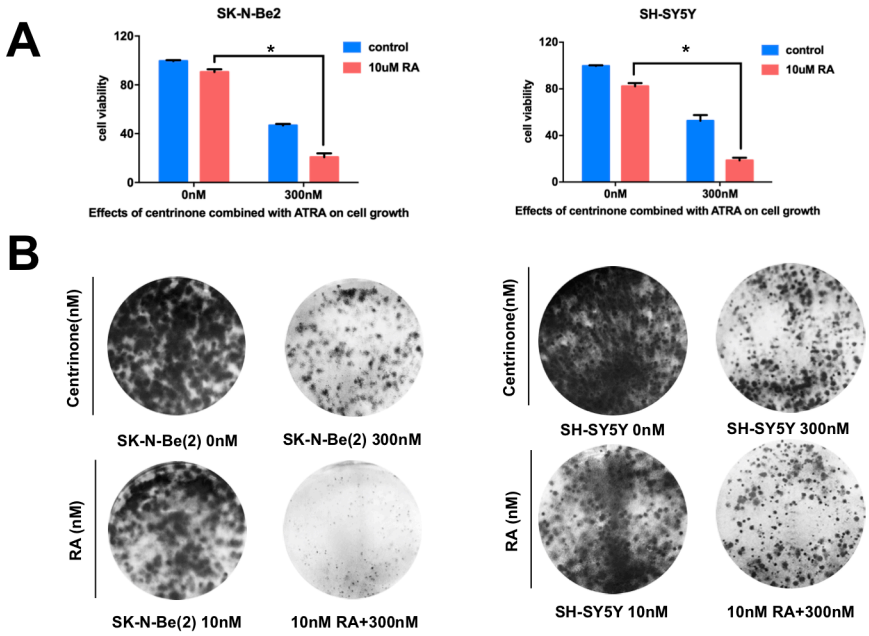
Results

This study showed the Centrinone can inhibit cell proliferation, centriole duplication, and promote cell
apoptosis and DNA damage. Importantly, The combination between Centrinone and Isotretinoin results in more durable and synergistic tumor regression than either single agent alone.

Summary/Conclusions

Therefore, targeting PLK4 in combination with Isotretinoin may result in increased antitumor efficacy in Neuroblastoma.

Fig3



Type Basic

Presentation Preference Traditional poster

Abstract nr. 74

Prospective germline next-generation sequencing in pediatric patients with neuroblastoma identifies frequent alterations in genes involved in DNA damage repair

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

The role of germline *ALK* and *PHOX2B* variants in predisposition to neuroblastoma (NB) is well-established. Alterations in genes associated with other syndromes, including RASopathies, Fanconi anemia and Li–Fraumeni, have also been detected. Emerging data suggests potential roles for DNA damage repair (DDR) pathway genes in NB predisposition. The use of Next Generation Sequencing (NGS) facilitates the unbiased detection of both known and novel variants as well as the identification of tumour DNA mutational signatures. Mutational signature 3 has been strongly associated with defects in homologous recombination repair of DNA double strand breaks.

Aims

Report our single centre experience of comprehensive sequencing of germline and tumour DNA in patients with neuroblastoma, including some with features of genetic predisposition.

Methods/Materials

Patients with newly diagnosed and relapsed malignancies were referred to our institutional pediatric oncology NGS (SickKids Cancer Sequencing (KiCS)) program. Germline DNA was sequenced using a custom pediatric cancer panel targeting 15,000 exons across 864 genes (1000X coverage). Variant pathogenicity was classified according to ACMG criteria and evaluated by our multi-disciplinary molecular tumor board. Rare variants of uncertain significance (VUS) with supportive corresponding somatic data, patient phenotypes, literature and/or in-silico functional analyses were termed “VUS with limited evidence for pathogenicity” (VUS-LEP). The same custom exon panel and whole genome sequencing (WGS) was performed on tumour DNA whenever available. Mutational signatures were assessed through WGS for the presence of SBS3 (single base substitution signature 3).

Results

Analyses have been completed for 45 NB patients. Patient history was retrospectively categorized as “high genetic predisposition risk” (HGPR) in 15/45 patients based on ≥1 of the following criteria: family history of NB and/or significant family history of other cancer(s); patient with NB and another malignancy; NB with congenital abnormalities; multifocal NB. Germline pathogenic (P), likely pathogenic (LP) variants or VUS-LEP in a known cancer predisposition gene were identified in 7/15 HGPR patients (46.6%), predominantly in DDR-related genes (*PALB2, BRCA1/CHEK2, CHEK2/PALB2, NF1, DICER1, MITF, EZH2/SMARCA4*). No *ALK* or *PHOX2B* germline variants were identified for the five patients with NB family history. For the 30 non-HGPR pts, germline P, LP variants or VUS-LEP in DDR genes were most common and detected in 8/30 pts (26.6%), in genes including *BAP1*, *BARD1* (n=2), *BLM*, *BRCA2, CHEK2, RAD51,* and *RAD51D/NBN*. In this non-HGPR population, we also identified a germline *TP53* P variant (n=1), a *MUTYH* P variant (n=1), a *FH* LP variant (n=1) as well as germline VUS-LEPs in *EZH2* (n=1) and *ERCC2* (n=1). Preliminary analyses of mutational signatures on tumour DNA (n=26) reveal the presence of SBS3 in a high proportion of tumours (15/26), with an enrichment of samples carrying germline variants in DDR genes.

Summary/Conclusions

Prospective sequencing identified frequent germline variants in NB pts, predominantly in genes involved in DDR, regardless of classification as HGPR. We report a high incidence of SBS3 in neuroblastoma tumour samples. Further studies are needed to correlate genomic variants and mutational signature patterns. Our findings have implications for treatment recommendations as well as for appropriate genetic counselling.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 75

Genome methylation defines EZH2 inhibitor sensitivity in neuroblastoma

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Topic Gene regulatory networks and epigenetics: from basic science to therapy

Background/Introduction

Polycomb repressive complex 2 (PRC2) is a multiprotein chromatin-modifying complex that is essential for vertebrate development and differentiation. PRC2 is composed of a trimeric core of SUZ12, EED, and EZH1/2, which catalyze the methylation of histone H3 at lysine 27 (H3K27me1/2/3). EZH2 is a H3K27 methylase and a target of cancer epigenetic treatments. We recently reported the oncogenic roles of EZH2 in NB and NTRK1 (TRKA) epigenetic regulation by EZH2 in the MYCN-amplified aggressive neuroblastomas (NB) (Li et al., ONCOGENE, 2018). Several EZH2 inhibitors (EZH2is) have been developing and the effects of them on NB cells have studied. These studies indicated that the combination of multiple drugs with EZH2 inhibitors will be required for curative NB therapy (Chen L et al., J Clin Invest. 2018; Henrich et al., Cancer Res 2018; Li et al., ONCOGENE, 2018).

Aims

To improve EZH2i treatments for curative NB therapy, we investigated EZH2i-sensitivity biomarkers by using aggressive NB model cell lines.

Methods/Materials

We performed transcriptome and methylome analysis by using EZH2i-sensitive and –resistant NB

cells. A microarray analysis was performed using the Agilent platform of 8 × 60 K design ID G4851B (Agilent Technologies, USA). A DNA methylation analysis of clinical data was performed using the Infinium HumanMethylation450 BeadChip Kit (Illumina, USA). The Combination Index (CI) was calculated by the Chou-Talalay (2010) method using CompuSyn (http://www.combosyn.com/).

Results

Here, we investigated the effects and function of small molecule EZH2i on aggressive NB model cell lines. We examined the antitumor effect of EZH2i using WST assay and colony formation assay. By EZH2i EPZ-6438, suppression was observed in SK-N-SH and SK-N-BE cells (sensitive cells) dose-dependently, whereas it was not observed in IMR32 and NGP cells (resistant cells). FACS analysis showed apoptosis and G0/G1 cell cycle arrest in sensitive cells. Transcriptome analysis and GSEA indicated significant changes were observed in the gene set related to cell cycle arrest and differentiation in the sensitive cells. We selected genes induced at mRNA level by EPZ-6438 only in the sensitive cells and confirmed tumor suppressor function in NB cells. Almost of the EPZ-6438-induced gene promoters were marked by H3K27me3 in *MYCN*-amplified NB cell lines. Interestingly, a part of the EPZ-6438-induced gene promoters have CpG islands methylated in NB tumor samples (Henrich et al., Cancer Res 2018; Li et al., ONCOGENE, 2018). Further, combination of EPZ-6438 and 5-aza-deoxycitide, suppressed proliferation in the EPZ-6438-resistant 3 NB cell lines effectively. These inhibitors suppressed the resistant NB cell proliferation synergistically as CI was less than 1.0. Transcriptome/methylome analysis of the EPZ-6438 and 5-aza-deoxycitide-treated IMR32 cells revealed the combinational epigenetic regulation of the tumor suppressors and oncogene expression.

Summary/Conclusions

Finally, methylome analysis of the promoters can be a biomarker of EPZ-6438-included epigenomic treatments for unfavorable NB patients. We will present the responsible CpG island methylation as biomarkers for the application of EZH2i/DNMTi combination therapy.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 76

Single-cell RNA-sequencing of peripheral neuroblastic tumors identifies an aggressive transitional cell state at the junction of an adrenergic-mesenchymal transdifferentiation trajectory

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

Peripheral neuroblastic tumors (PNTs) represent a spectrum of tumors derived from the neural crest comprising four histological variants, including neuroblastoma, ganglioneuroblastoma nodular, ganglioneuroblastoma intermixed, and ganglioneuroma. Emerging evidence from recent studies propose that tumor plasticity is a feature of neuroblastic cells. In these studies neuroblastomas were subtyped according to a two-group classification conforming to either an adrenergic/noradrenergic state or a mesenchymal/neural crest cell state. Importantly, cell state was seen to be relevant for therapeutic efficacy, with mesenchymal neuroblastic cells more resistant to conventional anti-cancer therapies. Whilst these studies have demonstrated the importance of tumor heterogeneity and cell plasticity in neuroblastoma, bulk-tissue profiling is still compromised by averaging the individual impact of cell types within the tumor microenvironment, potentially hindering the identification of other cell types.

Aims

To elucidate the underlying tumor heterogeneity present across the spectrum of PNT subtypes, with regard to: 1) neuroblastic cell types and 2) tumor microenvironmental cell types.

Methods/Materials

We performed Smart-seq2 based single-cell transcriptomic analysis of 5309 cells from seven PNTs including 3 neuroblastomas, 2 ganglioneuroblastoma nodular, 1 ganglionneuroblastoma intermixed and 1 ganglioneuroma. To investigate neuroblastic cell types, we first separated malignant and non-malignant cells using inferred copy number profiles and then performed trajectory analyses to identify putative differentiation trajectories and associated gene signatures. We then utilized publicly available neuroblastoma RNA-seq and H3k27ac ChIP-seq datasets to decipher epigenetic regulatory mechanisms influencing these gene signatures and then gauge prognostic significance. To identify tumor microenvironmental cell types we clustered and then classified non-malignant cells using canonical gene expression markers, using immunohistochemistry on tumor sections to further validate these findings.

Results

We demonstrate extensive transcriptome heterogeneity within and between individual PNTs. Trajectory modelling showed that malignant neuroblasts move between adrenergic and mesenchymal cell states via a novel state that we termed a “transitional” phenotype. Transitional cells are characterized by gene expression programs linked to a neurodevelopment, and aggressive tumor phenotypes such as rapid proliferation and tumor dissemination. Among primary bulk tumor patient cohorts, high expression of the transitional gene signature was highly predictive of poor prognosis when compared to adrenergic and mesenchymal expression patterns. High transitional gene expression in neuroblastoma cell lines identified a similar transitional H3K27-acetylation super-enhancer landscape. Additionally, examination of PNT microenvironments, found that neuroblastomas contained low immune cell infiltration, high levels of non-inflammatory macrophages, and low cytotoxic T lymphocyte levels compared with more benign PNT subtypes.

Summary/Conclusions

Collectively, our study reveals the extensive transcriptomic heterogeneity in PNTs. Description of a novel transitional cell type at the junction of an adrenergic-mesenchymal transdifferentiation

trajectory supports the concept that PNTs have marked phenotypic plasticity that is relevant for patient prognosis. This is an important consideration for patient risk stratification and the development of new therapies for PNTs.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 77

Polycomb EZH1 regulates cell cycle/drug sensitivity of neuroblastoma cells in concert with MYCN

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Topic Gene regulatory networks and epigenetics: from basic science to therapy

Background/Introduction

Polycomb repressive complex 2 (PRC2) is a multiprotein chromatin-modifying complex that is essential for vertebrate development and differentiation. PRC2 is composed of a trimeric core of SUZ12, EED, and EZH1/2, which catalyses the methylation of histone H3 at lysine 27 (H3K27me1/2/3). Recently, the role of PRC2, especially EZH2-related functions has been received a lot of attentions in NB tumorigenesis (Chen L et al., J Clin Invest. 2018; Laurel T. B-E et al., EJC. 2017; Tsubota S et al., Cancer Res. 2018; Li Z et al., ONCOGENE. 2018). Enhancer of zeste 1 polycomb repressive complex 2 subunit (EZH1), a homolog of EZH2, has different biochemical activities and effects on chromatin modification compared to EZH2.

Aims

We studied the EZH1 roles in NB biology by EZH1 knockdown and inhibitor treatments by using transcriptome and comprehensive chromatin modification analyses for development of a new epigenetic NB therapy.

Methods/Materials

Transcriptome analysis were performed using the Agilent platform of 8 × 60 K design ID G4851B.

qChIP and ChIPseq experiments were done according to the previous paper (Li Z et al., ONCOGENE. 2018). To evaluate synergistic efficiency of UNC1999 and 5-FU to suppress NB cells, the Combination Index (CI) was calculated by the Chou-Talalay (2010) method using CompuSyn (http://www.combosyn.com/).

Results

In the present study, we found that EZH1 depletion in *MYCN*-amplified neuroblastoma cells resulted in significant cell death as well as xenograft inhibition. EZH1 depletion decreased the level of H3K27me1; the interaction and protein stabilization of MYCN and EZH1 indicated the cooperative roles in epigenetic transcriptional regulation. Transcriptome analysis of EZH1-depleted cells resulted in up-regulation of neural differentiation-related pathways, such as “Nervous system development (GO:0007399)”, and “Neuron projection development (GO:0031175)”; down-regulation of the cell cycle progression-related pathways, such as “DNA recombination (GO:0006310)”, “cell cycle (GO:0007049)”, and “mitotic cell cycle (GO:0000278)”. In particular, GSEA revealed down-regulation of reactome E2F-mediated regulation of DNA replication along with key genes of this process, *TYMS, POLA2*, and *CCNA1*. *TYMS* and *POLA2* were transcriptionally activated by MYCN and EZH1-related epigenetic modification. ChIPseq/qChIP analysis showed the MYCN/EZH1 co-localization, which indicated that EZH1 and MYCN bind to the *TYMS* promoter regions in *MYCN*-amplified NB cell lines. Further, luciferase experiments using the *TYMS* promoter region confirmed MYCN-related *TYMS* induction. Treatment with the EZH1/2 inhibitor UNC1999 also induced cell death, decreased H3K27 methylation, and reduced the levels of TYMS and POLA2 in NB cells. Previous reports indicated neuroblastoma cells are resistant to 5-fluorouracil (5-FU) and *TYMS* (encoding 5-FU-target thymidylate synthetase) has been considered the primary site of action for folate analogues. Intriguingly, UNC1999 treatment significantly sensitized MYCN-amplified neuroblastoma cells to 5-FU treatment.

Summary/Conclusions

Taken together, EZH1 plays roles as an inhibitor of neuronal differentiation and as a transcriptional activator for thymidylate synthetase (TYMS), in cooperation with MYCN in NB. EZH1/2 inhibition may be an effective strategy for development of a new epigenetic treatment for NB and combination therapy with 5-FU seems to be promising for *MYCN*-amplified NBs.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 78

Pilot clinical trial of DNA vaccination against neuroblastoma

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

Effective treatment of high risk neuroblastoma (NB) remains a serious problem for pediatric oncology. A promising therapy option for this group is immunotherapy, including antitumor vaccination. We launched a pilot clinical trial of combined DNA vaccine, preceded by a long period of preclinical *in vitro* and *in vivo* studies in the animal model.

Aims

to check the safety and immunogenicity of combined DNA vaccination against neuroblastoma

Methods/Materials

DNA vaccination requires complete immune recovery, the absence of a bulky tumor, the time for the induction of an immune response, and the absence of immunosuppressive immunotherapy during the vaccination period. Considering this, we decided to include patients in remission 2-4 months after the completion of relapse chemotherapy. Each patient receives the vaccine against three NB-associated antigens of 6 candidates: tyrosine hydroxylase, Phox2B, Survivin, MAGE-A1, MAGE-A3, PRAME. IHC and RQ-PCR confirmation determines the choice of antigen for each patient. The genetic design of the vaccine consists of fusion of antigen and immune-enhancer – the potato virus X coat protein (PVXCP) gene.
The vaccine includes two ways of administration. An injectable vaccine based on the DNA-polyethyleneimine conjugate (PEI) is administered intramuscularly. Linear PEI-20kDa was chosen as optimal for *in vivo* vaccination according to the results of our preclinical tests. Conjugation is carried out by mixing a DNA and PEI solutions in a mass ratio of DNA:PEI = 1.2-1.3 (400 µg DNA/500 µg PEI).
The oral form of the vaccine is delivered by live bacteria. The attenuated strain SS2017 (*aroA*$^{-}$, *guaAB*$^{-}$) was obtained by further modification of the original vaccine strain *Salmonella enterica* serovar typhimurium SL7207. One dose includes 10^{10} CFU carrying plasmid DNA vaccine in suspension with 50% glycerol in a gelatin capsule. Three vaccination courses are carried out, with three introductions of a new antigen in each.
Before and during vaccination, accompanying chemotherapy is conducted, including

cyclophosphamide 300 mg/m^2 once three days before vaccination, lenalidomide 25 mg/day starting 7 days before the first vaccination, propranolol and celecoxib according to the metronome therapy regimen.
Monitoring includes: assessment of minimum residual disease (MRD) by RQ-PCR; determination of circulating tumor cells ($CD45^-$, $GD2^+$, $CD81^+$, $CD9^+$, $CD56^+$); production of INF-γ by lymphocytes in the presence of PVXCP protein or peptide library of the corresponding antigen by ELISPOT.

Results

The protocol and ethics committee were approved on January 9, 2019. To date, 4 patients have received a full course of vaccination. Patients tolerated the vaccine well without adverse symptoms. All four are in remission with negative MRD in the bone marrow. Two of them (B1, K3) are free of tumor by MRT/MIBG scintigraphy. 6 months after vaccination, patient B1 had isolated CNS relapse. M4 – had a residual MIBG-positive site in paravertebral area. Both foci of the tumor were completely surgically removed and pronounced CD8 infiltration was observed. Immune response was negative for patient K3, other three patients had pronounced CD4 immune response against the PVXCP protein and positive CD8 to the antigens peptide library.

Summary/Conclusions

Combined vaccination of DNA-PEI injection and oral bacteria was safe and immunogenicity against NB antigens was proved

Type Translational

Presentation Preference Traditional poster

Abstract nr. 79

Evaluation of the Effect of Trial Participation on Outcomes for High-Risk Neuroblastoma Patients: A Report from the International Neuroblastoma Risk Group Project

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Topic Other

Background/Introduction

Therapeutic trials allow for groundbreaking advancements in care, leading to new treatment options and superior survival outcomes. Even for those assigned to standard of care, participants in therapeutic trials may benefit by tight adherence to best practices per study requirements. We hypothesized neuroblastoma patients enrolled on therapeutic trials would have improved outcomes compared to biology trial patients. Historically, African Americans and other minorities have been underrepresented in therapeutic trials. Therefore, we speculated a low proportion of African American patients would enroll on neuroblastoma therapeutic trials.

Aims

We sought to determine the associations of demographics, clinical phenotype, and tumor biology with therapeutic trial participation and if trial participation impacted event-free survival (EFS).

Methods/Materials

The INRG database restricted to high-risk patients enrolled on an upfront CCG, POG, or COG therapeutic or biology trial, diagnosed from 1991-2016, was analyzed. Patients were dichotomized as either enrolled on an upfront therapeutic trial or a biology trial only. Differences between clinical and tumor factors between groups were assessed by t-tests and chi-squared tests. As outcomes for patients enrolled on ANBL0532 were not available, patients were classified pre-2006 by treatment Era (1: 1991-1998 and 2: 1999-2006). Five-year EFS was estimated using the Kaplan-Meier method and groups were compared with the log-rank test.

Results

The analytic cohort included 5,334 high-risk patients. Of these, 2,199 were enrolled on an upfront therapeutic trial (73% on CCG3891, A3973, or ANBL0532) and 3,135 were enrolled on biology trials only (94% on ANBL00B1 or POG9047). The enrollment of African American patients on therapeutic trials was similar to US census data (14.8% and 14.1%, respectively). Compared to patients enrolled only on biology trials, those enrolled on a therapeutic trial were more likely to have INSS stage 4 disease (87.0% vs. 81.6%, $p<0.01$), unfavorable histology (95.7% vs. 93.8%, $p=0.01$), and undifferentiated or poorly differentiated tumors (97.2% vs. 95.0%, $p<0.01$). There were no detectable differences according to age, sex, MYCN-amplification, MKI, or ploidy. There was no difference in EFS between patients according to therapeutic trial enrollment or not for either Era 1 ($p=0.1$) or Era 2 ($p=0.2$). A subgroup analyses of patients diagnosed between 1991 and 1996 showed no differences in 5-year EFS for those on the control arm of CCG3891 (n=284, EFS 25.2%, 95%CI 20.6-30.9%) compared to those only on biology trials (n=220, EFS 30.7%, 95% CI 25.2-37.6%) ($p=0.3$). A similar analysis comparing all patients enrolled on A3973 (n=440, EFS 39.0%, 95%CI 34.7-43.9% to biology trial patients diagnosed between 2001 and 2005 (n=547, EFS 41.8%, 95% CI 37.7-46.4%) also failed to detect a difference ($p=0.3$).

Summary/Conclusions

The clinical characteristics and tumor biology of patients enrolled on therapeutic trials are similar to those who only participate in biology trials. Therapeutic trial participants reflect the demographics of the North American population, with high representation of African American patients. The similarity in outcomes between patients enrolled on therapeutic trials and those only registered on biology trials likely reflects a close adherence to contemporary best clinical practices for all patients regardless of trial participation.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 80

The functional annotation and exploration the SOX11 enhanceosome

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Topic Gene regulatory networks and epigenetics: from basic science to therapy

Background/Introduction

Neuroblastoma (NB) is the most common extra cranial solid tumor during early childhood, arising from progenitors of the sympathetic nervous system. We identified the adrenergic specific transcription factor *SOX11* as a lineage dependency factor and a putative master regulator of epigenetic plasticity in NB. The expression of lineage identity genes is typically regulated by cell type specific enhancers thereby acting as putative cell-specific vulnerabilities that may be targeted while minimizing harm to the normal development of tissues.

Aims

We aimed to gain insight into the long-range control of *SOX11* expression in NB cells as a first step towards cell type specific therapeutic targeting of SOX11-driven neuroblastoma.

Methods/Materials

We analyzed H3K27ac data to map noncoding regulatory enhancer regions in the vicinity of the *SOX11* locus. Chromatin conformation analysis (4C-seq) additionally shows evidence for looping contacts between the *SOX11* promotor region and the *SILC1* locus, a lncRNA downstream of

SOX11. For further exploration of the function of SILC1, chromatin isolation by RNA purification (ChIRP) is performed to identify lncRNA bound DNA sequences. Functional inhibition of the *SILC1* locus is explored using dCas9-KRAB silencing in NB cells.

Results

We identified a super-enhancer in a large gene poor region, 1.1 Mb distal to the *SOX11* locus. This region coincides with the previously annotated lncRNA coined *SILC1* which was shown to be implicated in neurite outgrowth and neuron regeneration. *SILC1* was found to be strongly expressed in adrenergic NB cells while absent in mesenchymal NB cells and highly correlated to *SOX11* expression levels in both NB cell lines and tumors. Also, in the normal developing sympathetic lineage, *SILC1* expression pattern closely resembled that of *SOX11*. Using 4C-seq, we observed looping in this highly active region between the *SOX11* and *SILC1* promoter in adrenergic NB cells while absent in the mesenchymal NB cell line SH-EP. We are currently unraveling the role of *SILC1* using ChIRP sequencing and a dCas9-KRAB silencing system.

Summary/Conclusions

In conclusion, our data suggests that SILC1 plays a crucial role in assisting the function of the transcription factor SOX11 thus offering an entry point for targeted therapy for SOX11-driven neuroblastoma.

Type Basic

Presentation Preference Rapid Fire Poster Presentation

Abstract nr. 81

Antibodies from mice cured of melanoma identify >3000 antigens; several are homologs for proteins found in HLA-clefts on human neuroblastomas

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

Background/Introduction: C57Bl/6 mice bearing the $GD2^{pos.}$ B78 melanoma were cured of large (~$200mm^3$) tumors by a combination of 12-Gy radiotherapy + intratumoral anti-GD2/IL2 fusion protein (the hu14.18-IL2 immunocytokine)[1,2]. Cured mice showed memory by rejecting a B78 as well as a $GD2^{neg.}$ B16 melanoma rechallenge[1,2]. Sera from cured mice, showed strong binding to B16 melanoma (by flow cytometry), compared to background binding by naïve mouse sera.

Aims

Aims: We seek to comprehensively identify antigens that are recognized by antibodies induced during the immuno-therapeutically driven rejection of large neuroectodermal origin cancers in mice, and determine their relevance to human neuroblastoma antigens.

Methods/Materials

Methods/Materials: Sera from 6 naïve mice and 7 cured mice that rejected rechallenge were evaluated on a high-density peptide array, reflecting the entire murine proteome (~8.5 million 16-mer peptides, reflecting the full proteome of ~56,000 uniprot IDs, ~22,000 genes) using technology developed by Nimble Therapeutics[3]. This methodology should allow detection of any peptide-based epitope that is ≤16 amino acids.[3]

Results

Results: These immune sera from individual mice show clear binding to over 20,000 separate probes, derived from ~6500 separate genes. 6777 probes were each co-recognized by 2 separate immunotherapeutically cured immune mice (corresponding to ~3000 genes). The epitopes demonstrating the most robust binding by multiple mouse sera are being identified/characterized. We focused on 162 murine proteins that are the homologs of 162 human proteins from which peptides were highly represented in the clefts of MHC molecules on human neuroblastomas (also of neuro-ectodermal origin, like melanoma), as identified by mass spectrometry in the lab of John Maris (see Yarmarkovich M., ANR 2020). Of the murine proteins identified by the immune mouse sera, 34 were from this group of 162 human neuroblastoma homologs, including: Igfbpl1, Phox2b and Cdk1. In addition, of the unique murine genes identified by these immune mice, human homologs for 149 showed strong expression on human neuroblastoma and not normal tissues, as determined by comparison of RNA-seq data between 153 tumors from the TARGET database and 1641 normal tissues from the GTEx database. We are now focusing on the proteins showing the strongest binding, to characterize them and determine if any new targets may be identified. Future studies will perform similar analyses with immune sera from mice immunotherapeutically cured of neuroblastoma.[4]

Summary/Conclusions

Summary/Conclusions: Our data suggest that:

Antigens detected prominently by sera of cured mice may correspond to homologous human antigens that could be recognized by immunotherapy-treated patients;

Acquisition of strong antibody responses to certain antigens shared by several tumors might potentially serve as an early serum biomarker for an effective clinical immunotherapeutic response;

Antigens strongly detected by immune mouse sera may correspond to homologous human antigens, that may be considered as future targets for creation of mAb or engineered T cell receptor binders for therapeutic development.

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Type Basic

Presentation Preference Oral presentation

Abstract nr. 82

Role of Clinical Trial Nurses in Humanized Bispecific Antibody (Hu3F8-BsAb) Therapy

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

Anti-GD2 immunotherapy has improved progression-free survival rates of patients with metastatic Neuroblastoma. The International Neuroblastoma Risk Group found that the 5-year post-relapse overall survival for stage 4 Neuroblastoma was 8% (Journal of clinical oncology 2011;29:208-13). Novel treatment approaches are therefore warranted.
A Phase I/II study of humanized 3F8 bispecific antibody (Hu3F8-BsAb) in patients with relapsed/refractory neuroblastoma, osteosarcoma, and other GD2(+) solid tumors opened in 2019 (NCT03860207). Hu3F8-BsAb has two specificities: Hu3F8 which targets the cell surface disialoganglioside GD2, and huOKT3 (anti-CD3) which binds and activates T-cells. This hu3F8-BsAb has shown potent anti-tumor activity in *in vitro* and *in vivo* preclinical animal models.

Aims

The aim of this report is to describe the role of Clinical Trial Nurses (CTNs) in facilitating the conduct of the above study in both inpatient and outpatient settings.

Methods/Materials

We reviewed the role of CTNs in the care of patients enrolled on this clinical trial and in protocol education.

Results

CTNs play an active role in all aspects of this clinical trial, from protocol activation, patient screening and consent, treatment, toxicity management, relevant data capture, and coordination of

care.
Protocol activation encompasses the development of study tools such as order sets, patient consents and other materials. Prior to and during execution of the phase I/II trial, CTNs assist in the development of education materials for clinicians for adherence to protocol requirements and to update them on toxicities. CTNs coordinate the periodic distribution of this information to nurses, nurse practitioners, and physicians caring for enrolled patients. CTNs are responsible for real-time assessment of toxicities. This includes recognition and management of adverse events, anticipated and unanticipated, specifically cytokine release syndrome and neurotoxicity. Issues related to the clinical trial are discussed in weekly clinic meetings.
Patients are screened for eligibility by the CTN and the consenting physicians of the trial. CTNs ensure that every patient and guardian have a clear understanding of the nature of clinical trials, specific study rationale, planned treatment interventions, the risks and potential benefits of the treatment, and follow-up requirements. The CTN is present at each infusion to ensure protocol compliance, help with management of adverse reactions and update relevant drug and trial information for practitioners. As part of protocol compliance, CTNs ensure accurate collection of blood and tissue samples for pharmacokinetics and biologic correlate studies which require coordination with the clinical and research laboratories. The first six patients on Hu3F8-BsAb were treated successfully without dose limiting toxicities. This supports a continuation of dose escalation in this trial.

Summary/Conclusions

The key to the successful implementation of this Hu3F8-BsAb trial is close coordination and communication between CTNs, nurses, PIs, physicians, and the patient/family, to maximize compliance, safety, quality of clinical and research data, and adherence to institutional and FDA guidelines. For novel agents such as bispecific antibodies where toxicities in children are unknown, CTNs should provide the conduit for information transfer and data capture vital for reaching trial intended endpoints.

Type Clinical

Presentation Preference Traditional poster

Abstract nr. 83

GD2 is a Macrophage Checkpoint Molecule and Combined GD2/CD47 Blockade Results in Synergistic Effects and Tumor Clearance in Xenograft Models of Neuroblastoma and Osteosarcoma

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

The use of anti-GD2 antibodies for neuroblastoma (NBL) has resulted in enhanced survival, but many patients still relapse and ultimately die of their disease. Additionally, despite expression of GD2 on osteosarcoma (OS), anti-GD2 antibodies have not proven widely effective in that disease. Enhancing the efficacy of anti-GD2 antibodies could result in improved patient outcomes.

Aims

CD47 is the dominant “Don’t Eat Me” signal expressed by cancer cells to inhibit macrophage phagocytosis. Blocking CD47 with antibodies leads to phagocytosis of tumor cells. A recent trial of anti-CD47 combined with rituximab (anti-CD20) resulted in a high complete response rate in patients with B cell lymphoma. Using the same rationale, that disinhibiting a macrophage in the presence of a tumor targeting antibody would result in enhanced anti-tumor activity, we studied the combination of CD47 blockade with anti-GD2 antibody.

Methods/Materials

In vitro phagocytosis assays were carried out using human macrophages derived from human monocytes. In vivo studies were carried out in immunodeficient mice with an intact myeloid compartment. These mice were xenografted with human tumors and treated with anti-CD47 antibody, anti-GD2 antibody, or a combination of both antibodies. Tumor growth was monitored by bioluminescence or caliper measurement.

Results

We found a striking synergy between dinutuximab and anti-CD47 *in vivo*. This combination led to the complete clearance of both orthotopic and metastatic models of NBL. Additionally, the combination significantly delayed the growth of OS xenografts, resulting in enhanced survival, whereas single agent anti-GD2 or anti-CD47 had no anti-tumor activity. Finally, in a murine model of metastatic pulmonary OS, the combination of anti-GD2/CD47 led to a near elimination of all metastatic burden.

To understand the biologic basis for the synergistic effects observed, we studied both the effects of GD2 crosslinking on tumor cells and the effects of GD2 blockade on macrophages. When NBL cells are treated with dinutuximab *in vitro*, a portion of the cells die, and those that survive upregulate surface calreticulin, an important pro-phagocytic ("Eat Me") signal that stimulates macrophages to remove dying cells. Therefore, GD2 ligation results in cellular processes that drive macrophages to phagocytose tumors.

We measured *in vitro* phagocytosis of NBL cells and observed a synergistic effect in the presence of anti-GD2/CD47 compared to the single agents. However, when we combined anti-CD47 with a tumor specific antibody recognizing B7-H3, there was no synergy. We therefore hypothesized that GD2 itself is inhibitory to macrophages. We have identified the ligand for GD2, a molecule expressed on macrophages known to inhibit phagocytosis. Therefore, GD2 is a macrophage checkpoint capable of suppressing tumor cell phagocytosis.

Summary/Conclusions

In summary, we have identified a novel combination of anti-GD2 and anti-CD47 antibodies that is highly effective in xenograft models of NBL and OS and will soon be tested in children. Additionally, we have shown that GD2 itself is a macrophage checkpoint or "Don't Eat Me" signal. This finding may explain the high levels of GD2 expressed on NBL and OS as a mechanism for immune evasion.

The use of anti-GD2 antibodies for neuroblastoma (NBL) has resulted in enhanced survival, but many patients still relapse and ultimately die of their disease. Additionally, despite expression of GD2 on osteosarcoma (OS), anti-GD2 antibodies have not proven widely effective in that disease. Enhancing the efficacy of anti-GD2 antibodies could result in improved patient outcomes.

GD2 is a Macrophage Checkpoint Molecule and Combined GD2/CD47 Blockade Results in Synergistic Effects and Tumor Clearance in Xenograft Models of Neuroblastoma and Osteosarcoma

Type Translational

Presentation Preference Oral presentation

Abstract nr. 84

MCM2 and CA9 are novel potential targets for neuroblastoma pharmacological treatment

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Despite survival rates of Neuroblastoma (NB) patients have been improved significantly by advancements in cancer treatment in recent years, high-risk neuroblastoma (HR-NB) remains associated to high mortality. In order to investigate NB cell differentiation and to overcome the lack of effective pharmacological treatments for HR-NB, the development of in vitro and in vivo models that better recapitulate the disease are required.

Aims

We employed the NDM29-based model in order to identify genes resulting progressively up-regulated proceeding from the non-malignant stage of mature neuron-like cells to the fully malignant stage and those up-regulated during cell differentiation exhibiting the highest expression level in the differentiated non-malignant cells.

Methods/Materials

In the present study, human neuroblastoma cancer cells were genetically engineered to overexpress NDM29, a non-coding RNA whose increased synthesis causes the differentiation toward a neuronal phenotype. According to the different numbers of extra copies integrated and the different efficiency of expression, these clones express NDM29 RNA at different levels and exhibit a directly correlated level of differentiation/maturation toward a neural phenotype. This in vitro model encompasses NB cell differentiation stages and can be used to identify potential novel pharmacological targets.

Results

This analysis allowed us to identify (by low-density RT-PCR arrays) two gene sets, one over-expressed during NB cell differentiation and the other up-regulated in more malignant cells. Challenging two HR-NB gene expression datasets, we found that these two sets of genes are related to high and low survival, respectively. Mouse NB cisplatin-treated xenografts allowed the identification of two genes within the list associated to the malignant stage (MCM2 and CA9) whose expression was positively correlated with tumor growth. Thus, we tested their pharmacological targeting as a potential therapeutic strategy. We measured mice survival and growth rate of human NB xenografts treated with cisplatin in the presence of MCM2/CA9 inhibitors (ciprofloxacin and acetazolamide).

Summary/Conclusions

The results obtained show that NDM29-dependent multistep NB differentiation model recapitulates several features of NB cell differentiation and is suitable to identify novel prognostic genes and their inhibitors thus providing novel potential therapeutic molecules to be further investigated in preclinical studies. In our experimental setting, two gene lists represent pro-malignant and pro-differentiation sets where the pro-malignant genes might represent novel targets for innovative NB therapies. We observed that, *in vivo*, the pharmacological inhibition of MCM2 and/or CA9 is efficacious to inhibit/block tumor growth and increases cisplatin activity, supporting the possible testing of these two genes as targets for NB therapy.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 85

Role of Rho-GTPases signalling in Neuroblastoma

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Topic Other

Background/Introduction

Neuroblastoma (NB) is the most frequent extracanial solid tumour in childhood, causing 15% of all paediatric oncology deaths. It arises in the embryonic neural crest due to an uncontrolled behaviour of sympathetic nervous system progenitors giving rise to heterogeneus tumours. The disease is characterized for its variable clinical presentation, which ranges from spontaneous regression to uncurable aggressive metastasic tumours. During embryonic development, neural crest cells migrate throughout the embryo and ultimately differentiate into multiple cell types such as sympathetic neurons, glial derivatives or pigment cells.
Failures on neural crest cells migration and differentiation processes could result in the development of NB. Recent genomic analysis has identified Rho GTPase signaling pathway as one of the most frequently mutated in neuroblastoma tumours. Rho-GTPases proteins act as molecular switches cycling between GDP inactive state and GTP active state and regulated by multiple positive and negative factors. This family of proteins plays a key role in many physiological and pathological processes like cell proliferation, cell migration or differentiation. As a result, their deregulation could be having an impact on aggressive, resistant or metastatic NB.

Aims

To describe the consequences of Rho-GTPases signalling deregulation in high risk NB

Methods/Materials

Extensive expression studies were performed using “R2 plataform” (http://r2.amc.nl). Quantitative PCR was used to analyse Rho-GTPases gene expression in CHLA-20, CHLA-255, IMR-32 and SK-N-SH NB cell lines. NB cell lines stably expressing relevant Rho GTPases were generated. Cell viability, cell differentiation and xenografts assays were carried out using CHLA 20 and CHLA 255 NB cell lines, in immunocompromised mice.

Results

In silico expression studies show general deregulation of many GTPases in high risk NB patients. RhoB, RhoC, RhoF, RhoH, Cdc42, RhoJ, RhoQ, Rnd2, Rnd3, Rac1, Rac2, RhoU, RhoBTB1/2 seemed to be downregulated in high risk cases, meanwhile RhoA, RhoD, Rnd1, Rac3, RhoG, RhoV were upregulated in 3 and 4 stages, compared to 1 and 2 stages. Moreover, low levels of RhoB and RhoU determined poor overall survival in NB patients. Rho-GTPases expression like RhoB or Rac3 were also found significantly deregulated in NB relapses. In addition, some RhoGTPases were enriched in particular NB cell populations. Some Rho-GTPases modulators and effectors were also deregulated in high risk NB, correlating with Rho-GTPases expression. Rho-GTPases expression in CHLA 20, CHLA 255, IMR-32 and SK-N-SH NB cell lines were particularly low. Cdc42 overexpression in CHLA 20 and CHLA 255 NB cell lines decreased cell proliferation compared to parental cell lines. In addition, glial differentiation was promoted in Cdc42 overexpressing NB cell lines. Cdc42 overexpressing cell lines gave rise to lower tumour incidence and smaller size. These tumours showed lower expression of proliferative markers, like Ki67, comparing to control xenografts.

Summary/Conclusions

The Rho-GTPases signalling pathway is altered in high risk NB cases, leading us to think of their possible role in aggressive NB. Specially, the overexpression of the Rho-GTPase Cdc42 in NB cells causes a decrease in cell proliferation *in vitro* and *in vivo*. In addition, Cdc42 promotes cell differentiation in NB cell lines.

Type Basic

Presentation Preference Traditional poster

Abstract nr. 86

Relative bioavailability and comparative pharmacokinetics of a novel 13-cis-retinoic acid oral liquid formulation versus drug extracted from capsules: a randomised, open label, multi-dose, cross-over clinical trial in children with neuroblastoma

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Topic Other

Background/Introduction

In the last decade, the administration of 13-cis-retinoic acid (13-CRA) differentiation therapy in high risk neuroblastoma (HR-NBL) patients with minimal residual disease has been proven to be effective, and reported to prevent tumour relapse after myeloablative therapy. 13-CRA is now established as standard treatment alongside immunotherapy and is administered orally using the licensed capsule form. As few young children are capable of swallowing 13-CRA capsules, carers often have to administer the drug by first extracting liquid out of the capsule. This method of administration is associated with up to 20-fold variability in patient exposure, potentially increasing the risk of treatment failure. Furthermore, the practice of extracting drug from capsules puts mothers of childbearing age at risk of accidental exposure with 13-CRA, which can cause birth defects in unborn babies. Nova Laboratories Limited have developed a convenient, ready-to-use, multi-dose, oral liquid formulation of 13-CRA, which has now been investigated in a clinical trial.

Aims

The aim of the current trial was to investigate the comparative pharmacokinetics, safety, tolerability and palatability of 13-CRA oral liquid (test) and 13-CRA extracted from capsules (reference), in patients aged from 0 months to < 21 years.

Methods/Materials

This was a multi-centre, open label, randomised, multiple dose, cross-over pharmacokinetic study. The decision to initiate oral 13-CRA therapy was independent of the study protocol and was

conducted in patients requiring at least two 14-day treatment cycles of 13-CRA. 13-CRA was prescribed at a dose of 200mg/m^2/day (if bodyweight ≥ 12kg) or 160mg/m^2 (if body weight ≤12kg) for both test and reference product. Blood samples for pharmacokinetic analysis were taken 0.5, 1, 1.5, 2, 3, 4, 5, 6 and 24-48 hours post-dose, on Day 1 and Day 14 of each of the two treatment cycles. Primary end-points were population pharmacokinetics (of both 13-CRA and 4-oxo-13-CRA metabolite); secondary end-points were safety, tolerability and palatability of the formulations.

Results

22 patients consented and 20 completed the study, across 12 sites in the UK. 12 patients received the treatment via naso-gastric tubes, 8 orally. The pharmacokinetics of 13-CRA were adequately described by a one-compartment linear model. Drug formulation had an effect on the relative bioavailability and absorption kinetics of 13-CRA. Absorption of the oral liquid was best described by transit compartments, indicating a delay in absorption. No such delay was observed for the capsule. The first order absorption parameter for the oral liquid was 3-fold higher than the capsule, indicating a faster absorption compared to the capsule, once the delay period is over. Relative bioavailability was estimated to be 1.23 (23% increase for oral liquid vs capsule), although the CI was wide (0.81 – 1.65). 22 serious adverse events were reported (2 SUSARS) but there were no safety concerns during the study and no differences between the two formulations. Palatability feedback was good for the oral liquid and parents preferred the treatment to the capsule formulation.

Summary/Conclusions

The pharmacokinetics, safety, tolerability and palatability of a novel oral liquid formulation of 13-CRA compare favourably to 13-CRA extracted from the licensed capsule formulation.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 87

The prognostic impact of chromosome 11q loss in stage 4S neuroblastoma

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Genomic loss of chromosome 11q is known to be associated with poor outcome in neuroblastoma. According to the INRG risk classification, stage 4S patients harboring loss of chromosome 11q are considered to be at high risk, however, this classification was developed on few patients only.

Aims

We here aimed to assess the prognostic impact of 11q loss in a large cohort of patients with stage 4S.

Methods/Materials

Patients with stage 4S disease and lacking *MYCN* amplification who had been registered in the German neuroblastoma trials NB95-S, NB97 and NB2004 between 1995 and 2016 were included in the analysis. The copy number status of chromosome 11q at diagnosis was investigated by interphase fluorescence in situ hybridization (FISH) analysis in a dual color procedure, with DNA probes covering the region of 11q22.3 and centromere as control. At least 50 cells per slide were analyzed. Loss of 11q (deletion or imbalance) was defined according to the international

consensus criteria.

Results

Tumor samples of 165 neuroblastoma patients with stage 4S disease were analyzed for 11q copy number status. The median follow-up of patients was 9.6 years (0.15-25 years). Loss of chromosome 11q was found in 10 of the 165 tumors (6.1%; deletion, n=5; imbalance, n=4; deletion and imbalance, n=1). Median age at diagnosis was 5.3 months for patients with 11q aberrations (range, 0 – 10.6 months) and 3.2 months for patients without 11q aberrations (range, 0 – 11.2 months; p=0.098). Metastatic pattern did not differ between the groups. Tumor progression was observed in 4/10 patients with 11q loss (40%), and in 27/155 patients with normal 11q (17%). Five-year event-free survival was 0.70 ± 0.15 in patients with 11q loss and 0.83 ± 0.03 in patients with normal 11q (p=0.065). Five-year overall survival was high in both groups (11q loss, 0.90 ± 0.10; 11q normal, 0.93 ± 0.02; p=0.26). Interestingly, metastatic progression to stage 4 disease was observed for all four patients with 11q loss who had an event. By contrast, only 10 of the patients with normal 11q had metastatic progression into stage 4 disease (p=0.005). Accordingly, metastasis-free survival differed significantly between the groups (0.70 ± 0.15 vs. 0.93 ± 0.02; p<0.001).

Summary/Conclusions

Loss of chromosome 11q is rare in stage 4S neuroblastoma and does not define high-risk disease *per se*. The relatively high frequency of metastatic progression into stage 4 disease in this subgroup, however, suggest that additional factors associated with 11q loss may drive the clinical course of the disease.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 88

ARNT-dependent HIF-2 transcriptional activity is not sufficient to regulate downstream target genes in neuroblastoma

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Hypoxia-inducible factor (HIF)-2alpha associates with poor outcome in neuroblastoma and glioblastoma, and gain-of-function mutations in the *EPAS1* gene (encoding HIF-2alpha) have been reported in paragangliomas and pheochromocytomas. Specific targeting of a druggable hydrophobic pocket in the HIF-2alpha PAS-B domain with PT2385 have demonstrated promising clinical results for clear cell renal cell carcinoma (ccRCC).

Aims

Investigate the effect of PT2385-mediated inhibition of ARNT dependent HIF-2 activity in neuroblastoma.

Methods/Materials

Neuroblastoma patient-derived xenograft (PDX) cells were treated with PT2385 and analyzed for HIF-2-dependent gene expression, HIF activity, HIF-2alpha protein localization, response to chemotherapy and orthotopic tumor growth *in vivo*.

Results

We detected high levels of HIF-2alpha protein in perivascular niches in neuroblastoma PDXs *in vivo* and at oxygenated conditions in PDX-derived cell cultures *in vitro*, particularly in the cytoplasmic fraction. Nuclear HIF-2alpha expression was reduced following PT2385 treatment, but surprisingly, virtually no effects on tumor growth *in vivo* or expression of canonical HIF downstream target genes *in vitro* were observed. In coherence, RNA sequencing of PT2385-treated PDX cells revealed a virtually unaffected transcriptome. Treatment with PT2385 did not affect cellular response to chemotherapy. In contrast, HIF-2alpha protein knockdown resulted in

profound downregulation of target genes.

Summary/Conclusions

The lack of effect from PT2385 treatment in combination with high cytoplasmic HIF-2alpha expression at normoxia suggest that HIF-2alpha have additional roles than acting as an ARNT dependent transcription factor. It is important to further unravel the conditions at which HIF-2alpha has transcriptional and non-transcriptional roles in neuroblastoma.

Type Translational

Presentation Preference Traditional poster

Abstract nr. 89

Nivolumab and dinutuximab beta in refractory neuroblastoma

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

Already at first diagnosis, more than 50% of patients present with widespread or advanced localized neuroblastoma are at high risk of treatment failure. In these patients, event-free and overall survival after multimodal conventional therapy have been improved by the addition of dinutuximab beta, an anti-GD_2 antibody. The prognosis of children and adolescents with refractory or relapsed neuroblastoma remains poor, however. In the past decade, immunotherapy approaches with checkpoint inhibitors for patients with malignant diseases have gained increasing interest.

Aims

Evaluate the tolerability and response to treatment in patients with refractory neuroblastoma under the novel immunotherapeutic approach of nivolumab with dinutuximab beta.

Methods/Materials

Two patients with refractory neuroblastoma, a 9-year old girl and a 26-year old man, were treated with nivolumab (3mg/kg BW every second week) and dinutuximab beta (100mg/m² BSA every 6 weeks) for 10 months and 6 months, respectively. MRI and mIBG or PET-CT were checked every 3-5 months.

Results

Both patients had been treated with any available conventional therapies in the past including treatment with dinutuximab beta + interleukin-2. After the primary diagnosis of neuroblastoma in 2014, the girl had reached a first remission in 2015, but relapsed in January 2017. Combined

treatment with chemotherapy and dinutuximab beta from December 2017 to September 2018 had resulted in transient improvement, but then again in progressive disease. The young man´s primary diagnosis was in April 2010. He experienced local relapses in 2011, 2012 and 2014. In June 2018, a metastatic relapse and a large abdominal tumor bulk were found. After transient improvement with chemotherapy, tumor surgery and radiotherapy, progressive skeletal metastases were observed in May 2019. As both patients still had a satisfactory quality of life, treatment with dinutuximab beta and nivolumab was initiated. Seven months after starting this therapy, the girl achieved a second complete remission. She is living without disease 3 months after the end of therapy. In the second patient, the soft tissue lesions have disappeared, and his skeletal lesions have regressed after 6 months of therapy.

Summary/Conclusions

The novel combination of the anti-GD_2-antibody dinutuximab beta with the checkpoint inhibitor nivolumab was capable of inducing a complete and a very good partial remission in two heavily pre-treated patients with refractory neuroblastoma. Further trials are warranted to clarify the role of this novel approach in a larger number of patients.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 90

Prp19 is an independent prognostic marker and promotes neuroblastoma progression by regulating the YAP signaling pathway

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Topic Other

Background/Introduction

Neuroblastoma is the most common extracranial solid tumor in children. Although the overall prognosis of neuroblastoma patients has significantly improved, the prognosis of high-risk patients remains poor. Pre-mRNA processing factor 19(Prp19) was previously reported to involve in tumor progression. However, the Prp19 expression and functions remain elusive in neuroblastoma.

Aims

To identify the functions and mechanisms of Prp19 in neuroblastoma.

Methods/Materials

The correlation among clinical characteristics, survival and expression of the Prp19 were assessed using a neuroblastic tumor tissue microarray and was validated in two independent patient datasets. The role of Prp19 in neuroblastoma cell proliferation, migration and invasion was examined by overexpression or downregulation of Prp19. Gene expression regulations of Prp19 were analyzed by RNA-sequencing and bioinformatics.

Results

Neuroblastic tumor tissue microarrays and two independent validation datasets indicated that Prp19 was associated with high-risk markers and served as a prognostic marker for worse clinical outcome in neuroblastoma. Prp19 promoted proliferation, migration and invasion of neuroblastoma cell lines (SK-N-BE (2) and SK-N-AS). KEGG analysis and differential gene expression analysis showed that the expressions of YAP, a key member of the Hippo-YAP pathway, and its downstream genes were significantly inhibited after downregulation of Prp19. Further experiments showed that Prp19 regulates YAP expression by pre-mRNA splicing of YAP. Downregulating Prp19 may reduce the expression of YAP and consequently inhibit cell proliferation, migration and invasion in neuroblastoma.

Summary/Conclusions

Prp19 is associated with high-risk markers and poor prognosis in neuroblastoma and may promote neuroblastoma progression by regulating RNA splicing of YAP.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 91

The Yes-Associated Protein suppresses Harakiri to promote therapy resistance under tumor environmental stress in high-risk neuroblastoma

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

High-risk neuroblastomas (NB) display more genomic alterations at relapse than at diagnosis, including increased transcriptional activity of the Yes-Associated Protein (YAP), a component of the Hippo signaling network. YAP is a transcriptional co-activator that binds to TEA Domain family transcription factors, regulating genes associated with organ growth, cell renewal, and survival. While increased YAP transcriptional signatures in NB portend a poor prognosis, its functional role in NB is not well characterized. Given the lack of YAP or upstream Hippo pathway mutations in relapsed NBs, it is unclear when YAP activity increases, at or before tumor recurrence, and whether it contributes to relapse.

Aims

Our work aims to characterize the functional role of YAP in NB to elucidate how YAP may contribute to tumor recurrence and be therapeutically targeted.

Methods/Materials

Patient-derived xenografts (PDXs) from the same patient’s diagnostic and relapsed tumor were evaluated by RT-qPCR for YAP and downstream targets. Therapy naïve PDXs were grown in *NSG* mice, treated with vehicle or 5 days of topotecan/cyclophosphamide (Topo/Cy), and evaluated for YAP protein. Gain and loss of function approaches were used to perturb YAP expression to analyze YAP effects on cell proliferation *in vitro*, tumor growth *in vivo*, and therapy response in both settings. NB cells with and without YAP were grown as neurospheres in

neurobasal media and YAP expression, YAP-regulated stemness genes, and neurosphere formation were evaluated. RNA sequencing (seq) of SK-N-AS shYAP#2, shYAP#5 versus SK-N-AS control vector was analyzed for changes in gene expression. NLF shYAP and control vector cells were subjected to normal or serum starvation (0.1%FBS) conditions and Harakiri (HRK) expression and apoptosis were quantified.

Results

Our results show that YAP expression and transcriptional activity are increased in relapsed versus diagnostic matched NB PDXs. One cycle of Topo/Cy *in vivo* causes immediate increase in YAP expression in previously YAP-null NB PDX tumors. Neurosphere conditions induce YAP expression and YAP knockdown inhibits stemness gene expression as well as colony formation. ShRNA inhibition of YAP does not affect cell proliferation or therapy response *in vitro*, yet strongly suppresses NB tumor growth and sensitizes xenografts to chemotherapy *in vivo*. RNA seq of SK-N-AS shYAP versus control vector, focusing on genes regulating tumor environment, showed YAP significantly suppresses HRK, a BH3 protein known to activate mitochondrial apoptosis in response to nutrient deprivation. We confirmed HRK expression increases in NB-shYAP cells and decreases in NB cells transduced to overexpress YAP. Notably, HRK expression and intrinsic apoptosis significantly increase in response to serum starvation only when YAP is genetically inhibited. Importantly, HRK is decreased in the relapsed NB PDX compared to the diagnostic paired PDX, and HRK decreases in xenografts after Topo/Cy treatment, validating this reciprocal YAP-HRK relationship *in vivo.*

Summary/Conclusions

YAP may drive the aggressive nature of recurrent neuroblastoma through modulation of *in vivo* stress conditions, promoting tumor growth and therapy resistance via inhibition of HRK. This supports YAP as a logical therapeutic target and HRK as a potential novel tumor suppressor in neuroblastoma.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 92

Biomarkers of a Potent Allogeneic Ex Vivo Expanded Gamma Delta T Cell Product for Neuroblastoma Immunotherapy

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

High-risk neuroblastoma (NB) patient survival significantly improved following introduction of the therapeutic anti-GD2 antibody dinutuximab, increasing interest in other immunotherapies, like adoptive transfer of cellular products. Adoptive cell therapy has focused on αβ T cells, which so far, have been unsuccessful for solid tumors. γδ T cells are an innovative and potentially superior product as they are MHC independent, directly cytotoxic to tumor cells, and lack the alloreactivity of αβ T cells. Previously, we demonstrated γδ T cells expanded from NB patient apheresis products effectively kill NB cell models. However, NB patient marrows are heavily pretreated and residual apheresis products are often depleted. We, therefore, propose expanding from healthy donors to yield a more robust cell therapy product and provide ample supply to meet the demands of our planned first-in-human Phase 1 clinical trial of expanded γδ T cells, which will be combined with temozolomide and dinutuximab for patients with recurrent NB.

Aims

This work aims to potentiate the clinical effectiveness of allogeneic *ex vivo* expanded γδ T cells by defining biomarkers for the most potent donor products.

Methods/Materials

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood of healthy donors and expanded following our GMP-certified, serum free protocol using zoledronate and IL-2. Expansion products were αβ T cell depleted and immunophenotyped for γδ T cell and Natural Killer (NK) cell content prior to assaying cytolytic potential against IMR-5 NB cells. Negative selection was performed for γδ-TCR or CD56 expression to test the cytotoxic potential of γδ T cells and NK cells alone.

Results

Healthy donor PBMC expansions yield cellular products predominantly populated with γδ T cells in addition to NK cells. The γδ T cell-to-NK cell ratio varies donor-to-donor and may depend on the NK cell content pre-expansion. This ratio remains relatively stable through one freeze-thaw cycle. All expanded γδ T cell products effectively kill NB cells *in vitro*. Notably, across 15 expansions, products with a final NK cell content >50% demonstrated the most potent anti-NB cell death (>70-80% at 5:1, effector:target). However, only 5/15 expansions reached a threshold of >50% NK, suggesting most expanders yield γδ T cell-dominant populations. Depletion of γδ-TCR+ cells from the expanded product resulted in a highly potent NK cell population, while depletion of CD56+ cells resulted in a γδ T cell population with reduced killing potential compared to the complete product. It remains unclear how CD56+ γδ T cells contribute to the potency of the overall product or how different NK cell content influences dinutuximab responses, as γδ-TCR+ T cells after expansion express high CD16.

Summary/Conclusions

Not all healthy donors expand cytolytic lymphocytes equally nor are their products equally potent. CD56 expression on both NK and γδ T cells may serve as a biomarker of cytolytic potential. We are determining NB responses to CD56 enriched and depleted products combined with dinutuximab, and distinguishing if CD56 expression pre-expansion predicts potency post-expansion. Concomitantly, we will determine if CD56 may be induced *ex vivo* to derive the most potent γδ T/NK cellular therapeutic to-date.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 93

Oncogenic ALK signaling represses the pro-differentiation DLG2 tumor suppressor to promote an undifferentiated state in neuroblastoma

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Topic Neuroblastoma as developmental disease

Background/Introduction

High-risk neuroblastomas (NBs) typically display an undifferentiated/poorly differentiated morphology. Here we report a novel mechanism by which gain of function activity of the Anaplastic Lymphoma Kinase (ALK) Receptor Tyrosine Kinase (RTK) can counteract differentiation of neuroblastoma cells. ALK activity inhibits the expression of a previously described tumor suppressor gene, *DLG2*. In keeping with a role in NB, expression of DLG2 is low in high risk NB and associated with worse prognosis of these patients. We show here that DLG2 drives the neuronal differentiation process and identify the molecular mechanisms that down-regulate the expression of DLG2 by ALK. *DLG2* is expressed in the ‘bridge signature’ that represents the transcriptional transition state when neural crest cells or Schwann Cell Precursors (SCP) become chromaffin cells of the adrenal gland. A synergistic increase in neuronal differentiation, close to 100%, is observed when DLG2 is expressed with known inducers of differentiation such as retinoic acid (RA) or nerve growth factor (NGF). This is reflected in the robust androgenic signature that is observed in NB cells expressing DLG2, in comparison with the more mesenchymal signature seen in cells treated with RA. Taken together, our data show that ALK activation suppresses expression of the DLG2 tumor suppressor to promote an undifferentiated phenotype in neuroblastoma.

Aims

To reveal the development of neuroblastoma

Methods/Materials

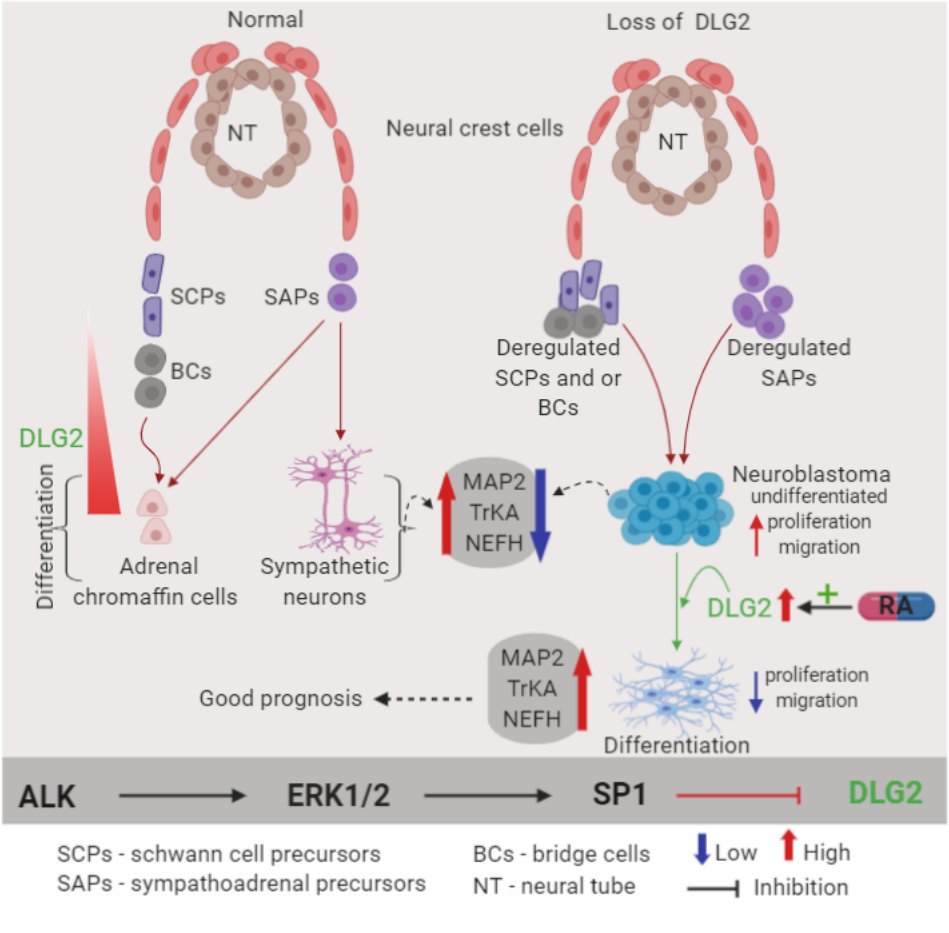
biochemistry, cell culture, xenograf, NGS

Results

Taken together, our data show that ALK activation suppresses expression of the DLG2 tumor suppressor to promote an undifferentiated phenotype in neuroblastoma.

Summary/Conclusions

In summary, our data suggest that an important role for oncogenic ALK-ERK1/2-SP1 signaling may be the maintenance of an undifferentiated state of transformed NC-derived progenitors that is achieved by repression of DLG2. The importance of SP1 and DLG2 in this process highlighted by the findings that restoring DLG2 expression, e.g. by targeting SP1, spontaneously drives NB differentiation. While targeting SP1 presents challenges, these results motivate further exploration of SP1 as therapeutic target as well as evaluation of DLG2 and SP1 as prognostic markers for NB. Finally, our data also suggest that further exploration of other 'bridge genes' may help to understand the mechanisms underlying the differentiation of SCPs and SA cells and their contribution to NB



ALK regulate DLG2
Type Basic

Presentation Preference Oral presentation

Abstract nr. 94

Anti-GD2 therapy in high-risk and relapsed/refractory neuroblastoma in Taiwanese pediatric patients: NTUCH experience

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

High-risk neuroblastoma treated with anti-GD2 antibody showed better overall survival. In Taiwan, the experience of anti-GD2 therapy was limited. Here we reported the experience of anti-GD2 therapy in our hospital.

Aims

We reported the experience of anti-GD2 antibody therapy in our hospital.

Methods/Materials

We review the chart of the neuroblastoma patients treated with anti-GD2 regimen retrospectively.

Results

Six patients received anti-GD2 therapy in our hospital in the past 3 years. Two patients were in relapse/refractory status, one was in second complete response status and three patients were in first complete response status under maintain therapy. Two patients received anti-GD2 antibody therapy combined with chemotherapy. Five of six patients(83%) continue complete response till now, mean event free time is 21.6 months(2~38 months). One patient shows progression disease and dropped out after 3 courses of the anti-GD2 therapy. Among the side effect, fever is the most common side effect (79%). Capillary leakage syndrome (CLS) with desaturation, tachypnea, wheezing, stridor and edema was the second common (38%). Pain was controlled well by morphine. No patient died in the treatment, and they all recovered from symptomatic treatment or by slowing down the rate of medication infusion.

Summary/Conclusions

Anti-GD2 therapy seemed to be safe and effective to high-risk neuroblastoma and relapsed neuroblastoma. The most common effect is fever which usually happen within 48 hours after

infusion. CLS is another noticeable side effect. CLS becomes worse in the last courses in our experience. The development of Human anti-mouse antibody might be considered.

Type Clinical

Presentation Preference Traditional poster

Abstract nr. 95

Combined replenishment of miR-34a and let-7b inhibits neuroblastoma growth by down-modulation of MYCN, LIN28B and ALK expression in pre-clinical models

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

Neuroblastoma (NB) is a malignant tumor of the sympathetic nervous system that substantially contributes to childhood cancer mortality, mostly due to toxicities and/or chemo-resistance, especially in high-risk patients with relapsed disease. The design of novel drugs to target specific molecular alterations in NB becomes imperative to provide more effective therapies and to ameliorate patients' outcomes. The dysregulated expression of *MYCN*, *ALK* and *LIN28B* as well as the aberrant under-expression of two microRNAs, miR-34a and let-7b are known to drive NB tumorigenesis by maintaining an 'oncogene addiction'.

Aims

Due to the ability of miRNA-mimics to recover the tumor suppressor functions of miRNAs which are depleted into cancer cells, in this study we have developed a safe and effective delivery system, selectively targeted to NB cells, to restore the correct activity specific miRNAs in clinically relevant mouse models of NB.

Methods/Materials

To evaluate the functional effects of miRNA replenishment and the down-modulation activity over *MYCN*, *LIN28B* and *ALK*, four NB cell lines, IMR-32, HTLA-230, LAN-5 and SH-SY5Y, characterized by different genetic alterations and/or over-expression of these key oncogenes, were chosen. Following *in vitro* cell transfection with ten candidate tumor suppressor miRNA-mimics, functional testing led to the selection of miR-34a and let-7b as the best ones. To investigate the therapeutic efficacy of restoring the tumor suppressor functions miR-34a and let-7b in pre-clinical mouse models, we set-up and characterized a novel formulation of GD_2-targeted liposomes entrapping miR-mimics for systemic administration of miR-34a and let-7b, individually and in combination. RT–qPCR and IHC techniques were adopted for gene/protein expression analyses. Systemic toxicity and immune-response were evaluated by serum chemistry analyses. Significance of experimental data and mice survival were evaluated by specific statistical tests.

Results

The replenishment of miR-34a and let-7b by NB-targeted nanoparticles, individually and more powerfully in combination, significantly reduced cell division, proliferation, neo-angiogenesis, tumor growth and burden, and induced apoptosis in orthotopic xenografts and improved mice survival in pseudo-metastatic models. These results represent the functional consequences of an efficient and cooperative down-modulation of MYCN transcription factor and its down-stream targets, *ALK* and *LIN28B,* effected by miR-34a and let-7b, which reactivate the physiological regulatory networks and lastly lead to a favorable therapeutic response. Our targeted liposomes hold physical-chemical features in terms of very high and stable entrapment efficiency, long-lasting stability and very effective binding to NB cells and they do not induce any acute or chronic toxicity nor immune-stimulation, thus resulting safe and efficient carriers for miR-mimics.

Summary/Conclusions

Our findings demonstrate that the combined replacement of miR-34a and let-7b has a promising therapeutic efficacy in itself. Moreover, as miRNA expression affects the response of cancer cells to cytotoxic therapy, the reactivation of crucial pathways following miR-34a and let-7b replenishment by our NB-targeted liposomal formulations is expected to enhance drug sensitivity, providing a viable tool for future clinical applications as adjuvant therapy for high-risk NB patients.

Type Translational

Presentation Preference Traditional poster

Abstract nr. 96

Contribution to metastasis of undifferentiated cells in neuroblastoma

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

Neuroblastoma (NB) is the most common extracranial solid tumour occurring in childhood. This disease arises from the components of the neural crest of the sympathetic nervous system and presents a high clinical heterogeneity that ranges from spontaneous regression to aggressive metastasis. Cellular heterogeneity has also been observed, which includes at least two types of tumour cells with divergent gene expression profiles and phenotypes: undifferentiated stem-like mesenchymal cells (MES) and compromised adrenergic cells (ADRN). The undifferentiated cell population seems to exhibit stem cell properties and it has been suggested that they contribute to NB malignization by evading intensive multimodal therapy. Therefor they might be the responsible of tumour relapses metastasis formation and poor clinical outcomes. The formation of these aggressive metastases takes place thanks to a multi-stage process in which cell migration is essential.
Migratory cancer cells undergo dramatic molecular and cellular changes by reshaping their cell-cell and cell-matrix adhesion, as well as their cytoskeleton. Changes in cell shape are translated and are indicative of biochemical signals that can modulate cell phenotype and biological properties. These shape changes are indicative of the behavior and evolution of the cells. In fact, it has been shown that cell morphology regulates biological processes, such as proliferation, differentiation and stem cells fate.

Aims

To study the specific adhesive and migratory properties of undifferentiated neuroblastoma cells.

Methods/Materials

Cultures are enriched in undifferentiated cells by tumorespheres in defined culture conditions and FACS sorting from NB cell lines and patient derived cells. Adhesion and spreading assay, cell

morphology assay, migration analysis and membrane dynamics analysis, were performed.

Results

The results obtained show that undifferentiated NB cells have greater adhesion and spreading capacity than the rest of tumour cells. They also present defined morphological features (smaller area, less perimeter, greater form factor, greater solidity) and a lower number and length of neurites. In addition, they migrate at a faster speed, and more persistently. Undifferentiated cells also presented higher levels of actin membrane dynamics.

Summary/Conclusions

With the results obtained, it can be concluded that the most undifferentiated cells from the tumour, have great cellular plasticity, indicated by a particular cytoskeletal morphology and structure. In addition, they are characterized by having a greater migratory capacity than the cells forming the bulk of the tumour. This preliminary results suggests that undifferentiated NB cells could have important metastatic characteristics.

Type Basic

Presentation Preference Traditional poster

Abstract nr. 97

The R2 Platform as a Resource for Neuroblastoma Data Analysis and Visualization

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Over the past decade, the R2 platform has proven to be a useful and ever-expanding resource for the Neuroblastoma community. As science progresses and novel technologies become available, so does the development of the R2 platform continues to expand.

Aims

Methods/Materials

The R2 program, rooted in neuroblastoma research, has a user-friendly interface enabling a wide range of interconnected analyses, which can be harnessed by users with limited or no bioinformatics training. The web-based genomics analysis and visualization platform has been greatly extended over the past period. Novel analyses, easier workflows, and new types of omics data have been introduced.

Results

Aside from mRNA expression, copynumber, SNP, Exome and whole genome sequencing data we have greatly extended R2 with intuitive tools for the analysis of single cell, epigenome and ChIPseq analyses. Various different technologies can be combined in integrative analyses and visualizations.
We have added many histone modification profiles of neuroblastoma cell lines and tumor samples. On these profiles peak calling has been performed as well as super enhancer analyses if applicable.

Interactive plots, like ROSE SE visualizations, genome browser views and heatmaps of aligned peaks provide accessible entry points for further exploration of one, or a series of profiles. In addition, integration with other genomic features, such as chromatin states, transcription factor binding sites, gene expression patterns help to test hypotheses. Genome reconstruction has also been added to the repertoire. Finally, Tools to work with single cell sequencing data are now being implemented and added to the repertoire.
Other additions: The popular tSNE dimensionality reduction algorithm has been added and allows for interactive definition of patient subgroups on the basis of the underlying genomics data. UMAP dimensionality reduction is being implemented.
Improved expression signature generation, coupled to various downstream analyses now allows the users to get a better understanding of their cohorts or experiments. The introduction of data scopes, creates easier workflows and allows for more focused analyses within R2.

Summary/Conclusions

R2 remains a valuable resource for high throughput data of Neuroblastoma. The R2 program and database has been used in more than 1200 PubMed listed publications and is publicly accessible at http://r2.amc.nl. R2 assists researchers in the identification of important genes and biological processes in neuroblastoma.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 98

Embryonic mutations reveal independent tumour formation in bilateral neuroblastoma

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Topic Neuroblastoma as developmental disease

Background/Introduction

Multifocal cancer is most typically regarded as comprising primary tumours with metastatic deposits. However, in some forms of childhood cancer, synchronous lesions located in separate anatomical regions may represent independent oncogenic events as has been described in Wilms' tumour and retinoblastoma. Bilateral/Multifocal neuroblastoma is a well recognised but little-studied phenomenon.

Aims

We aimed to study two cases of neuroblastoma with bilateral adrenal tumours to determine their respective embryological origins and thereby distinguish two potential models: separate events initiating tumourigeneses versus a common precursor.

Methods/Materials

Two patients presenting with bilateral adrenal neuroblastoma tumours underwent diagnostic biopsies and/or surgical resection of the primary tumours. Whole-genome sequencing was performed on DNA derived from each tumour and from peripheral blood of the child and of both parents. Using a validated variant-calling pipeline we determined mutations of all classes, subdivided into inherited germline, *de novo* germline, mosaic (embryonic), and somatic variants. From these catalogues of mutations, we reconstructed the phylogenetic development of the respective tumours.

Results

In each case, we identified germline variants predisposing to neuroblastoma (SMARCA4 and CHEK2 in one family and PHOX2B in the second). In each case, analysis of *de novo* mosaic mutations in blood and tumour indicated separate embryonic origins with no evidence of somatic mutations in common. Remarkably in the first case the two embryonic lineages evolved in parallel into high risk tumours with apparent identical second hits on cytogenetic analysis, which sequencing revealed to have no molecular features in common.

Summary/Conclusions

Our findings show that bilateral neuroblastoma in our two cases are independent neoplasms that separated early in embryogenesis, thus providing direct phylogenetic proof of the suspicion that some multisite tumours in children do not represent metastatic disease. Whilst parallel convergent development of different clones within a tumor is a well-described phenomenon, the development of independent synchronous tumors that mirror each other is an unprecedented observation. We speculate that the pre-existing two oncogenic germline mutations forced tumor formation down a restricted conglomeration of required second hits. As whole-genome DNA sequencing is becoming a routine clinical test, our analytical strategy may guide treatment and provide further insights into the early embryonic development of childhood cancer.

Coorens

, University of Cambridge

Embryonic mutations reveal independent tumour formation in bilateral neuroblastoma

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Type Translational

Presentation Preference Oral presentation

Abstract nr. 99

Detection of actionable genetic alterations in cell-free DNA of neuroblastoma patients enrolled in the MAPPYACTS study

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Cell-free DNA (cfDNA) is increasingly used for molecular characterization of cancer, enabling both the identification of molecular alterations and correlation with disease burden, but to date such data has not been used in a clinical setting in pediatric cancers at relapse, such as Neuroblastoma (NB).

Aims

MAPPYACTS (NCT02613962) is a molecular profiling trial for children and adolescents with recurrent/refractory malignancies, with a relapse-specific, on-purpose tumor biopsy/surgery studied by whole exome sequencing (WES, 150x, tumor/germline) and RNAseq, and treatment recommendations provided by a clinical molecular tumor board based on the molecular tumor

profile. Within an ancillary study, in addition to the molecular characterization of the tumor, plasma was collected at the time of tumor biopsy to determine the feasibility of molecular characterization based on the study of cfDNA.

Methods/Materials

Among 500 patients enrolled in MAPPYACTS, for 254 patients with extracranial solid tumors, including 44 neuroblastoma (NB) patients, cfDNA was extracted from plasma (volume 300-2000 µl) using a Qiagen® cfDNA kit. CfDNA WES was performed using a Roche Medexome® modified protocol, with 100bp paired-end sequencing (Novaseq®) for an expected coverage of 100x. Alignment to the reference genome was done using bowtie2. Copy number profiles were generated using Sequenza and Facets, enabling also the determination the fraction of circulating tumor DNA (ctDNA) in the total cfDNA. Single nucleotide variations (SNVs) were called with GATK Haplotypecaller & Genotyper; variants with a coverage of at least 20x and 2 alternate reads were retained. SNVs were confirmed using Integrative Genomics Viewer (IGV). Results were compared both to the germline and the primary tumor to determine somatic and potentially ctDNA-specific alterations.

Results

cfDNA amounts were higher in NB patients than in other pediatric cancer patients (NB patients: mean 144 ng/mL of plasma (range 7-1338); other cancer patients: mean 46 ng/ml (range 0-475), t.test=0.017). CfDNA analysis was deemed contributive in 27 of 44 cases, based on the detection of either somatic copy number changes, or SNVs, or both, with 3 cases being contributive only in plasma but not in the analyzed tumor material.
Actionable genetic alterations were detected in 20 of 44 cfDNA samples and included copy number alterations (for instance *CDK4-MDM2* amplification, *CCND1* amplification or *CDKN2A* deletion) and/or SNVs (for instance *ALK*, *SMARCA4* or *ATRX* mutations). A total of 87 actionable alterations were detected, with 46 common to cfDNA and tumor.Eleven actionable alterations were specific to tumor, whereas 30 were observed only in cfDNA but not in the tumor, including alterations targeting *BRCA2* or *ATRX*.

Summary/Conclusions

In NB patients enrolled in the MAPPYACTS trial, cfDNA WES enabled contributive results in 27 of 44 (61%), and 20 of 27 (74%) of the contributive samples presented at least one actionable molecular target, of which 30 of 87 (34%) were cfDNA-specific, highlighting the importance of spatial genetic heterogeneity in NB. These results provide proof of principle for the feasibility of cfDNA analysis in particular for NB patients within pediatric precision medicine trials.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 100

Liquid biopsy in neuroblastoma patients using Tumor Epitope Detection in CD14 /CD16 Macrophages (EDIM)

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Epitope Detection In Macrophages (EDIM blood test) has been described to investigate characteristics of solid tumors in the peripheral blood of patients. This blood test allows detection and analysis of the tumor proteins transketolase-like 1 (TKTL1, activation of aerobe glycolysis) and Apo10 (DNaseX, proliferation defect) in a non-invasive way by isolating intracellular tumor epitopes in peripheral blood macrophages after phagocytosis of tumor cells. The ganglioside GD2 is highly expressed in neuroblastoma and thus possibly represents an attractive additional specific biomarker for the EDIM blood test.

Aims

Aim of this study was to evaluate the role of TKTL1, Apo10, and GD2 as potential targets for EDIM liquid biopsy and as biomarkers for diagnosis, monitoring, and disease progression in neuroblastoma.

Methods/Materials

Expression levels of TKTL1 and Apo10 in neuroblastoma tumor samples (*n*=6) and cell lines (LAN-1, IMR-32, SK-N-BE, LS, SH-SY5Y) were analyzed using RT-PCR and flow cytometry. Macrophages from the peripheral blood of patients with neuroblastoma (*n*=38) and from healthy individuals (control group, *n*=37) were labeled with $CD14^{+}/CD16^{+}$, then analyzed using flow cytometry, and assessed for the presence of intracellular epitopes of TKTL1, Apo10, and GD2 using the EDIM blood test technology. LAN-1 cells were co-cultured with blood macrophages in order to verify phagocytosis of tumor compounds as underlying mechanism for the EDIM technology.

Results

Expression levels of TKTL1 and Apo10 were elevated in all investigated neuroblastoma tumor samples, neuroblastoma cell lines showed differential TKTL1 and Apo10 expression levels. A significant upregulation of TKTL1, Apo10, and GD2 in peripheral blood macrophages co-incubated with LAN-1 neuroblastoma cells was observed compared to peripheral blood macrophages alone or peripheral macrophages incubated with DMEM medium. Using flow cytometry we identified tumor epitopes in peripheral blood macrophages of the investigated neuroblastoma patients (TKTL1 36/38, Apo10 34/38, GD2 16/19). Analysis of tumor epitopes in the macrophages of the control group showed negative results for TKTL1 (0/37) and Apo10 (0/37), as well as positive values for GD2 in only 2/22 patients. Expression scores of these three epitopes in the EDIM blood tests were able to significantly differentiate between neuroblastoma patients and healthy blood donors.

Summary/Conclusions

Analyzing tumor epitopes in CD14+/CD16+ labeled peripheral macrophages using the EDIM blood test seems to be a promising non-invasive tool for liquid biopsy in neuroblastoma patients. Further studies are currently being undertaken evaluating a potential risk stratification at initial diagnosis, determination of tumor biology, treatment monitoring, and early detection of tumor relapses.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 101

Targeting Fatty Acid Transport in MYCN-amplified Neuroblastoma.

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

MYCN amplification occurs in half of high-risk neuroblastoma (NB) patients and strongly correlates with disease progression and treatment failure. Hence, there is an unmet need to identify novel MYCN-dependent pathways and develop effective therapies for high-risk patients. The MYC oncogene is well documented as a master regulator of cell metabolism to support tumor growth. However, how MYCN reprograms NB tumor metabolism and its impact on tumor growth remain elusive.

Aims

The overall goal of our study was to identify novel MYCN-driven metabolic alterations that contribute to NB oncogenesis. By selectively targeting specific metabolic dependencies, we will be able to identify innovative and effective therapeutic approaches for high-risk disease.

Methods/Materials

Global metabolomics profiling was performed in NB cells upon changes in MYCN expression (MYCN Tet-On and Sh MYCN systems) and primary patient tumors (MYCN-amplified, MNA, n=18; and non MYCN-amplified tumors, non MNA, n=18). Stable isotope D_2O and palmitate[U-13C] were employed to trace fatty acid (FA) synthesis and uptake in multiple MYCN models. The dependency on exogenous FAs and the expression of transmembrane FA transporters were evaluated in NB cells and patient samples. ChIP-qPCR and luciferase assays were used to determine MYCN direct regulation of distinct FA transporters. The effect of FA transport on tumor growth was evaluated via a small molecule inhibitor of FA uptake in multiple pre-clinical models of NB.

Results

MYCN activates lipid metabolism in both NB cells and primary tumors, resulting in accumulation of diglycerides and triglycerides ($p \leq 0.05$), which are vital in maintaining lipid homeostasis and tumor survival. Moreover, MYCN drives both FA synthesis and FA uptake in MYCN3 (MYCN Tet-On) and SK-N-AS MYCN-ER cells. Importantly, FA uptake is necessary for MNA cell survival, as deprivation of exogenous FAs blocks cell proliferation and induces apoptotic cell death ($p<0.01$). To determine how MYCN regulates FA uptake, we screened (by mRNA expression and MYCN binding) seven major transmembrane FA transporters and found that MYCN directly binds to the promoter of FA transport protein 2 (*FATP2*) and remarkably upregulates its transcription (>10 fold change). Clinically, *FATP2* expression is higher in MNA and stage 4 patients, and predicts poor clinical outcome in independent patient cohorts (patient n=932, $p<0.0001$). Pharmacological inhibition of FATP2 via the small molecule CB5 inhibits FA uptake, selectively suppresses cell growth without affecting normal cells, and sensitizes cells to conventional therapies. Notably, FATP2 inhibition significantly blocks the growth of orthotopic xenografts derived both from MNA cells ($p=0.026$) and TH-MYCN$^{+/+}$ tumors ($p=0.013$).

Summary/Conclusions

Our data reveal a novel metabolic dependency of MNA tumors: MYCN activates lipid metabolism and specifically drives FA uptake to support tumor growth. Pharmacological inhibition of FA uptake effectively blocks tumor growth and sensitizes NB cells to conventional therapy.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 102

Characterization of the immunosuppressive microenvironment during MYCN-driven oncogenesis.

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Tumors evolve an immunosuppressive microenvironment that prohibits the immune system's natural ability to recognize and destroy tumor cells. The MYC oncogene drives both metabolic rewiring and immune evasion by targeting both innate and adaptive immune regulators. MYCN amplification occurs in almost 50% of high-risk neuroblastoma (NB) patients and associates with repressed cellular immunity. However, it remains elusive how MYCN regulates the immunosuppressive microenvironment during NB progression.

Aims

The overall goal of this study was to characterize the immune landscape of NB tumors at different stages of MYCN-driven oncogenesis, and determine the immune events driving tumor development. We will further determine how MYCN modulates the immunosuppressive microenvironment to promote tumor growth and develop effective therapeutic approaches to revert MYCN-driven immunosuppression.

Methods/Materials

The TH-MYCN GEM model, with targeted MYCN expression in the developing neural crest, was used to study immune cells within the microenvironment during NB development. Week 4 and week 6 were defined as early- and late-phase time points of tumor development depending on MRI imaging and tumor weight. Mice (n=4 at week 4 and n=8 at week 8) were perfused to deplete circulating immune cells. Tumor-infiltrating immune cells were isolated by percoll gradient centrifugation, stained and fixed before flow cytometry analysis of T cell and myeloid-derived suppressor (MDSC) cell types. In parallel, global metabolomics profiling (DiscoveryHD4 Platform,

Metabolon Inc.) was performed to understand the metabolic landscape of MYCN-driven tumors at the same time points.

Results

Tumors in TH-MYCN mice initiate along the paraspinal region by week 4 (average: 10 mm^3, 0.06 g) and rapidly grow by week 6 (average: 1160 mm^3, 2.0 g). There was no difference in infiltrating cytotoxic T cells ($CD8^+$) between week-4 and week-6 tumors (3.18% vs. 5.07% of total tumor-infiltrating cells). However, week-6 tumors had lower percentage of CD69, a T cell activation marker, suggesting that T cells are less active at later stages. We did not observe a significant difference in the population of regulatory T cells ($CD4^+$ $Foxp3^+$) or tumor-associated macrophages ($CX3CR1^{hi}$ $F4/80^{hi}$ $MHCII^{hi}$) between week-4 and week-6 tumors. However, we found that a specific sub-population of MDSCs cells, the polymorphonuclear MDSC (PMN-MDSCs), was remarkably enriched in tumors of week-6 compared to those of week-4 (63.63% vs. 2.77% $CD11b^+$ $Ly6G^{hi}$ $Ly6C^{lo}$ $CX3CR1^{lo}$ $F4/80^{lo}$ $MHCII^{lo}$ cells). In parallel, we have identified distinct metabolic nodes that were highly enriched in week-6 tumors: polyamine (putrescine and spermidine, $p<0.05$), aspartate (N-acetylaspartate and N-acetyl-aspartyl-glutamate, $p<0.0001$), and lipid (acyl carnitines and ceramides, $p<0.05$) metabolism.

Summary/Conclusions

We have demonstrated a significant enrichment of PMN-MDSCs during tumor progression, suggesting a potential link between PMN-MDSC and NB oncogenesis. We are currently characterizing the role of PMN-MDSC in suppressing T cell functions and promoting oncogenesis. We will further test our hypothesis that the MYCN-directed metabolic pathways form a permissive microenvironment for the development of high-risk tumors.

Type Basic

Presentation Preference Traditional poster

Abstract nr. 103

Haploidentical stem cell transplantation and subsequent immunotherapy with anti-GD2 antibody for patients with relapsed metastatic neuroblastoma

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

Pediatric patients with relapsed metastatic neuroblastomas have a poor prognosis and additional therapeutic strategies are needed. We present results of a phase I/II-trial analysing subsequent immunotherapy with anti-GD2mAb (CH14.18/CHO) following HLA mismatched, haploidentical stem cell transplantation (SCT).

Aims

Methods/Materials

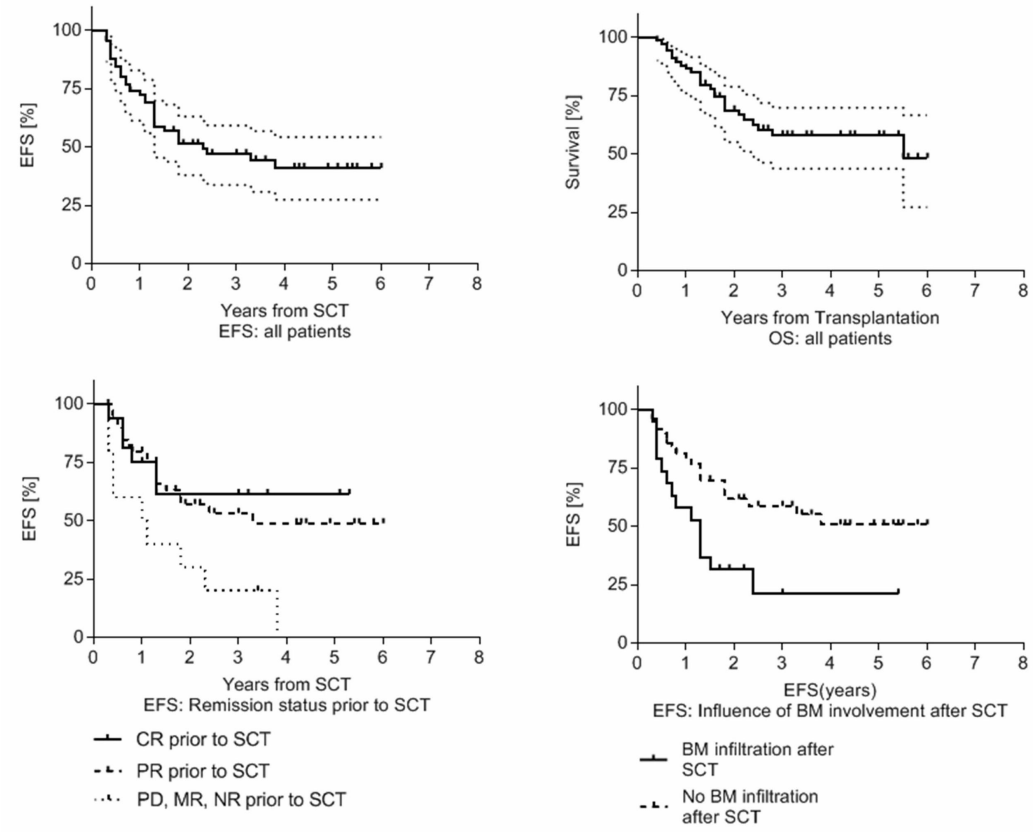
T- and B-cell depleted stem cells from parental donors were used in combination with Melphalan140mg/m², Thiotepa10mg/kg, Fludarabin160mg/m², and ATG-F. Infusions with CH14.18/CHOmAb were started on day 60-180 post transplant: 6 cycles with 20mg/m²/day x 5 days; in cycles 4-6, $1x10^6$ U/m² Interleukin 2 (IL2) was given additionally. The disease status was evaluated with whole body MRI, MIBG and BM-aspirates.

Results

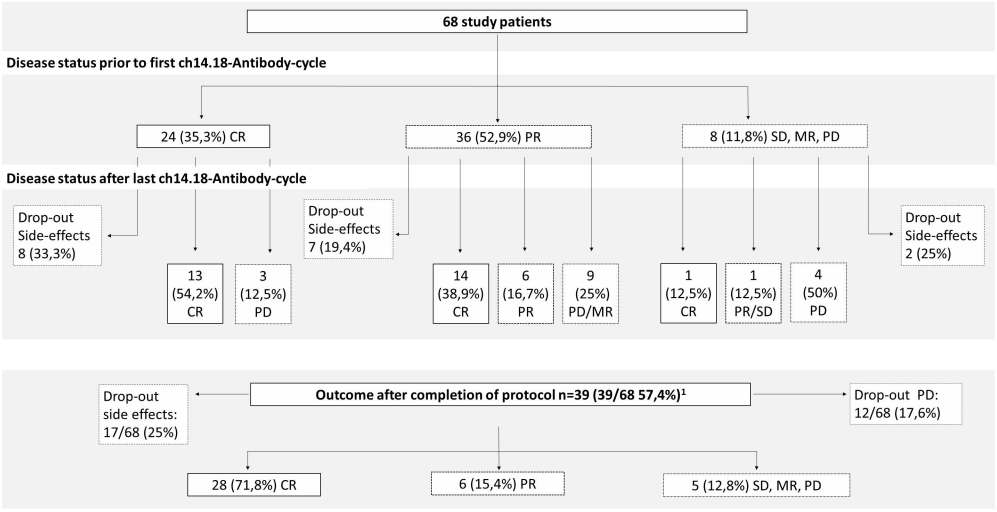
68 patients with 1^{st} to 5^{th} metastatic relapse were enrolled, relapse treatment comprised induction chemotherapy and surgical intervention and local irradiation if possible. 70,1 % of patients received a backbone of temozolomide+irinotecan in combination with Topotecan/Cyclophosphamide/Etoposide based cycles.
The median time from the start of relapse therapy to HSCT was 7 months.
Stem cell transplantation was well tolerated without TRM, primary engraftment occurred in 64 patients (95,5%), the median time to an ANC > 0.5 x 10^9/l was 11 days.
Induction of relevant late onset acute GvHD (during antibody treatment) was seen in two patients, one steroid-sensitive stage III GvHD of the GI-tract, one stage II GvHD of the GI-tract. Frequent side effects of antibody treatment were pain, fever, and CRP elevation; rare side effects were SIRS (n=7) seizures (n=3), and accommodation disturbances (n=8).
Thirteen of twenty-four patients (54,2%) who achieved CR already prior to antibody treatment, could maintain a complete remission (CR), 20/36 patients (55%) with partial remission prior to antibody treatment achieved CR or could significantly reduce their tumour load after antibody treatment. 2/8 patients (25%) with NR (stable disease/mixed resp.) responded only transiently.
In all patients the number of patients with CR increased from 24 before immunotherapy to 28 after the last treatment cycle.
Thus, success of treatment, defined as stable disease or improvement after the last treatment cycle, was shown in 58,8%. Overall survival at 2 and 3 years was 68,6% and 58%. Event free survival at 2 and 3 years was 51,2% and 46,9% (median follow up: 2.5 years).
Significant factors of influence on OS and EFS were: remission status prior to SCT and bone marrow (BM) involvement after SCT.
(3-year-EFS for patients in CR vs. PR vs. NR: 61,36 %, 53,2%, 20% p=0,02 (CR/PD), p=0,006 (PR/PD); BM+ vs. BM-: 21,1% / 58,7%, p=0,007)
(3-year-OS for patients in CR vs. PR vs. NR: 62,8 %, 69,1%, 20% p=0,03 (CR/PD), p=0,0002 (PR/PD); BM+ vs. BM-: 31,1% / 65,7%, p=0,04)

Summary/Conclusions

CH14.18/CHO infusions after haploidentical SCT appear to be feasible without increased risk of inducing GvHD. In patients with detectable tumour burden an immunological and clinical response could be shown due to combination of mAb and immune system of donor origin. Side effects of the treatment were tolerable and well manageable.
In summary, for patients with refractory/relapsed NBL, haploidentical SCT plus ch14.18 plus Il2 can induce long-term remissions with favourable EFS.



Event-free and overall survival estimated by the Kaplan-Meier method.



CR: Complete remission, PR: Partial remission (incl. VGPR), SD: Stable disease (or non-response), MR: Mixed response, PD: Progressive disease
[1] not success of treatment

Response of study patients

Type Clinical

Presentation Preference Oral presentation

Advances in Neuroblastoma Research (ANR) Meeting
ANR 2020
Amsterdam
11-14
May
www.anr2020.org

Abstract nr. 104

RESULTS OF HIGH-DOSE TREOSULFAN-CONTAINING REGIMEN IN PATIENTS WITH HIGH-RISK NEUROBLASTOMA

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

High-dose chemotherapy is reduced the risk of relapse in high-risk neuroblastoma (NB). Busulfan-containing regimens are considered to be the standard, while the efficacy of treosulfan has not yet been defined.

Aims

The aim of the study was to analyze the results of therapy of high-risk NB using treosulfan/ melphalan conditioning regimen.

Methods/Materials

171 patients with high-risk NB were treated for the period 01.2012-12.2018 (84 months). Patients were stratified and treated according to the modified German NB2004 protocol. Patients with *MYCN*-amplified stage 1 were included. 29 (17%) patients didn't receive hematopoietic stem cell transplantation (HSCT). Among 142 transplanted patients high-dose preparative regimens included carboplatin/etoposide/melphalan (CEM) (till June 2013) in 28 (19.7%) cases and treosulfan/melphalan (TreoMel) (since July 2013) in 114 (80.3%). Since July 2014 patients with clear MIBG-positive residual primary tumor and/or metastases prior to HSCT received ^{131}I-MIBG-therapy. Database was locked on 20.12.2019.

Results

114 patients who received Treo/Mel were included in the analysis. Male: female ratio was 1.15:1. The median age at the diagnosis was 33.4 months (range 1.3-169.6). Age was older than 12 months in 100 (87.7%) and 18 months in 93 (81.5%) cases. Adrenal glad/retroperitoneum was the primary site in 99 (86.8%) cases. *MYCN* amplification was observed in 53 (46.4%) cases. 95 (83.3%) patients had stage 4 NB. Distribution of patients according to the response prior to HSCT was as follows: complete – 24 (21.0%), very good partial – 34 (29.8%), partial – 46 (40.4%), mixed response – 5 (4.4%), stable disease – 5 (4.4%).

Median follow-up time for all patients was 36.7 months (range 5.6-74.8), for alive patients 43.5 months (range 10.7-74.8). 3-year and 5-year EFS was 42.9% (95%CI 33.0-52.8) and 38.0% (95%CI 27.7-48.3). 3-year and 5-year OS - 71.5% (95%CI 62.4-80.6%) and 61.4% (95%CI 50.3-72.6). One patient died as a result of transplant-related complications. There were no cases of veno-occlusive disease, including patients who underwent MIBG-therapy.

Cumulative incidence of relapse at 36 and 60 months was 56.2% (95%CI 47.1-67.1) and 61.1% (95% CI 51.7-72.4), transplant-related mortality (TRM) 0.9% (95%CI 0.1-6.3).

Univariate analysis showed significantly better EFS in children < 18 months (3-year EFS 75.9% (95%CI 57.5-94.3) vs. 35.0% (96%CI 24.3-45.7) in patients > 18 months, p=0.01) and patients with stage 1-3/4S (82.6% (95%CI 64.4-100.0) vs. 34.9% (95%CI 24.4-45.3) in stage 4, p=0.004). Topography of the primary tumor and response prior to HSCT didn't impact EFS. Multivariate analysis showed that stage was the independent prognostic factor for EFS (HR 3.4, 95% CI 1.2-9.7).

The only factor that impacted the OS was stage: 3-year OS was 89.5 (95%CI 75.7-100.0) in patients with stage 1-3/4S versus 67.8% (95%CI 57.5-78.2) in stage 4 (p=0.02). Age at diagnosis,

topography of the tumor and response didn’t impact OS.
Presence of *MYCN* amplification significantly decreased EFS (p=0.008) and OS (p=0.001) in stage 4 patients with know *MYCN* status (n=91).

Summary/Conclusions

We presented the results of treatment of a large cohort of high-risk NB patients who received treosulfan-containing conditioning regimen. Treosulfan-melphalan regimen was associated with low TRM and safe use in patients who had received MIBG-therapy. Direct comparison with therapeutic programs based on busulfan-containing conditioning regimen is not possible due to different induction chemotherapy in NB2004 and SIOPEN protocols. Additional studies are needed to assess the long-term prognosis of patients who received treosulfan.

Type Clinical

Presentation Preference Rapid Fire Poster Presentation

Abstract nr. 105

Composite pheochromocytoma with low-grade neuroblastoma in a four-year-old boy.

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

The simultaneous development of two tumors in different layers of the adrenal gland is extremely rare. Single clinical observations of adult patients aged 23 to 68 years with a composite adrenal tumor have been published in the literature. In the described cases in adult patients, this was mainly a combination of pheochromocytoma with ganglioneuroma or ganglioneuroblastoma, while in children it was pheochromocytoma with low-grade or undifferentiated neuroblastoma

Aims

rare clinical observation of a bilinear tumor

Methods/Materials

We present a clinical case of a 4-year-old patient with a typical clinical presentation of pheochromocytoma. At the end of August, the patient was admitted to the clinic with complaints of high blood pressure (150/100 mm Hg), headaches, tachycardia, sweating and neuropsychic agitation. Abdominal cavity CT showed a solid mass of 8.1 * 7.6 * 7.9 cm (in size), formed from the

left adrenal gland. During MIBG I^{123}-scintigraphy no active tumor tissue of neurogenic nature was detected. The bone marrow examination from 5 points haven't revealed any tumor cells. Urine test showed increased levels of renin, methanephrine and normetanephrine. According to the results of a comprehensive examination, a diagnosis of pheochromocytoma was established. At the first stage a surgical treatment was performed - left-sided adrenalectomy with retroperitoneal lymphatic dissection. The clinical presentation of pheochromocytoma was stopped after surgery. Histological conclusion: composite pheochromocytoma (13 points by PASS) with low-grade neuroblastoma with high MKI. FISH reaction - 1q23 locus deletion detected.10 days after surgery, the child was comprehensively examined. PET-CT, abdominal cavity ultrasound, MIBG I^{123}-scintigraphy and bone marrow examination revealed disseminated disease progression with metastases in the location of the removed tumor, retroperitoneal lymph nodes, supra- and infraclavicular lymph nodes, posterior mediastinum and both lungs

Results

Currently, the patient is receiving program treatment according to the high-risk neuroblastoma treatment protocol.

Summary/Conclusions

The prognosis in patients with a composite tumor is extremely unfavourable. Relapse occurs at least after 3 months completion of treatment. Patients died from a progressive disease and lack of response to treatment. However, for a more accurate assessment of the cancer outcome, further long-term and large-scale studies of this rare disease are required.

Type Clinical

Presentation Preference Traditional poster

Abstract nr. 106

Management of Acute Toxicities of Naxitamab (Hu3F8): A Model for Infusion Nursing at the Bedside

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

Anti-GD2 monoclonal antibody (MoAb) therapy is an integral part of treatment for patients with high-risk neuroblastoma. The humanized anti-GD2 MoAb (naxitamab) is given as a 30-90 minute infusion in the outpatient setting at Memorial Sloan Kettering Cancer Center (MSKCC), unlike dinutuximab (Ch 14.18), which is given inpatient over 10-20 hours. Adverse events encountered during or soon after the infusion include pain, allergic reactions, respiratory distress, lethargy, and hypotension. Treatment is administered by infusion nurses trained specifically in naxitamab therapy. Other nursing personnel involved include nurse practitioners and bedside nurses. Nursing autonomy emphasizes timely intervention within specific guidelines using best clinical judgment and medical knowledge to manage adverse events at the bedside.

Aims

The aim of this report is to describe the role of the infusion nurse in the outpatient administration of naxitamab.

Methods/Materials

Infusion nurses are required to receive formal training over a 12-week period on the management of naxitamab adverse events. They familiarize themselves with therapeutic interventions which include administration of hydromorphone, racemic epinephrine, intramuscular epinephrine, levalbuterol, naloxone, inhaled oxygen, diphenhydramine and IV fluid boluses as clinically indicated. Detailed order sets governing the use of the above medications are prepared by nurse practitioners. Infusion nurses are assigned to each patient receiving naxitamab and remain at the

bedside for the duration of the treatment and immediate recovery period. Within the framework of nursing guidelines, they assess and manage adverse events using these medications throughout the infusion. A primary nurse model is utilized to develop familiarity with individual patient adverse reactions. Infusion nurses are assisted by bedside nurses who provide pre-infusion management, assist during the infusion, and provide post-management care. When the need for naloxone, epinephrine, or fluid boluses arises, infusion nurses call upon nurse practitioners or attending physicians for consultation and advice. In the absence of the above adverse events, infusion nurses exercise autonomy in the management of naxitamab infusion. In rare occasions when cardiopulmonary support is needed, patient care will transition to attending staff. Patients remain in the clinic until all adverse events have subsided and are cleared for discharge by infusion nurses, nurse practitioners or attending physicians.

Results

Up to 5 infusion nurses can treat up to 8 patients/day while maintaining safety and patient flow in the outpatient setting. More than 250 patients have been treated at MSKCC with >1000 infusions in the outpatient setting from 2011-2019. There was no drug-associated mortality, and post-treatment inpatient admission rates were <1%. Coordination, cooperation and trust have enabled infusion nurses to consistently make appropriate clinical judgements. Having dedicated trained infusion nurses at the bedside, with nurse practitioner and attending physician back-up, allows for rapid and consistent responses to clinical changes in the patients' status, and the ability to treat multiple patients in the same day.

Summary/Conclusions

Trained infusion nurses can administer naxitamab safely in the outpatient setting and may provide a viable nursing model for testing future biologics with predictable and severe acute side effects.

Type Clinical

Presentation Preference Traditional poster

Abstract nr. 107

A novel multimeric IL15/IL15Ra-Fc complex to enhance neuroblastoma immunotherapy

Author - Xu, Hong, Memorial Sloan Kettering Cancer Center, NEW YORK, USA (Presenting Author)
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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

The role of T cells and NK cells in controlling a wide spectrum of human cancers is proven. The success requires their continual in vivo persistence and their ability to traffic to tumor sites. Yet, there is no clinically approved cytokine available that will expand, maintain, and activate effector T cells or NK cells, other than IL-2 which has many undesirable toxicities. IL-15 has diverse immunologic effects, and plays an important role in the development, homeostasis and function of memory CD8+ T cells, NK cells, and other immune cells. A soluble IL15/IL15Rα complex, using the sushi domain of the IL15Rα and the N72D mutation in IL15 (IL15-N72D/IL15Rα-Sushi-Fc, ALT-803), has been proven effective in preclinical and clinical trials. GD2 is one of the proven neuroblastoma antigens for immunotherapy, and 3F8 mAbs and hu3F8-BsAb have shown robust anti-tumor activities against neuroblastoma and other GD2+ tumors both in vitro and in vivo.

Aims

To develop a novel IL15/IL15Rα complex in order to achieve better in vivo efficacy than ALT-803 without the disadvantage of introducing mutations in IL15 which could potentially induce neutralizing antibodies.

Methods/Materials

Instead of using Sushi domain (SU) of IL15Rα (aa1-66) in the complex, we used full length ectodomain (FL) of IL15Rα (aa1-175) fused to the CH2-CH3 of human IgG1 Fc region. Both WT and N72D mutant IL15 (MUT, to enhance affinity for IL15βγc), in complex with either FL or SU domain of IL15Rα were made for comparison. All four complexes (WT-FL, MUT-FL, WT-SU and MUT-SU) showed >90% purity and stability with storage.

Results

We successfully engineered a full length IL15Rα construct (WT-FL) that allowed the IL15/IL15Rα

complex to form spontaneous trimers of dimers (6 IL15 + 6 IL15Rα). The complex was able to stimulate PBMC-derived T cells and NK cells with augmented in vitro tumor cytotoxicity, increased expression of surface markers such as stimulatory receptors, cytotoxic proteins both intra-cytoplasmically and on the cell surface. Furthermore, when compared to MUT-SU (ALT-803), the WT-FL complex showed substantially longer serum half-life, with considerable enhancement of anti-neuroblastoma potency in the presence of NK + 3F8 mAbs, or T cells + hu3F8-BsAb, in multiple xenograft models.

Summary/Conclusions

The increase in serum half-life and enhancement of anti-tumor effect was substantial when the WT-FL complex is administered subcutaneously, a convenient route for clinical application. The trimeric nature of this complex is the key to its superior biologic properties. This is novel, especially given the previous concern that such constructs could be inhibitory instead of stimulatory on immune cells. These results support further evaluation of this complex for clinical development.

Type Translational

Presentation Preference Traditional poster

Abstract nr. 108

A novel multimeric IL15/IL15Ra-Fc complex to enhance neuroblastoma immunotherapy

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

The role of T cells and NK cells in controlling a wide spectrum of human cancers is proven. The success requires their continual in vivo persistence and their ability to traffic to tumor sites. Yet, there is no clinically approved cytokine available that will expand, maintain, and activate effector T cells or NK cells, other than IL-2 which has many undesirable toxicities. IL-15 has diverse immunologic effects, and plays an important role in the development, homeostasis and function of memory CD8+ T cells, NK cells, and other immune cells. A soluble IL15/IL15Rα complex, using the sushi domain of the IL15Rα and the N72D mutation in IL15 (IL15-N72D/IL15Rα-Sushi-Fc, ALT-803), has been proven effective in preclinical and clinical trials. GD2 is one of the proven neuroblastoma antigens for immunotherapy, and 3F8 mAbs and hu3F8-BsAb have shown robust anti-tumor activities against neuroblastoma and other GD2+ tumors both in vitro and in vivo.

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Results

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stimulate PBMC-derived T cells and NK cells with augmented in vitro tumor cytotoxicity, increased expression of surface markers such as stimulatory receptors, cytotoxic proteins both intra-cytoplasmically and on the cell surface. Furthermore, when compared to MUT-SU (ALT-803), the WT-FL complex showed substantially longer serum half-life, with considerable enhancement of anti-neuroblastoma potency in the presence of NK + 3F8 mAbs, or T cells + hu3F8-BsAb, in multiple xenograft models.

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The increase in serum half-life and enhancement of anti-tumor effect was substantial when the WT-FL complex is administered subcutaneously, a convenient route for clinical application. The trimeric nature of this complex is the key to its superior biologic properties. This is novel, especially given the previous concern that such constructs could be inhibitory instead of stimulatory on immune cells. These results support further evaluation of this complex for clinical development.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 109

Strong prognostic ability of LDH and serum ferritin in neuroblastoma: overall and in high-risk patients after 2009 - An International Neuroblastoma Risk Group (INRG) Project

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

Age, *MYCN* status, presence of metastases, and histology have been used as neuroblastoma risk factors for decades. Lactate dehydrogenase (LDH) and serum ferritin are reproducible, easily obtained from a blood sample, and prognostic of outcome, though not formally used in risk stratification except in one German trial. LDH is a marker of tissue injury, and ferritin is a marker of acute inflammatory reaction or cancer.

Aims

To determine the prognostic strength of LDH and ferritin, overall, within high-risk (INSS stage 4 disease and were ≥18 months old at diagnosis), and by era, using the INRG Data Commons.

Methods/Materials

Children diagnosed with neuroblastoma (1990–2016; all stages) were categorized into LDH (n=8,867) and ferritin (n=8,575) risk groups using event-free survival (EFS). Cox models compared the prognostic strength of LDH and ferritin to age, *MYCN* status, and presence of metastatic disease. Eras were defined based on major changes in therapeutic strategy (Era I:

1990-1996, Era II: 1997-2009, Era III: 2010-2016).

Results

LDH and ferritin had non-linear relationships with outcome. The group with highest LDH had the worst EFS: overall cohort (5-year EFS) [100-899 IU/L: 76±0.6%; (0-99 and 900-1399 IU/L): 60±1.2%; ≥1400 IU/L: 36±1.2%; p<0.0001], and high-risk cohort post-2009 (3-year EFS) [117-381 IU/L: 67±8.9%; 382-1334 IU/L: 58±4.4%; (0-116 and ≥1335 IU/L): 46±3.9%; p=0.003]. The group with highest ferritin had the worst EFS: overall cohort (5-year EFS) [1-29 ng/mL: 87±0.9%; (0 and 30-89 ng/mL): 74±0.8%; ≥90 ng/mL: 48±0.9%; p<0.0001], and high-risk cohort post-2009 (3-year EFS) [1-53 ng/mL: 71±9.3%; (0 and 54-354 ng/mL): 55±4.7%; ≥355 ng/mL: 34±6.1%; p=0.0008]. In a multivariable model adjusting for age, *MYCN* status, and INSS stage 4 disease, LDH and ferritin maintained independent prognostic ability [p<.0001; adjusted hazard ratios (HRs) (95% CI): 1.7 (1.5-1.9), 2.3 (2.0-2.7), respectively].
EFS/OS improved steadily over time: 60±0.7%/67±0.7% in Era I, to 67±0.5%/75±0.5% in Era II, to 70±1.0%/79±1.0% in Era III. Over time, LDH maintained prognostic strength, attenuated slightly in Era III (HR=3.3, high-risk; HR=1.7, intermediate-risk) compared to Era I (HR=3.9, high-risk LDH; HR=1.8, intermediate-risk LDH). Ferritin gained prognostic strength over time, with larger HRs in Era III (HR=8.6, high-risk; HR=3.5, intermediate-risk) than in Era I (HR=5.6, high-risk; HR=2.4, intermediate-risk). Within the high-risk cohort over time, LDH had similar prognostic strength in all three Eras; similarly for ferritin.

Summary/Conclusions

LDH and ferritin are strongly prognostic in neuroblastoma, overall, within high-risk in all eras, and within high-risk patients treated post-2009 with modern therapy. Risk stratification should utilize the most strongly prognostic factors, whatever they may be; to date LDH and ferritin have been overlooked. LDH and ferritin have maintained prognostic strength over time, possibly because treatment assignment has not been made on the basis of LDH and ferritin. LDH and ferritin show promise for a) identifying an ultra-high risk cohort; and, b) risk stratification in low-/middle-income countries who lack access to *MYCN* status and histology data. Our descriptive analysis provides justification for routine collection of LDH and ferritin on frontline studies. LDH and ferritin should be tested in future prognostic comparisons in evolving neuroblastoma risk stratification.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 110

Harnessing copper as a novel immune checkpoint therapy for neuroblastoma

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

Clinical evidence shows that the immune system is unable to guarantee long-lasting control of neuroblastoma (NB), suggesting the existence of mechanisms allowing tumor evasion of host immunity. Cancer immune evasion is recognised as a central hallmark of tumor development. One mechanism that cancer cells use to protect themselves from attack by immune cells is the over-

expression of the Programmed Death Ligand 1 (PD-L1). The immune checkpoint protein Programmed Death receptor 1 (PD-1) expressed by lymphocytes instructs T-cells not to attack any cell expressing PD-L1, including tumor cells. Immune checkpoint inhibitors acting through the PD-L1/PD-1 axis have shown efficacy in some chemotherapy-resistant adult cancers, generating interest that these agents may also be helpful to treat certain refractory paediatric malignancies such as NB. However, the factors regulating PD-L1 expression in NB cells are poorly understood and acquired resistance to checkpoint inhibitors is also emerging to limit clinical benefit. Understanding the regulation of PD-L1 in NB is of utmost importance to optimize novel strategies and improve cancer immunotherapies.

Aims

Our recent preliminary research has identified elevated levels of copper (Cu) in NB tumors compared to normal tissues, with Cu levels strongly influencing the expression of PD-L1 in primary NB. The aim of this study is to evaluate whether copper chelation therapy can inhibit PDL-1 expression in NB and enhance immune responses, reducing tumor growth and increasing survival in vivo.

Methods/Materials

Experiments were performed using primary NB tissue and three NB cell lines: SK-N-FI, SH-SY5Y, and SK-N-BE(2)C. Protein expression of PD-L1 and copper transporter 1 (CTR1) was determined via western blot. Intracellular copper was measured by Inductively coupled plasma mass spectrometry (ICP-MS). Flow cytometry was used to analyse tumor-infiltrating lymphocytes and natural killer (NK) cells. An RNAseq analysis elucidated the mechanism of copper chelating agents on PD-L1 expression. In vivo activity of these agents was assessed in a syngeneic NB (TH-MYNC) mouse model.

Results

Tissue microarrays from neuroblastoma patient tumors revealed a significant correlation between CTR1 and PD-L1 expression ($p=0.00014$). Media supplementation with copper enhanced PD-L1 expression at both the mRNA and protein levels in NB cell lines. mRNA profiling indicated both copper and IFNy treatment produced similar effects on several key pathways involved in PD-L1 expression and tumor immune evasion. Gene set enrichment analysis (GSEA) from the top 1000 dysregulated pathways, revealed 326 upregulated and 350 downregulated pathways through copper or IFNy stimulation in NB cells. Among these, we identified four signalling cascades involved in tumor immunity: response to cytokines; regulation of cytokine production; IL6/JAK/STAT3 signalling; and TNF-a signalling via NF-kB. These cascades are all key regulators of PD-L1 expression. Conversely, copper chelators were observed to inhibit STAT3 and EGFR phosphorylation and promote ubiquitin-mediated PD-L1 degradation. Furthermore, use of copper chelators in vivo significantly increased the number of tumour-infiltrating CD8+ T and NK cells, slowed tumor growth and improved survival of TH-MYCN mice.

Summary/Conclusions

This study provides the first evidence of the crucial role of copper in modulating PD-L1 expression and its contribution to tumor immune evasion. Moreover, it raises the potential of repurposing

clinically available copper chelating agents to enhance anti-tumor immunity. This novel immunotherapy could target NB and a wide spectrum of other copper-dependent cancers.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 111

GSK-3ß inhibition by 9-ING-41: A Potential Therapy for High-Risk Neuroblastoma.

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

High-risk neuroblastoma (NB) is characterized by poor differentiation and chemoresistance. Thus, there is an urgent need for new effective therapeutic agents. Glycogen synthase kinase 3β (GSK-3β), a serine/threonine kinase with roles in neuronal progenitor homeostasis and cell metabolism, has been identified as a potential target in NB. 9-ING-41 is a small-molecule inhibitor of GSK-3β which has exhibited anti-cancer activity and no significant attributable toxicity in an ongoing phase I/II study in adults with refractory hematological malignancies and solid tumors (NCT03678883).

Aims

The specific goals of our study were to: 1) determine the anti-tumor activity of 9-ING-41 in multiple pre-clinical models of NB, and 2) identify the transcriptional programs activated by 9-ING-41.

Methods/Materials

The effect of 9-ING-41 on NB cell proliferation, apoptosis, and tumorigenic activity was determined by MTT, caspase 3, and clonogenic assays in multiple MYCN-amplified (MNA) and no-MNA cell lines. Protein expression of pro- and anti-apoptotic proteins upon 9-ING-41 treatment was assessed by western blotting. Genetic depletion of p53 in p53 wild-type lines was obtained via lentiviral-mediated shRNA. To elucidate the mechanism of action of 9-ING-41, transcriptomic analysis (RNA-seq) was performed in MNA cells upon 9-ING-41 treatment at different time points (8 and 24h). Lastly, the anti-tumor activity of 9-ING-41 was determined in MNA orthotopic cell-derived xenografts.

Results

9-ING-41 blocks cell proliferation and promotes apoptosis to a greater extent in MNA cells (IC_{50}: 0.2 μM, 5 fold-caspase activation) compared to no-MNA cells (IC_{50}: 0.98μM, < 2 fold-caspase activation). 9-ING-41 also remarkably suppresses colony formation independently of MYCN status. Because we observed high apoptosis in MNA cells which retain wild-type p53, we asked whether 9-ING-41 could stimulate p53 signaling. Western blotting analysis of MNA cell lines upon treatment with 9-ING-41 showed a clear stabilization of p53 and p53-responsive proteins (p21 and Bax), suggesting that 9-ING-41 effectively activates p53 downstream signaling. Supporting this finding, genetic depletion of p53 in MNA cells completely abrogates the effects of 9-ING-41 on cell proliferation and apoptosis, confirming that these effects are indeed p53-dependent. RNA-seq analysis identified a total of 452 genes differentially expressed upon 9-ING-41 at 24h (FDR < 0.05, FC ≥ 1.5). Hallmark pathway enrichment analysis determined the top enriched pathways as the ones associated with p53 ($-\log_{10}$ P=29.3), TNFalpha signaling, hypoxia, apoptosis, and cell cycle regulation. Notably, 9-ING-41 as single agent (40mg/Kg i.p. 5 days/week for three weeks) effectively blocked the tumor growth of MNA xenografts (p=0.03). Lastly, 9-ING-41 was able to sensitize NB cells to conventional therapies, such as irinotecan (IC_{50} irinotecan: 0.94μM; combo: 0.16μM) and temozolomide (IC_{50} temozolamide: 74μM; combo: 12μM).

Summary/Conclusions

9-ING-41 has significant pre-clinical activity in NB. Mechanistically, 9-ING-41 activates p53-driven apoptosis and upregulates gene expression programs promoting cytokine signaling, hypoxia, apoptosis, and cell cycle regulation. These data confirm that GSK-3β inhibition is a potentially effective therapeutic approach for high-risk NB, and provide a strong rationale for the inclusion of patients with advanced NB in clinical studies of 9-ING-41. These data also support the conduct of clinical studies of 9-ING-41 combined with irinotecan or temozolamide.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 112

Combination therapy with the CDK7 inhibitor and the tyrosine kinase inhibitor exerts synergistic anticancer effects against MYCN-amplified neuroblastoma

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Topic Gene regulatory networks and epigenetics: from basic science to therapy

Background/Introduction

The most common cause of death from diseases in children is cancer. Neuroblastoma is accountable for approximately 8% of all paediatric cancers but 15% of all paediatric cancer mortality.(Maris et al., 2007; Matthay et al., 2016) Neuroblastoma arises from embryonic sympathoadrenal lineage of neural crest cells and is commonly situated within the adrenal medulla, head, chest, neck and pelvis.(Brodeur, 2003). *MYCN* oncogene amplification occurs in approximately 25% of human neuroblastoma tissues and correlates with poor patient prognosis.(Maris et al., 2007; Matthay et al., 2016)

MYCN oncogene encodes N-Myc oncoprotein, a Myc family transcription factor important for cell cycle progression, cell proliferation, resistance to apoptosis *in vitro* as well as neuroblastoma formation and progression *in vivo*.(Gustafson and Weiss, 2010; Huang and Weiss, 2013) While N-Myc presents as a therapeutic target, Myc oncoproteins are considered “undruggable” as they

have no active site for ligand binding by small molecule compounds.(Beltran, 2014; Dang, 2013) As such, targeting N-Myc down-stream targets, N-Myc protein stability or *MYCN* gene transcription provides an attractive approach for inhibiting the N-Myc oncogenic pathway.
MYCN oncogene transcription is regulated by super-enhancers and cyclin dependent kinase 7 (CDK7).(Chipumuro et al., 2014) CDK7 binds to Transcription Factor II Human and regulates transcriptional initiation, pausing and elongation by phosphorylating the serine 5 and serine 7 residues of the C-terminal domain of RNA polymerase II at super-enhancer-associated oncogene loci.(Chipumuro et al., 2014; Christensen et al., 2014; Kwiatkowski et al., 2014). The covalent CDK7 inhibitor THZ1 binds to the cysteine 312 residue adjacent to the ATP cleft in the canonical kinase domain of CDK7 protein, suppresses CDK7 function, RNA polymerase II phosphorylation and the transcription of super-enhancer-associated oncogenes including *MYCN*, *MYC* and *BLC2*, and induces cancer cell growth inhibition.(Chipumuro et al., 2014; Christensen et al., 2014; Kwiatkowski et al., 2014; Nagaraja et al., 2017; Rusan et al., 2018; Wong et al., 2017b)
Receptor tyrosine kinases are a family of enzymes which are key regulators of cell proliferation, differentiation, metabolism, survival and apoptosis.(Lemmon and Schlessinger, 2010) Tyrosine kinases are activated due to ligand binding or constitutively activating mutations and induces phosphatidylinositol 3-kinase (PI3K) activation, leading to glycogen synthase kinase 3b inactivation, c-Myc and N-Myc protein dephosphorylation at threonine 58, and c-Myc and N-Myc protein stabilization.(Gustafson and Weiss, 2010)
In this study, we have performed drug screening and identified TKIs, including ponatinib and lapatinib, as the approved oncology drugs which exerted the best synergistic anticancer effects with the CDK7 inhibitor THZ1 against *MYCN*-amplified neuroblastoma cells. Combination therapy with THZ1 and ponatinib or lapatinib synergistically reduced gene expression of the protein kinase *PNUTS* and thereby reduced N-Myc protein but not mRNA expression, and synergistically induced *MYCN*-amplified neuroblastoma cell apoptosis.

Aims

We aimed to screen the US Food and Drug Administration-Approved Oncology Drugs Set V from the National Cancer Institute, and identified tyrosine kinase inhibitors (TKIs), including ponatinib and lapatinib, as the Approved Oncology Drugs exerting the best synergistic anticancer effects with THZ1 in *MYCN*-amplified neuroblastoma cells. We then validated the synergistic anti-cancer effects and to identify the underlying mechanisms of action of THZ1 and RTK inhibitor combination therapy against MYCN-amplified neuroblastoma.

Methods/Materials

Cell culture

BE(2)-C cells were cultured in Dulbecco's modified Eagle's medium (DMEM) and Kelly and CHP134 cells were cultured in Roswell Park Memorial Institute Medium (RPMI) 1640, all supplemented with 10% fetal calf serum. BE(2)-C cells were obtained from Barbara Spengler (Fordham University, New York, NY) 20 years ago, and Kelly and CHP134 cells were purchased from the European Collection of Cell Cultures in 2010 (Sigma, Sydney, Australia). All cell lines were maintained in a humidified incubator at 37^0C and 5% CO_2 in air. The identity of cell lines was confirmed in 2016, 2017 and 2018 by small tandem repeat profiling conducted at the Garvan Institute of Medical Research or Cellbank Australia.

Alamar blue assays

Alamar blue assays were performed as we described.(Sun et al., 2017; Sun et al., 2015) Briefly,

cells were transfected with control or PNUTS siRNAs for 96 hours, or treated with vehicle control or anticancer agents for 72 hours. The cells were incubated with Alamar blue (Invitrogen) for the last five hours, and cell culture plates were read on a microplate reader at 570/595 nm. Results were quantified according to the optical density absorbance units and expressed as percentage changes in the number of cells, relative to control siRNA-transfected or vehicle control-treated samples.

Drug screening

For primary screening, *MYCN* oncogene-amplified BE(2)-C neuroblastoma cells were seeded into 96-well plates and treated in triplicates with vehicle control, the CDK7 inhibitor THZ1 at 16nM which reduced the number of BE(2)-C cells by approximately 30% on its own, the US Food and Drug Administration (FDA)-Approved Oncology Drugs (AODs) Set V from National Cancer Institute at 1μM, or combination for 72 hours. Cell viability was determined by Alamar blue assays. Synergistic/additive interaction between THZ1 and the AODs was examined by fractional product method and expressed as R values.(Greco et al., 1995)

AODs which reduced the number of viable BE(2)-C cells by ≥ 90% on their own and AODs which synergized with THZ1 in reducing the number of viable BE(2)-C cells with a R value of less than 0.7 were shortlisted for secondary drug screening. BE(2)-C cells were treated with a range of doses of THZ1, the shortlisted AODs, or combination of THZ1 and the AODs for 72 hours, followed by Alamar blue assays. To determine if the effect of THZ1 and the AOD were synergistic or additive, Bliss-additivity was calculated with Bliss-additive formula.(Bliss and Bartels, 1946) If the actual combination effect was greater than that of the predicted additivity, THZ1 and the AOD exerted synergistic anticancer effect. Synergy was further validated by calculating combination indexes (CIs) for effective doses for 75% and 90% cell number reduction with the Chou-Talalay method(Chou and Talalay, 1984) and CompuSyn software (Combosyn Inc, http://www.combosyn.com/), where CI <1, 1 or >1 indicates synergistic, additive and antagonistic effect, respectively.

Apoptosis analysis

Neuroblastoma cells were transfected with control siRNA, PNUTS siRNA-1 or PNUTS siRNA-2 for 72 hours, or treated with vehicle control, 32nM THZ1, 250nM ponatinib, 1000nM lapatinib, THZ1 plus ponatinib, or THZ1 plus lapatinib for 72 hours. Cells were then stained with Annexin V-FITC and propidium iodide, followed by flow cytometry analysis of cells positively stained by Annexin V-FITC and/or propidium iodide, using FACS Canto Flow Cytometer (BD Biosciences, Franklin Lakes, NJ). The percentage of Annexin V and/or propidium iodide positively stained cells was analyzed with FlowJo Version 10 (TreeStar Inc., Ashland, OR) as we described.(Sun et al., 2015)

siRNA transfection

Neuroblastoma cells were plated into 6 well plates or T25 flasks and transfected with siRNAs using Lipofectamine 2000 (Life Technologies, Grand Island, NY) according to the manufacturer's instructions and as we described previously.(Liu et al., 2014; Wong et al., 2017a) RNA or protein were harvested for RT-PCR and immunoblot analyses.

Plasmid transfection

pCMV6-entry empty vector and pCMV6-entry PNUTS expression constructs were purchased from Origene (Origene, Rockville, MD). Neuroblastoma cells were transfected with the constructs using Lipofectamine 2000 reagent (Life Technologies) according to the manufacturer's protocol and as we described.(Sun et al., 2017; Sun et al., 2015)

Real time reverse transcription PCR (RT-PCR)

RNA was extracted from cells with RNeasy Plus Mini kit (Qiagen, Hamburg, Germany) and quantified with a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA), according to the manufacturer's instructions. RT-PCR was performed using Moloney murine

leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA) for cDNA synthesis and gene specific primers and Power SYBR Green Master Mix (Invitrogen) as the fluorescent dye for PCR in Applied Biosystems 7900 (Applied Biosystems, Grand Island, NY). All primers were synthesized by Sigma (Sigma). The comparative threshold cycle (Ct) method(Schmittgen and Livak, 2008) was used to evaluate fold changes in target genes, relative to the housekeeping gene actin.

Immunoblot

Neuroblastoma cells were lysed in RIPA buffer (150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-Cl pH 7.5) containing protease inhibitors (Sigma, St Louis, MO) and phosphatase inhibitors (Roche, Penzberg, Germany). Protein was extracted, quantified with the Bicinchoninic Acid Assay kit (Pierce, Rockford, IL), and loaded onto sodium dodecyl sulphate-polyacrylamide gels, followed by electrophoresis and transfer to nitrocellulose membranes. After blocking, membranes were probed with rabbit anti-PNUTS (1:1,000) (Bethyl Laboratories, Montgomery, TX) and mouse anti-N-Myc (1:1000) (Santa Cruz Biotechnology) antibodies. The membranes were then incubated with a goat anti-rabbit or goat anti-mouse antibody conjugated to horseradish peroxidase (1:10000) (both from Santa Cruz Biotechnology), and protein bands were visualized with SuperSignal (Pierce). The membranes were finally probed with an anti-actin antibody (1:30000) (Sigma) as loading controls.

Affymetrix microarray differential gene expression study

BE(2)-C neuroblastoma cells were treated with vehicle control, THZ1, ponatinib, lapatinib, THZ1 plus ponatinib, or THZ1 plus lapatinib. Six hours later, RNA was extracted from the cells with RNeasy Mini kit (Qiagen), and differential gene expression was investigated using Affymetrix Arrays (Affymetrix, Santa Clara, CA), as we described.(Liu et al., 2014; Sun et al., 2017; Sun et al., 2015) Microarray data were analyzed in R (http://www.r-project.org/) with bioconductor package (http://www.bioconductor.org/), and normalized with GenePattern software (Version 3.9.0 Broad Institute) and LimmaGP modules (Version 20.0) available at (https://pwbc.garvan.org.au/gp/), and deposited at Gene Expression Omnibus website (Series GSEXXXXX).

Statistical analysis

Experiments for statistical analysis were performed at least 3 times. Data were examined with Graphpad Prism 6 program and expressed as mean ± standard error. Differences were analyzed for statistical significance with one-way *ANOVA* among groups or two-sided unpaired *t* test for two groups.

Results

We screened the Approved Oncology Drugs Set and identified 17 AODs which reduced cell viability by ≥ 90% on their own and the 5 AODs which synergized with THZ1 were then subjected to secondary screen. BE(2)-C cells were treated with vehicle control, a range of dosages of THZ1, the AODs, or combination, followed by Alamar blue assays. Synergy or additivity of the THZ1 and AOD combination therapies was calculated using the Bliss-additivity model.(Bliss and Bartels, 1946) The synergy/additivity analysis showed that vincristine and doxorubicin, two first line chemotherapy drugs for treating neuroblastoma patients, did not synergize with THZ1; that the other DNA topoisomerase II inhibitors, the other microtubule inhibitors, the MEK inhibitor, the gene transcription/histone deacetylase/RNA synthesis inhibitors as well as the proteasome and protein translation inhibitors did not considerably synergize with THZ1; and that the TKIs ponatinib, lapatinib and nilotinib synergized with THZ1. The primary and secondary screens therefore identify the TKIs as the AODs exerting the best synergistic anticancer effects with THZ1 against *MYCN*-amplified neuroblastoma.

We next examined whether TKIs and THZ1 exerted synergistic anticancer effects in a range of *MYCN*-amplified neuroblastoma cell lines with little toxicity to normal cells. *MYCN*-amplified KELLY and CHP134 neuroblastoma cells were treated with vehicle control, a range of doses of THZ1, ponatinib, lapatinib, nilotinib, THZ1 plus ponatinib, lapatinib or nilotinib for 72 hours, followed by Alamar blue assays. Combination effect analysis with the Bliss-additivity model demonstrated that combination therapy with THZ1 and ponatinib, lapatinib or nilotinib synergistically reduced the number of viable Kelly and CHP134 cells. In addition, combination index (CI) values for reducing BE(2)-C, Kelly and CHP134 neuroblastoma cell viability by 75% and 90% by the combination therapies were generated by Compusyn(Bijnsdorp et al., 2011), and the CI values further confirmed that combination therapy with THZ1 and ponatinib, lapatinib or nilotinib synergistically reduced the number of viable BE(2)-C, Kelly and CHP134 cells with CI values < 0.5 in all cases.

Ponatinib and nilotinib both target the tyrosine kinase Bcr-ABL(Rosti et al., 2017) while lapatinib targets the tyrosine kinase ERBB2 and EGFR.(Rimawi et al., 2015) As the concentrations for achieving considerable synergistic anticancer effects with THZ1 was higher for nilotinib (up to 8μM) than ponatinib (up to 1μM) and lapatinib (up to 2μM), we used ponatinib and lapatinib for further studies. BE(2)-C and Kelly neuroblastoma and WI-38 fibroblast cells were treated with vehicle control, THZ1, ponatinib, lapatinib, or combination of THZ1 and ponatinib or lapatinib for 72 hours, followed by staining with the apoptosis marker Annexin V. Flow cytometry analysis showed that THZ1 and ponatinib, as well as THZ1 and lapatinib, synergistically induced apoptosis in 72% and 70% BE(2)-C and 66% and 58% Kelly neuroblastoma cells but not in WI38 fibroblasts. Taken together, the data suggest that THZ1 and the TKIs ponatinib and lapatinib synergistically induce apoptosis in neuroblastoma but not normal non-malignant cells.

THZ1 suppresses *MYCN* gene transcription by blocking CDK7 binding to the *MYCN* gene promoter.(Chipumuro et al., 2014) We examined whether THZ1 and the TKI ponatinib and lapatinib synergistically regulate N-Myc mRNA and protein expression. BE(2)-C and Kelly cells were treated with vehicle control, THZ1, ponatinib, lapatinib, THZ1 plus ponatinib, or THZ1 plus lapatinib for 48 hours. RT-PCR and immunoblot analyses showed that THZ1 alone reduced N-Myc mRNA and protein expression, and that combination therapy with THZ1 and the TKIs did not co-operatively reduce N-Myc mRNA expression, but synergistically blocked the expression of N-Myc protein. The data suggest that THZ1 and TKI combination therapy synergistically blocks N-Myc protein expression through a post-transcriptional mechanism.

To examine the mechanism through which THZ1 and the TKI ponatinib and lapatinib synergistically reduce N-Myc protein expression and neuroblastoma cell proliferation and survival, we performed Affymetrix microarray gene expression experiments in BE(2)-C cells after treatment with vehicle control, THZ1, ponatinib, lapatinib, THZ1 plus ponatinib or THZ1 plus lapatinib for 6 hours. Using two fold change as the cut-off point, 13 genes were found to be commonly down-regulated and one gene commonly up-regulated by both THZ1 and ponatinib as well as THZ1 and lapatinib combinations. Lysine rich coiled-coil 1 (KRCC1) and phosphatase 1 nuclear targeting subunit (PNUTS, also known as PPP1R10), the genes most synergistically downregulated by THZ1 and TKI combination therapies were selected for validation.

BE(2)-C and Kelly cells were treated with vehicle control, THZ1, ponatinib, lapatinib, THZ1 plus ponatinib, or THZ1 plus lapatinib for 48 hours. RT-PCR analysis showed that monotherapy with THZ1 reduced KRCC1 and PNUTS mRNA expression and monotherapy with ponatinib or lapatinib showed no effect, and that combination therapy with THZ1 and ponatinib or lapatinib synergistically and dramatically reduced KRCC1 and PNUTS mRNA expression. Consistent with the RT-PCR data, immunoblot analysis showed that combination therapy with THZ1 and ponatinib or lapatinib synergistically blocked PNUTS protein expression. Taken together, the data

demonstrate that the THZ1 and TKI combination therapy synergistically reduces KRCC1 and PNUTS expression in *MYCN*-amplified neuroblastoma cells.
PNUTS has recently been shown to directly binds to Aurora kinase A and c-Myc proteins and blocks c-Myc protein degradation.(Dingar et al., 2018; Wang et al., 2019) We therefore examined whether PNUTS regulates N-Myc protein expression and neuroblastoma cell proliferation and survival. Real-time RT-PCR and immunoblot analyses showed that transfection of BE(2)-C and Kelly cells with two independent PNUTS siRNAs, PNUTS siRNA-1 or PNUTS siRNA-2, efficiently knocked down PNUTS mRNA and protein expression, and considerably reduced N-Myc protein but not N-Myc mRNA expression. BE(2)-C and Kelly cells were next transfected with an empty vector or PNUTS expression construct. Immunoblot analysis demonstrated that PNUTS over-expression up-regulated N-Myc protein expression.
BE(2)-C and Kelly cells were then transfected with control siRNA or PNUTS siRNAs for 72 hours, followed by Alamar blue assays or staining with Annexin V and flow cytometry analysis of Annexin V positively stained cells. Alamar blue assays showed that knocking down PNUTS expression significantly reduced the number of viable BE(2)-C and Kelly neuroblastoma cells, and flow cytometry analysis of Annexin V stained cells showed that knocking down PNUTS expression led to BE(2)-C and Kelly cell apoptosis. Taken together, the data demonstrate that PNUTS is important for N-Myc protein expression and neuroblastoma cell proliferation and survival.

Summary/Conclusions

In summary, our screening of AODs identifies TKIs ponatinib, lapatinib and nilotinib as the AODs exerting the best synergistic anticancer effects with THZ1 against *MYCN* gene-amplified neuroblastoma cells. Combination therapy with THZ1 and the TKIs synergistically blocks *PNUTS* gene expression and N-Myc protein expression, and synergistically induces apoptosis in *MYCN*-amplified neuroblastoma but not normal non-malignant cells. PNUTS up-regulates N-Myc protein expression, and PNUTS knockdown induces *MYCN*-amplified neuroblastoma cell growth inhibition and apoptosis. As CDK7 inhibitors are currently under clinical evaluation in patients, our data suggest the addition of the TKIs in patients in clinical trials.

Type Basic

Presentation Preference Traditional poster

Abstract nr. 113

CASZ1 represses the core transcriptional regulatory circuitry in neuroblastoma via an interconnected feedback loop

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Topic Gene regulatory networks and epigenetics: from basic science to therapy

Background/Introduction

Neuroblastoma (NB) is neural crest-derived and commonly arises in the adrenal gland. NB core transcriptional regulatory circuitry (CRC) contains essential transcription factors (TFs) ISL1, HAND2, GATA3, PHOX2B and TBX2, and these TFs form an interconnected autoregulatory feed-forward loop to determine an adrenergic phenotype of NB. What TFs directly repress the NB CRC are unknown.

Aims

The zinc-finger TF CASZ1 orchestrates cell commitment in normal neuroblasts and functions as a Chr1p36 tumor suppressor in NB. Here we investigate whether CASZ1 regulates the CRC to affect NB cell identity.

Methods/Materials

CASZ1b overexpressing NB cell lines are established. Single-cell RNA-seq (scRNA-seq), bulk RNA-seq and ChIP-seq are performed to investigate the role of CASZ1 in controlling CRC.

Results

Interrogation of published scRNA-seq data of the developing adrenomedullary cells within the adrenals of murine embryos reveals that CASZ1 is heterogeneously expressed with low levels in the schwann cell precursors (SCPs), median levels in the bridge state cells and sympathoblasts, and high levels in more differentiated chromaffin cells. NB CRC components are highly expressed in sympathoblasts, but other types of adrenomedullary cells have variable mRNA levels. This suggests that together with CRC TFs, CASZ1 is involved in the regulation of SCPs stemness and chromaffin cell differentiation. Published microarray data analyses shows that levels of CASZ1 is 2-fold lower while levels of NB CRC components is 2-100x higher in NB compared to levels in the

adrenals ($p<0.0001$), suggesting that CASZ1 is silenced while CRC components are aberrantly upregulated in NB. Published ChIP-seq data analyses show that NB CRC components bind to CASZ1 gene locus. Knock-down of HAND2 in NB cells decreases HAND2 binding to CASZ1 gene locus resulting in a >2-fold increase of CASZ1 mRNA levels, indicating that the CRC represses CASZ1 expression. Consistently, scRNA-seq of NB cells reveals that CASZ1 is expressed in less than 5% of cells and CASZ1 expressing cells have lower levels of CRC components. Restoration of CASZ1b in SY5Y cells results in growth inhibition and neurite extension. Consistently, retinoids up-regulates CASZ1 and CASZ1 knock-down attenuates retinoids induced neuronal differentiation. RNA-seq results show that restoration of CASZ1b in NB results in a positive enrichment of neurotransmitter transporter, catecholamine secretion and neuron projection gene signatures ($p<0.002$), indicating a chromaffin cell differentiation. Importantly, CASZ1b restoration in NB cells down-regulates the expression of CRC components ($p<0.05$) and results in a negative enrichment of the NB adrenergic gene signature that determined by CRC ($p<0.001$). ChIP-seq results show that restoration of CASZ1b in SY5Y cells leads to an enrichment of CASZ1b binding within super enhancers (SEs) and typical enhancers (TEs) of NB CRC components which is accompanied by decreased H3K27ac signal mainly in TEs. Thus, CASZ1b directly represses CRC components through decreasing TEs activity.

Summary/Conclusions

Our study identifies that CASZ1 forms a negative feedback regulatory circuit with established NB CRC to switch the NB adrenergic phenotype to a more differentiated chromaffin cell phenotype. This leads to a novel model in which the CRC forms an interconnected feedback loop with a TF to control cell identity in health and disease.

Type Basic

Presentation Preference Oral presentation

Advances in Neuroblastoma Research (ANR) Meeting
ANR 2020
Amsterdam
11-14
May
www.anr2020.org

Abstract nr. 114

Efficacious targeting of TERT-rearranged neuroblastoma with BET bromodomain inhibitor and proteasome inhibitor combination therapy

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Topic Gene regulatory networks and epigenetics: from basic science to therapy

Background/Introduction

TERT gene rearrangement with transcriptional super-enhancers leads to massive *TERT* over-expression and neuroblastoma in 20-25% of high-risk neuroblastoma patients. Unfortunately, the majority of patients with this subtype of neuroblastoma die of the disease, as no targeted therapy is currently available.

Aims

To identify the BET bromodomain protein BRD4 as a critical regulator of *TERT* expression in *TERT* gene rearranged neuroblastoma cells; to identify the anticancer agent which exerts the best synergistic anticancer effects with the BET bromodomain inhibitor OTX015 against *TERT*-rearranged neuroblastoma; and to demonstrate the considerable anticancer effects of the combination therapy in mice xenografted with *TERT*-rearranged neuroblastoma cell lines or patient-derived xenograft (PDX) tumors.

Methods/Materials

The effects of BRD4 on TERT mRNA and protein expression, cell proliferation and survival were examined by RT-PCR, immunoblot and cell cycle analyses in *TERT*-rearranged neuroblastoma cells after transfection with control or BRD4 siRNAs or shRNAs. The US Food and Drug Administration-approved oncology drug library was screened to discover the proteasome inhibitor, carfilzomib, as the drug exerting the best synergistic anticancer effects with the BET bromodomain inhibitor OTX015 against *TERT*-rearranged neuroblastoma cells. The synergistic anticancer effects of OTX015 and carfilzomib were then examined by Alamar blue assays and flow cytometry analysis of apoptosis in *TERT*-rearranged neuroblastoma cell lines and PDX cells. Finally, mice were xenografted with *TERT*-rearranged neuroblastoma cell lines or PDX tumors, and the anticancer effects of OTX015 and/or carfilzomib were investigated in the mice.

Results

Knocking down BRD4 considerably reduced TERT mRNA and protein expression and telomerase activity in *TERT*-rearranged neuroblastoma cells, and led to *TERT*-rearranged neuroblastoma cell cycle arrest. By screening a library of the US Food and Drug Administration-approved oncology drugs, we identified the proteasome inhibitor carfilzomib as the drug exerting the best synergistic anticancer effects with OTX015 against *TERT*-rearranged neuroblastoma cells. OTX015 and carfilzomib synergistically induced apoptosis and synergistically reduced TERT protein expression in *TERT*-rearranged neuroblastoma cells with little toxicity against normal non-malignant cells, and the anticancer effect was substantially blocked by forced ectopic TERT over-expression. In mice xenografted with *TERT*-rearranged neuroblastoma cells, OTX015 and carfilzomib synergistically and considerably blocked TERT expression, induced tumor cell apoptosis, suppressed tumor progression and improved mouse survival. Importantly, forced TERT over-expression considerably blocked the anticancer effects of OTX015 and carfilzomib combination therapy in mice xenografted with *TERT*-rearranged neuroblastoma cells, and OTX015 and carfilzomib also considerably blocked tumor progression and improved survival in a PDX model of *TERT*-

rearranged neuroblastoma.

Summary/Conclusions

TERT gene expression in *TERT*-rearranged neuroblastoma is controlled by the BET bromodomain protein BRD4. OTX015 and carfilzomib combination therapy synergistically blocks TERT expression, induces *TERT*-rearranged neuroblastoma cell apoptosis, and blocks *TERT*-rearranged neuroblastoma progression *in vivo*. As OTX015 is currently in Phase II clinical trials and carfilzomib is an approved oncology drug, OTX015 and carfilzomib combination treatment is likely to be rapidly translated into the first clinical trial of targeted therapy for *TERT*-rearranged neuroblastoma patients.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 115

Bone marrow and peripheral blood express significantly correlated levels of seven neuroblastoma-associated mRNAs detected by droplet digital PCR

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

Background: Neuroblastoma (NB) is the most common extracranial solid tumor in children and is characterized by its extreme heterogeneity, ranging from spontaneous regression to malignant progression. More than 50% of high-risk NB patients experience tumor relapses. Minimal residual disease (MRD) is defined conceptually as residual tumor cells that persistently reside in cancer patients after local and systemic cancer therapies. It persists as cancer stem cells in primary tumor, circulating tumor cells in peripheral blood (PB), and disseminated tumor cells in bone marrow (BM). In NB patients, MRD is primarily responsible for tumor relapses and usually monitored in BM and PB. To improve the outcome of high-risk NB, more sensitive and accurate detection of MRD is needed to evaluate the treatment response and disease burden. Although a growing number of MRD detection assays based on either the same or distinct set(s) of NB-associated mRNAs (NB-mRNAs) for BM and PB have been reported, the correlation of NB-mRNAs expression between BM and PB remains elusive.

Aims

Aims: To determine the correlation of 7 NB-mRNAs expression detected by droplet digital PCR (ddPCR) between BM and PB in high risk NB patients.

Methods/Materials

Methods: 7 NB-mRNAs (CRMP1, DBH, DDC, GAP43, ISL1, PHOX2B, and TH mRNAs) expression in PB and BM samples from 17 high risk NB patients who were diagnosed and treated at Kobe Children's Hospital or Kobe University Hospital between June 2011 and January 2018 was determined by ddPCR. The samples were collected concurrently (within 3 days apart) during the entire course of treatment. Medical charts were retrospectively reviewed to retrieve clinical data (age at diagnosis, sex, BM infiltration at diagnosis, DNA ploidy and MYC-N status of the primary tumor). Correlation between BM and PB was assessed by Spearman's rank correlation coefficient.

Results

Results: Level of 7NB-mRNAs (defined as the combined signature) showed a significant positive correlation between BM and PB, but it was considerably higher in BM than PB. The level of each NB-mRNA in BM and PB was also significantly correlated between BM and PB. The significant correlation of 7NB-mRNAs expression was detected even in BM infiltration at diagnosis-negative patients. When the disease status (remission, stable, or progression) was evaluated for all pairs of BM and PB samples, the samples collected at remission and progression showed higher correlation than those at stable.

Summary/Conclusions

Conclusions: Level of 7 NB-mRNAs detected by ddPCR is approximately 100 times higher in BM than PB, but shows a significant positive correlation between BM and PB.

Type Translational

Presentation Preference Traditional poster

Abstract nr. 116

THE EVALUATION OF THE TREATMENT RESULTS ACCORDING TO THE RELATIONSHIP OF THE RENAL VESSELS AND TUMOR IN LOW-RISK PATIENTS WITH ABDOMINAL NEUROBLASTOMA – AN ANALYSYS OF THE PATIENTS WHO ENROLLED IN THE LOW-RISK PROTOCOL (JN-L-10) FROM THE JAPAN CHILDREN'S CANCER GROUP NEUROBLASTOMA COMMITTEE

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

Image-defined risk factors (IDRFs) have been used to evaluate surgical risks before any treatments. An INRG staging system was developed based on the clinical criteria and IDRFs. The Japan Children's Cancer Group (JCCG) Neuroblastoma Committee (JNBSG) conducted the JN-L-10 for low-risk patients using IDRFs as the main factor for determining treatment. According to the new guideline (NG) for assessing IDRFs (Brisse HJ, et al. Radiology, 2011), patients should be considered "IDRF-present", even if the tumor is in contact with the renal vessels only, despite being previously diagnosed as "IDRF-not present." However, this new definition may change the

IDRF status in a considerable number of patients.

Aims

We analyzed the treatment results of the patients who enrolled in the JN-L-10 according to the relationship between the renal vessels and tumor in order to clarify whether or not “contact with renal vessels” should be considered an IDRF.

Methods/Materials

Of the 60 patients enrolled in JN-L-10, the 37 with an abdominal tumor who had precise information available for IDRFs were selected in this study. Patients were divided into the 3 groups according to the relationship between the renal vessels and tumor: Group S (n=14), separated; Group C (n=16), in contact; Group E (n=7), encased. We analyzed the treatment results, including the surgical results, such as operation time, intra-operative blood loss and surgical complications. Data were shown as the median with the range.

Results

In Groups S, C and E, 5 (35.7%), 2 (12.5%) and 4 (57.1%) patients did not undergo any surgery other than a biopsy until the end of the treatment, 9 (64.3%), 13 (81.3%) and 0 (0%) patients underwent primary resection, and 0 (0%), 1 (6.3%) and 3 (42.9%) patients underwent resection after neoadjuvant chemotherapy, respectively. Therefore, 26 of 37 patients (9 in Group S, 14 in Group C, 3 in Group E) underwent surgery rather than a biopsy. In Groups S, C and E among these 26 patients, the operation time (minutes) was 161.5 (85-304), 168.5 (103-500) and 235.0 (207-268), and the intra-operative blood loss (ml) was 10 (0-25), 44 (5-941) and 103 (85-144), respectively. There were some trends, but no significant differences were noted among the three groups in the operation time or intra-operative bleeding. Surgical complications were observed in four patients, all in Group C (ages: 1, 4, 76 and 134 months). Three of these four patients underwent primary resection. The surgical complications were as follows: class IV intra-operative bleeding, partial renal atrophy, vena cava injury and adrenal vein injury.

Summary/Conclusions

Group E patients tended to have a longer operation time and greater intra-operative bleeding than other patients. Surgical complications were observed only in Group C patients. Although there was only one patient who suffered a complication directly related to the renal vessels, “contact with renal vessels” might be a potential surgical risk for abdominal neuroblastoma. Whether or not neoadjuvant chemotherapy can reduce the surgical risks in Group C patients should be prospectively investigated in a larger cohort study in the future.

Type Clinical

Presentation Preference Rapid Fire Poster Presentation

Abstract nr. 117

Single-cell transcriptomic analysis reveals the early separation of neuroblastoma fate in Th-MYCN mice

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

One of the challenging topics of neuroblastoma research is to understand the enigmatic phenomenon of “spontaneous regression”. Although its plausible mechanisms such as developmental programmed cell death following neurotrophin deprivation have been proposed, the precise molecular mechanisms driving this phenomenon remain a matter of speculation (Brodeur GM, *Cell Tissue Res*. 2018).

Aims

The aim of this study is to understand the cellular and molecular bases of neuroblastoma fate separation (i.e. either tumor growth or spontaneous regression) in the Th-MYCN mouse model and human specimens.

Methods/Materials

We investigated the early-stage tumor tissues in Th-MYCN mice and performed droplet-type single-cell RNA sequencing (scRNA-seq) by Chromium Single Cell 3’ assay (10x Genomics). We carried out single-molecule fluorescence in situ hybridization (smFISH) to validate the analysis. Besides, we also obtained scRNA-seq data from stage 4 and stage 4S human neuroblastoma specimens.

Results

Firstly, survival analysis showed that 80% of Th-MYCN hemizygous mice died of tumor at 10 to 20 weeks of age while 20% of them never developed neuroblastoma. Secondly, when we investigated the tumor origin of the mice histologically, all of them had clusters of neuroblastoma cells until 3 weeks of age. Therefore, although neuroblastoma cells appeared in all cases, whether they developed further into a tumor or disappeared was likely determined during early age. We assume that the latter fate was due to a spontaneous regression-like phenomenon in Th-MYCN mice. Thirdly, to understand the observed fate determination, we obtained single-cell transcriptomes of early neuroblastoma cells from the tissues of seven 3-week-old and one 6-week-old (tumor-developing) Th-MYCN mice. The analysis revealed distinct cell types in the tissues including $MYCN^{+}$ neuroblastoma cells, ganglion cells, glial cells and so on. Within $MYCN^{+}$ neuroblastoma cells, there were distinct sub-populations and the proportion of cells was different between individuals, suggesting that different fates were captured at the single-cell level. The sub-populations were characterized by differential expressions of target genes regulated by certain transcription factors. Lastly, smFISH analysis validated the results of single-cell analysis. Recently, we obtained scRNA-seq data from two stage 4 and two stage 4S human neuroblastoma specimens which were dissected at diagnosis. We are currently analyzing these data and trying to integrate the data from Th-MYCN mice and human neuroblastomas.

Summary/Conclusions

Single-cell transcriptomic analysis captured variations in neuroblastoma fates observed in early ages of Th-MYCN mice. Early-stage subpopulation of $MYCN^{+}$ neuroblastoma cells can be discriminated by the differential expressions of several genes. These data obtained from Th-MYCN mice and ongoing analysis of human neuroblastoma at single-cell resolution will possibly provide fundamental cellular and molecular bases of fate determination, i.e. spontaneous regression, in neuroblastoma.

Type Basic

Presentation Preference Rapid Fire Poster Presentation

Abstract nr. 118

A review of the 27K neuroblastoma PubMed abstracts: finding missing links of cellular heterogeneity with tumor origins through text-mining

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

A hallmark of neuroblastoma is its high cellular heterogeneity, both intra-tumor and inter-tumor despite the low mutational burden beyond copy number variants. MYCN is a key oncogene involved in this heterogeneity. One feature of the heterogeneity is the heavy metastasis burden for high-risk patients. The type of metastasis is very specific for neuroblastoma and cannot be directly compared to adult metastasized cancers. Neuroblastoma researchers might, therefore, be expected to communicate differently on concepts such as “metastasis” and “heterogeneity” in contrast to other (adult) cancer researchers.

Aims

Through the use of natural language processing (NLP), we aimed to map genes related to heterogeneity within neuroblastoma literature, to genes linked with the origin of the tumors. As a supporting aim, we aimed to extract genes discussed in the neuroblastoma literature, and link them to ‘factual’ neuroblastoma transcriptional data.

Methods/Materials

We collected 27.449 abstracts from a local PubMed repository and indexed it with the whoosh package. Using the gensim package, we generated embeddings for all words in abstracts containing “neuroblastoma”. In parallel, we processed transcriptomics from a neuroblastoma dataset. With the gene correlation-matrix, we formed a gene-gene network when a predefined minimum correlation was reached. We subsequently embedded the network with node2vec to vectors of the same size as the literature embeddings. Code for the analysis can be found in our broader text mining tool “sina” (search indirect nomenclature associations, https://github.com/dicaso/sina).

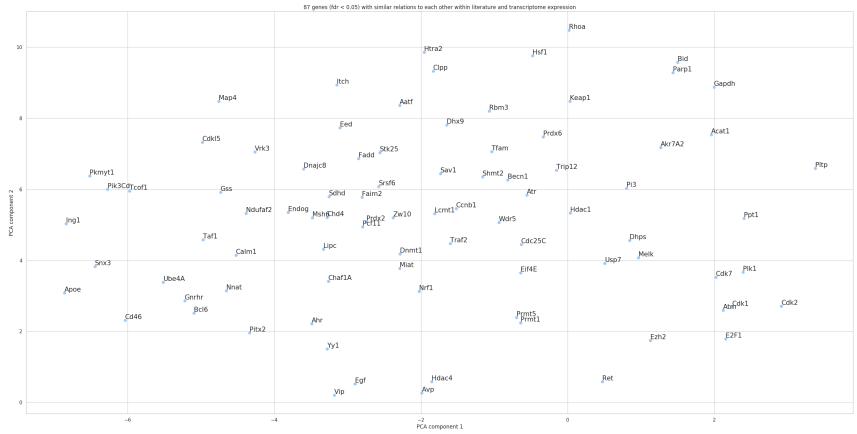
Results

In a multidimensional space, different word vector dimensions should correspond to different types of meaning (e.g. size of an entity, gender) that a computer algorithm can then subsequently use to make inferences. A well-known example within NLP is the ability to turn a “king”-vector into a

“queen”-vector by subtracting the “man”-vector and adding a “woman”-vector. In analogy, what we have done is investigating the extent to which such relations can be extracted from the neuroblastoma literature or vice-versa to what extent factual experimental data is shaping the correlations that can be extracted through text-mining. From 845 genes for which we were able to construct neuroblastoma embedding vectors, we found 178 genes that had a p-value < 0.05 for correlation between literature association with other genes and correlations with those genes in the transcriptional dataset, of which 87 with FDR < 0.05. Top correlating genes between literature and transcriptional correlations were WDR5, CLPP, CDK1, CDC25C, and PDRX2. The abstract figure shows the gene associations within the neuroblastoma literature. Within the full set of significant genes, Enrichr shows enrichment for SP1, E2F1, and TP53 targets. Subsequently, we linked these genes with significant embedded meaning, to heterogeneity and tumor origin of neuroblastoma using the literature embeddings.

Summary/Conclusions

Science progresses through analysis of correlations or mutual information between features such as genes, discovered in experimental results. Our work allows mapping the reported connections, directly back to experimental data to see which correlations are emphasized most in the literature. In the future, this might indicate underrepresented correlations worthy of further investigations.



87 genes (fdr < 0.05) with similar relations to each other within literature and transcriptome expression

Type Basic

Presentation Preference Oral presentation

Abstract nr. 119

Identification of deregulated ECM and Axon guidance pathways in ALK- overexpressing Neuroblastoma xenografts

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Neuroblastoma (NB) tumors are composed of both adrenergic (ADR) and mesenchymal (MES) cell types displaying plasticity. The Anaplastic Lymphoma Kinase (ALK) gene is frequently altered in NB through copy number changes and mutations, with $ALK^{-F1174L}$ and $ALK^{-R1275Q}$ being the most potent activating mutations. ALK thus represents a bona fide target for therapy.

Aims

Here we aim to identify common and ALK-status specific downstream target genes and signaling pathways activated by ALK-wt, $ALK^{-F1174L}$ and $ALK^{-R1275Q}$ activating mutations *in vivo.*

Methods/Materials

ALK-wt, $ALK^{-F1174L}$ and $ALK^{-R1275Q}$ were overexpressed in the human NB cell lines SK-N-Be2c (adrenergic and tumorigenic) and SH-EP (mesenchymal and weakly tumorigenic) using a lentiviral vector (pLIV). Transduced cells were further injected orthotopically (SK-N-Be2c) in the adrenal gland of athymic Swiss nude mice or subcutaneously (SH-EP) in NOD-SCID- mice. Tumor growth was followed by echography. Histologic analysis were performed on hematoxylin and eosin

stained tumors. Transcriptomic analyses were performed by RNA-seq (Illumina).

Results

The tumor growth capacity of SH-EP cells was highly increased by ALK$^{-F1174L}$, and to a lesser extent by ALK-wt overexpression. Histologic analysis revealed that ALK expression in SH-EP cells altered xenograft histology. Indeed, tumor cells of the SH-EP-control group (pLIV) displayed a MES-like phenotype, while ALK$^{-R1275Q}$ and ALK-wt tumors presented heterogeneous regions with either MES-like cells or differentiating neuroblasts, and ALK$^{-F1174L}$ expressing tumors were mainly composed of undifferentiated/poorly differentiated neuroblasts. In contrast, ALK expression had no impact on the *in vivo* growth properties of SK-N-Be2c cells and on tumor cell histology. RNAseq analyses revealed numerous differentially expressed genes ($|Log2(FC)| \geq 1$; adjusted p-value ≤ 0.05) in SH-EP and SK-N-Be2c ALK$^{-F1174L}$ (n=1371 and n= 963, respectively), ALK$^{-R1275Q}$ (n=1049 and n=102) and ALK-wt tumors (n=1639 and n=282) relative to controls. In addition, Gene Set Enrichment Analysis (GSEA) of the ADR and MES signature showed an enrichment of the adrenergic signature in SH-EP ALK$^{-F1174L}$ and ALK-wt tumors. Moreover, shared deregulated genes and pathways were identified in SH-EP and SK-N-Be2c ALK-overexpressing tumors. First, gene ontology (GO) term enrichment analysis revealed the deregulation of MAP kinase (MAPK/ERK) related genes in ALK-overexpressing tumors. Second, several deregulated pathways associated to the ECM and to axon guidance/neuronal differentiation in ALK-overexpressing tumors were identified. Finally, among these pathways, we observed specifically deregulated genes belonging to the collagen family, the semaphorin/plexin complex and to the ROBO family. Moreover, STRING analysis (interaction score: highest confidence 0.900) revealed high interactions across ECM and axon guidance/neuronal differentiation pathways.

Summary/Conclusions

These transcriptomic results highlight a possible role for ALK in downregulating biological pathways related to ECM and neuronal differentiation that are known to be involved in NB pathogenesis. Selected genes identified in these pathways will be further investigated in order to validate their implication in ALK-overexpressing NB tumors. The validation of new genes/signaling pathways would bring new information about the pathways activated through ALK deregulated signaling and highlight potential new targets for NB therapy.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 120

Fatty acid metabolism is essential for neuroblastoma

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

High-risk neuroblastoma frequently acquire therapy resistance and metastatic spread, with fatal clinical outcome. Moreover, these patients may suffer long-term side effects after treatment, including slowed mental and physical development. High MYC signaling or *MYCN* amplification are strongly correlated to poor prognosis and treatment failure in neuroblastoma patients. Alternative approaches, as differentiation-inducing therapy, are especially interesting for high-risk patients in which resistance to conventional chemotherapy arises. Lipids perform critical roles not only as energy fuels and cell membrane components, but also as signaling molecules regulating many cellular processes. We have previously shown that MYCN inhibition induces accumulation of neutral lipids in the cytoplasm of neuroblastoma cells, together with induction of neural differentiation (Zirath et al., PNAS 2013). We recently published that fatty acid ß-oxidation is a major source of energy production in neuroblastoma cells, and the inhibition of fatty acid oxidation *in vivo* with the small molecule etomoxir reduces tumor growth in a *MYCN*-amplified neuroblastoma xenograft model in mice (Oliynyk, Ruiz-Pérez et al., iScience 2019).

Aims

Here our objective is to analyze the crosstalk between lipid metabolism and cell differentiation regulated by MYC(N), to identify new therapeutic targets with potential use for induction of differentiation.

Methods/Materials

We have performed bioinformatics analysis of patient gene expression datasets, as well as metabolomics, Seahorse extracellular flux metabolic assays, cell and molecular biology techniques, and xenografts using *MYCN*-amplified and non-amplified cells in mice.

Results

The analysis of functional processes in a neuroblastoma patient gene expression dataset highlights lipid metabolism as one of the most relevant functions for this type of tumor. Proteomics of neuroblastoma cells shows that lipid metabolism and related signaling elements, as well as proteins involved in cell differentiation, are consistently affected by MYCN downregulation. Furthermore, high expression of acetyl-CoA carboxylase (*ACACA*) and fatty acid synthase (*FASN*), the enzymes involved in fatty acid synthesis, correlates to poor survival in neuroblastoma patients, suggesting fatty acid synthesis and degradation as promising targets for treatment. Metabolomic analysis shows that MYCN inhibition has an impact on the relative contribution of glucose- and glutamine-derived carbons to fatty acid synthesis, and to reduced fatty acid content in neuroblastoma cells. The inhibition of *de novo* fatty acid synthesis (targeting either ACC or FASN) triggers MYC(N) protein downregulation and differentiation of both *MYCN*-amplified and non-amplified neuroblastoma cells. Moreover, fatty acid synthesis inhibition has an impact in mitochondrial structure and function. The withdrawal of major nutrients as glucose and glutamine does not induce neuroblastoma differentiation, while the incubation in delipidized cell culture medium induces neurite outgrowth. Consequently, cell membrane-permeable fatty acids prevent the phenotypes observed, suggesting a very specific relationship of lipid metabolism with induction of differentiation. Inhibition of de novo fatty acid *in vivo* prevents the growth of *MYCN*-amplified and non-amplified neuroblastoma xenografts and induces expression of differentiation markers in the tumor cells.

Summary/Conclusions

Our work aims to elucidate the impact of lipid metabolism on neuroblastoma biology, with potential implications for metabolism-targeted and differentiation-based therapies. Here we show that targeting fatty acid metabolism could be an interesting therapeutic strategy for neuroblastoma treatment, with a special potential for induction of differentiation.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 121

Particular poor treatment outcome of high-risk patients with low affinity FCGR2A/3A and IL-2-related high regulatory T cell levels during dinutuximab beta long-term infusion in two independent cohorts

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

We previously reported the role of regulatory T cell (Treg) levels as well as FCGR polymorphisms in therapy outcome of pts treated with a long-term infusion (LTI) of the anti-GD_2 antibody (Ab) dinutuximab beta in combination with interleukin-2 (IL-2).

Aims

Here, we identified a subgroup of high-risk pts with poor treatment response by combining these parameters for the analysis of therapy outcome.

Methods/Materials

53 pts received up to 5 cycles (35 days (d)) LTI of 100 mg/m^2 ch14.18/CHO (d8-18) in combination with 6x10^6 IU/m^2 subcutaneous IL-2 (d1-5; 8-12), and 160 mg/m^2/d oral isotretinoin (d22-35) in a closed single-center program (APN311-303). Therapy-related Treg ($CD4^+$/$CD25^+$ /$CD127^-$) counts were determined by flow cytometry (d15, day 8 of Ab-infusion). FCGR2A-H131R- and FCGR3A-V158F polymorphisms were assessed with real-time PCR. The impact of all parameters on 4-year PFS was evaluated. To validate the results of the single-center program 124

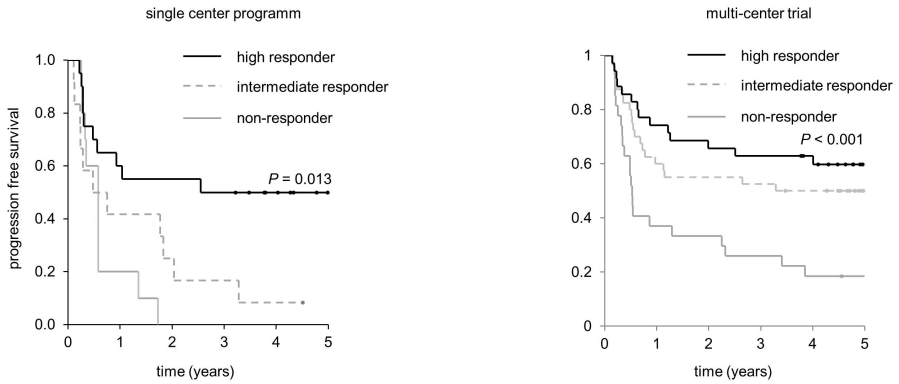
pts enrolled in a prospective open label multi-center SIOPEN Phase II clinical trial (EudraCT-Number: 2009-018077-31) were additionally analyzed.

Results

We identified pts with unfavorable high Treg levels (> 138 cells/µl) and low affinity FCGR2A/3A revealing a particular poor outcome (non-responder). These pts showed a PFS of 0% (95% CI[0; 0], n = 10) compared to 8% (95% CI [0; 0.24], n = 12) and 50% (95% CI [0.28; 0.72], n = 24) in pts with either one (intermediate responder; FCGR2A/3A high affinity or low Treg level) or both favorable parameters (high responder), respectively (P = 0.013). These observations could be confirmed in the SIOPEN trial showing a PFS of 19% (95% CI [0.04; 0.33], n = 27) in non-responder pts compared to 50% (95% CI [0.35; 0.66], n = 40) and 63% (95% CI [0.47; 0.78], n = 35) in intermediate and high responder, respectively (P < 0.001).

Summary/Conclusions

IL-2-dependent Treg counts and FCGR-polymorphisms could be utilized to identify pts with particular poor therapy outcome of pts treated with dinutuximab beta in combination with IL-2.



Impact of FCGR2A/3A polymorphisms and regulatory T cells on progression-free survival.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 122

4SC-205-mediated targeting of KIF11, a new prognostic marker and therapeutic target in neuroblastoma.

Author - Dr Segura, Miguel F, Vall Hebron Research Institute, Barcelona, Spain (Presenting Author)

Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

High-risk neuroblastoma tumors are highly proliferative and, therefore, need a robust cell division machinery. Furthermore, targeting cell division has been demonstrated to be effective in various preclinical studies in pediatric malignancies. Early phase studies of pharmacological inhibition of cell-cycle regulators in the treatment of cancer in children showed appreciable tolerability, and support the incorporation of combination approaches.

Preliminary results of our laboratory have shown a clear association of motor proteins expression (i.e. kinesins) with poor patient survival. In particular, we observed that tumors from patients in advanced stages of the disease or relapsed tumors, express high levels of KIF11 (kinesin family member 11), a mitotic spindle-specific kinesin which is essential for bipolar spindle formation and mitotic progression in human cells.

Loss of function experiments and pharmacological inhibition of KIF11 showed efficacy in patient-derived orthotopic xenografts (PDOX), suggesting that KIF11 is essential for the proliferation and viability of neuroblastoma cells, thus positioning KIF11 as a possible new therapeutic target for high-risk, refractory and/or relapsed neuroblastoma.

Aims

This project aims to define the contribution of KIF11 to the aggressiveness of high-risk neuroblastoma and evaluate its potential as a prognostic tool and therapeutic target. We will test the therapeutic potential of the new KIF11 inhibitor, 4SC-205 (currently in clinical trials for adult tumors), orally administered and highly specific for KIF11.

Methods/Materials

To analyze KIF11 mRNA expression levels and correlation with clinical parameters, we used the GSE49710 (SEQC, Revet et al, 2010, n=498) dataset. SiRNA, shRNA targeting KIF11 and a small-molecule inhibitor (i.e. 4SC-205, 40mg/kg three times/week) were used to investigate the effects of KIF11 inhibition in proliferation, apoptosis and cell cycle in multiple neuroblastoma cell lines *in vitro* and *in vivo* using PDOX.

Results

The expression analysis of KIF11 mRNA in NB patients revealed that expression levels above the median correlated with poor event-free and overall survival. Multivariate Cox regression analysis showed that KIF11 mRNA expression can be an independent prognostic factor for lower overall survival. Furthermore, genetic and pharmacologic KIF11 inhibition strikingly reduced cell proliferation and viability in multiple chemoresistant neuroblastoma cell lines. Specifically, KIF11 inhibition arrested neuroblastoma cells in G2/M-phase and triggered distinct molecular events compatible with mitotic catastrophe and apoptotic cell death. The oral administration of the KIF11 inhibitor 4SC-205 in mice bearing neuroblastoma PDOX significantly impaired tumor growth and triggered the expected molecular response in the tumors (e.g. increase in the phosphorylation of Histone 3). Moreover, 4SC-205 reduced metastatic burden and expanded the overall survival of mice with neuroblastoma liver metastasis.

Summary/Conclusions

Our findings suggest that the expression of KIF11 could be an independent prognostic factor in neuroblastoma and its pharmacological inhibition a potential new therapeutic strategy to treat high-risk neuroblastoma.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 123

Catecholamine metabolism in neuroblastoma differs from pheochromocytoma due to a reduced amount of neurosecretory vesicles and lack of PNMT expression

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Topic Other

Background/Introduction

Metabolism of catecholamines (CATs, dopamine (DA), norepinephrine (NE) and epinephrine (E)), and metanephrines (MNs; methoxytyramine (MT), normetanephrine (NMN), and metanephrine (MN)) remains enigmatic in patients affected by neuroblastoma (NB). NMN and especially MT are used as biomarkers of NB in plasma and urine. MNs are suspected to directly arise from the tumor, as extensively described for pheochromocytoma and paraganglioma (PHEO/PGL) two other neuroendocrine tumors secreting MNs.

Aims

To study and compare CAT metabolism and secretion in NB relative to PHEO/PGL.

Methods/Materials

NB patient-derived xenografts (PDX) were generated by subcutaneous injection of primary NB cells into athymic Swiss nude mice. CATs and MNs were measured by HPLC MS/MS in NB-PDX, NB, and PHEO/PGL tumor tissues, in primary and established cell lines *in vitro*, as well as in

plasma from mice and patients. Neuroendocrine markers were analyzed by immunoblotting, and the presence of neurosecretory vesicles within tumor cells was observed by electron microscopy.

Results

Due to difficulties in finding sufficient samples of NB biopsies, we first characterized CAT metabolism in six NB-PDX models as compared to NB patients. Plasma levels of MNs were comparable in NB-PDX-bearing mice and patients with NB. Also, the profile of individual MNs, characterized by a reduced level of MN relative to NMN and MT, previously identified in the plasma and urines of patients with NB and referred to as noradrenergic, was also conserved in the plasma of mouse with NB. Moreover, we observed that the intratumoral concentrations of MNs and CATs were similar in NB and NB-PDX tissues and displayed a noradrenergic profile as well. Phenylethanolamine N-methyltransferase (PNMT), the enzyme that converts NE to E, was undetectable by immunoblotting in primary NB tumors, NB-PDX and NB cell lines, which explains the noradrenergic CAT profile of NB.
Furthermore, comparison between plasma from patients with NB and PHEO revealed comparable levels of MNs, while CATs displayed a slight reduction (2.7x) in NB. Surprisingly, intratumoral concentrations of MNs and CATs in human primary NB and NB-PDX were extremely low contrasting with elevated values commonly observed in PHEO/PGL (~120 and ~3500 fold change, respectively). To understand the biological bases of this discrepancy, we analyzed the amount of neurosecretory granules and their associated markers. A low expression level of markers of neurosecretory vesicles, such as VMAT1/2, CHGRA/B, and SYP, was identified in NB relative to PHEO through investigation of published transcriptomic datasets (R2: Genomics Analysis and Visualization Platform (http://r2.amc.nl)). This was confirmed for SYP protein expression by immunoblotting. Moreover, a very low number of neurosecretory granules was observed in NB-PDX relative to PHEO.

Summary/Conclusions

Our results demonstrate that murine NB-PDX are relevant NB preclinical models reflecting the CATs and MNs metabolism of NB patients. The noradrenergic profile of CATs and MNs results from the lack of PNMT expression in NB. Moreover, conversely to PHEO/PGL where high amount of neurosecretory vesicles enable CATs storage, their low amount in NB cells do not protect CATs of being metabolized and lead to their rapid transformation in the cytoplasm into MNs that diffuse in blood.

Type Basic

Presentation Preference Rapid Fire Poster Presentation

Abstract nr. 124

A Novel Chimeric Antigen Receptor Targeting Anaplastic Lymphoma Kinase (ALK) for Childhood Cancer Immunotherapy

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

Manipulating patient T cells through the insertion of chimeric antigen receptors (CARs) has transformed the treatment of haematological malignancies by enabling T cell redirection. Success rates in solid tumours have been less efficacious. High risk neuroblastoma (NB) is the most common extracranial solid tumour of childhood accounting for ~15% of childhood cancer related deaths. Anaplastic lymphoma kinase (ALK), a tyrosine kinase receptor, is speculated to be involved in healthy neural development but also in NB progression. Both germline and somatic ALK mutations are implicated in constitutively active oncogenic signalling. Previous CAR T cells targeted against NB, such as GD2 and L1-CAM do not yet yield desired results, demonstrating the demand for novel targets.

Aims

To evaluate an ALK CAR as potential novel therapy based on *in vitro* cytotoxic activity, cytokine production and proliferative capabilities.

Methods/Materials

A panel of second generation ALK-specific CARs was initially investigated; consisting of five unique ALK single chain variable fragments (scFv), two stalks regions of different lengths and a CD28 costimulatory domain with CD3ζ, in order to determine a lead combination. Human primary αβ T cells were virally transduced to express the CAR on their surface and co-cultured with cell lines of varying ALK surface expression to determine *in vitro* cytotoxicity, cytokine production, short- and long-term proliferative capacity with and without antigen re-stimulation.

Results

All ALK CARs created were successfully expressed on the surface of αβ T cells and demonstrated robust cytotoxicity and antigen-dependant cytokine generation when co-cultured

with ALK positive cell lines but not with ALK negative cell lines. When NB cell lines were co-cultured, with lower ALK surface expression, cytotoxicity and the ability to produce cytokines by the ALK CARs was lowered in a correlative manner. Furthermore, all CARs demonstrated the ability to proliferate at short time points and produce IL-2 and IFNγ after re-stimulation. Despite this, their perseverance at longer time points appeared to be limited. Interestingly, differences in stalk regions appeared to influence whether CAR proliferation was antigen-dependant or antigen-independent.

Summary/Conclusions

We have successfully demonstrated that CAR T cells directed against ALK show clear antigen dependant cytotoxicity and cytokine production when ALK target expression is high. There was a clear correlative relationship between ALK surface density and the level of cytotoxic activity and cytokine production, shown when NB cell lines were utilised, suggesting ALK may be expressed below the threshold antigen density required to trigger responses. There is hope to combine to targeting of ALK with another NB associated antigen in a co-CAR system also.

Type Translational

Presentation Preference Traditional poster

Abstract nr. 125

Combining genomics and ultra-sensitive bone marrow assessment for risk stratification in high-risk metastatic neuroblastoma: a HR-NBL1/SIOPEN study

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

While genomic analysis and therapy response measurement is established clinical practice in hematological malignancies and various solid cancers, markers guiding risk stratification and therapy decisions for high-risk metastatic neuroblastoma (HR-NBL) are limited.

Aims

To analyze and show the prognostic relevance of an integrated analysis including genomic aberrations and highly sensitive bone marrow minimal residual disease (BM-MRD) quantification.

Methods/Materials

BM and tumor samples from high-risk metastatic neuroblastoma patients treated in the HR-NBL1/SIOPEN trial were analyzed for genomic aberrations using ultra-high-density SNP array or whole genome sequencing techniques. BM samples were searched and GD_2/CD56/DAPI-positive disseminated tumor cells (DTCs) and mononuclear cells (MNCs) were quantified by highly sensitive and automated immunofluorescence plus FISH (AIPF) method.

Results

The cohort included 226 patients with a 5-year event-free survival (5y-EFS) of 37±3% and a cumulative incidence of relapse (5y-CIR) of 61±4%. Multivariate analysis found the presence of telomere maintenance including aberrations in *TERT* and *ATRX* genes, and a gain on chromosome 1q significantly associated with an increased risk for relapse, as was post-induction BM-MRD positivity. A combined model resulted in a superior outcome for BM-MRD negative patients lacking adverse genomic markers (n=15) with a 5y-EFS of 87±9%. The presence of BM-MRD after induction therapy combined with any of the genomic markers (n=20) indicated dismal outcome with 5y-EFS of 5±5%.

Summary/Conclusions

The combination of genomic analysis and highly sensitive BM-MRD monitoring shows additive value that potentially enhance an early assessment of therapy response and risk of relapse, justifying further evaluation in prospective clinical trials.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 126

Novel strategies for delivering small RNAs to treat neuroblastoma

Author - Dr Boloix, Ariadna, Vall Hebron Research Institute, Barcelona, Spain (Presenting Author)
Topic Gene regulatory networks and epigenetics: from basic science to therapy

Background/Introduction

MicroRNAs are a class of small non-coding RNAs that are crucial regulators of gene expression and found to be deregulated in multiple human diseases such as cancer, both in adult and pediatric tumours. In particular, an overall reduction of miRNA expression has been shown to contribute to the progression and outcome of patients with neuroblastoma, one of the most aggressive pediatric solid tumours that could benefit from microRNA-restoration therapies. Nevertheless, miRNA-based therapies have not reached the market due, in part, to suboptimal biodistribution and short half-life in bloodstream. Nanomedicine promises the precise delivery of nucleic acids into tumours with reduced off-target toxicities. Hence, conjugation of miRNA to nanoparticles could be a good strategy to test anti-tumour effects of miRNAs *in vivo*. However, there is still a lack of a standard formulation for their clinical administration.

Aims

The aim of this project is to develop a novel nanocarrier able to deliver tumour suppressive miRNAs to treat high-risk-neuroblastoma tumours.

Methods/Materials

Novel unilamellar lipid-based nanoparticles were prepared using a compressed fluid-based technology named DELOS-SUSP process (Depressurization of an Expanded Liquid Organic Solution-Suspension) and further purified by diafiltration. The tumour suppressor miR-323a-5p was conjugated with nanovesicles through electrostatic interactions. The delivery of miR-323a-5p to target cells was measured by quantitative RT-qPCR. MiRNA target genes levels were monitored by RT-qPCR and western blot. Finally, the phenotypic effects of miR-323a-5p overexpression on cell proliferation were measured by crystal violet staining.

Results

We have synthetized a potential clinical formulation based on novel nanoparticles. Compared to other nanoparticles, such as liposomes, our nanovesicles are small (80-100nm), unilamellar, with high homogeneity and positive charge. These properties grant an excellent long-term colloidal stability and an efficient entrapment of small RNA (sRNA) in their surface. miR-323a-5p nanocomplexes showed efficient miRNA loading, resistance to nucleases, high transfection

efficiency and intracellular accumulation of mature miRNA forms. From the functional point of view, treatment of neuroblastoma cells with miR-323a-5p nanoconjugates induced downregulation of *bona fide* miR-323a-5p targets, such as CCND1 or CHAF1A, at mRNA and protein level. Finally, miR-323a-5p nanoconjugates were capable of halting the proliferation of neuroblastoma cells.

Summary/Conclusions

In summary, we developed a new nanomedicine composed by a tumour-suppressive microRNA and an innovative nanocarrier which together could be a potential tool for the treatment of high-risk neuroblastoma.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 127

A Novel Chimeric Antigen Receptor Targeting Anaplastic Lymphoma Kinase (ALK) for Childhood Cancer Immunotherapy

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

Manipulating patient T cells through the insertion of chimeric antigen receptors (CARs) has transformed the treatment of haematological malignancies by enabling T cell redirection. Success rates in solid tumours have been less efficacious. High risk neuroblastoma (NB) is the most common extracranial solid tumour of childhood accounting for ~15% of childhood cancer related deaths. Anaplastic lymphoma kinase (ALK), a tyrosine kinase receptor, is speculated to be involved in healthy neural development but also in NB progression. Both germline and somatic ALK mutations are implicated in constitutively active oncogenic signalling. Previous CAR T cells targeted against NB, such as GD2 and L1-CAM do not yet yield desired results, demonstrating the demand for novel targets.

Aims

To evaluate an ALK CAR as potential novel therapy based on *in vitro* cytotoxic activity, cytokine production and proliferative capabilities.

Methods/Materials

A panel of second generation ALK-specific CARs was initially investigated; consisting of five unique ALK single chain variable fragments (scFv), two stalks regions of different lengths and a CD28 costimulatory domain with CD3ζ, in order to determine a lead combination. Human primary αβ T cells were virally transduced to express the CAR on their surface and co-cultured with cell lines of varying ALK surface expression to determine *in vitro* cytotoxicity, cytokine production, short- and long-term proliferative capacity with and without antigen re-stimulation.

Results

All ALK CARs created were successfully expressed on the surface of αβ T cells and demonstrated robust cytotoxicity and antigen-dependant cytokine generation when co-cultured

with ALK positive cell lines but not with ALK negative cell lines. When NB cell lines were co-cultured, with lower ALK surface expression, cytotoxicity and the ability to produce cytokines by the ALK CARs was lowered in a correlative manner. Furthermore, all CARs demonstrated the ability to proliferate at short time points and produce IL-2 and IFNγ after re-stimulation. Despite this, their perseverance at longer time points appeared to be limited. Interestingly, differences in stalk regions appeared to influence whether CAR proliferation was antigen-dependant or antigen-independent.

Summary/Conclusions

We have successfully demonstrated that CAR T cells directed against ALK show clear antigen dependant cytotoxicity and cytokine production when ALK target expression is high. There was a clear correlative relationship between ALK surface density and the level of cytotoxic activity and cytokine production, shown when NB cell lines were utilised, suggesting ALK may be expressed below the threshold antigen density required to trigger responses. There is hope to combine to targeting of ALK with another NB associated antigen in a co-CAR system also.

Type Translational

Presentation Preference Oral presentation

Advances in Neuroblastoma Research (ANR) Meeting
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Abstract nr. 128

Minimal residual disease evaluation by droplet digital PCR and quantitative real-time PCR in high-risk neuroblastoma patients

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

More than half of highrisk neuroblastoma (NB) cases experience tumor relapse/regrowth due to chemoresistant residual disease. To achieve the long-term survival of high-risk NB patients, the disappearance and/or inactivation of minimal residual disease (MRD) is essential. Although the detection of several sets of NB-associated mRNAs (NB-mRNAs) by quantitative real-time PCR (qPCR) has been used for monitoring MRD in NB patients, the accurate evaluation of MRD remains a clinical challenge. Droplet digital PCR (ddPCR) is an adaption of qPCR that potentially provides more simple and reproducible detection of low-level of mRNAs but remains tested for MRD detection in NB patients.

Aims

To compare ddPCR with qPCR for the accurate evaluation of MRD in high-risk NB patients.

Methods/Materials

Bone marrow (BM) and peripheral blood (PB) samples were collected from 20 high-risk NB patients treated based on the high-risk NB protocols (JN-H-07, JN-H-11, JN-H-15) of the Japanese Children's Cancer Group (JCCG) Neuroblastoma Committee (JNBSG) at Kobe Children Hospital and Kobe University Hospital between June 2011 and January 2018. Expression of CRMP1, DBH, DDC, GAP43, ISL1, PHOX2B, and TH mRNAs were determined by ddPCR using a QX200 ddPCR system (Bio-Rad Laboratories, Hercules, CA) and qPCR using an ABI 7500 Fast Real-time PCR system (Applied Biosystems, Foster City, CA, USA). HPRT1 and B2M, GAPDH, PGK1 were used as endogenous references for ddPCR and qPCR, respectively. Level of each NB-mRNA (each signature) and 7NB-mRNAs (combined signature) was defined as the relative copy number of each NB-mRNA and the weighted sum of 7 relative copy numbers, respectively. For a direct comparison between ddPCR and qPCR, level of 7NB-mRNAs was defined as the geometric mean of 7 relative copy numbers for ddPCR and of 7 delta threshold cycle (dCt) values for qPCR, respectively.

Results

Among 208 BM and 67 PB samples analyzed in the present study, level of 7NB-mRNAs determined by ddPCR varied between individuals and collection time points but was significantly correlated with the treatment response and disease burden. Of 208 BM samples, 73 samples were collected during post-treatment follow-up period and their level of 7NB-mRNAs were determined by both qPCR and ddPCR. 17 relapse/regrowth samples expressed significantly higher level of qPCR and ddPCR 7NB-mRNAs than 56 non-relapse samples. To compare qPCR with ddPCR, their ROC curves were then plotted for level of 7NB-mRNAs. Whereas qPCR estimated area under curve (AUC) of 0.594 (<0.7), ddPCR did AUC of 0.706 (>0.7) with the significant accuracy.

Summary/Conclusions

The present study suggests that level of 7NB-mRNAs detected by qPCR and ddPCR is significantly associated with tumor relapse/regrowth of high-risk NB patients and ddPCR provides a better prognostic value than qPCR.

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Type Translational

Presentation Preference Oral presentation

Abstract nr. 129

DFMO can reduce anti-GD2 induced allodynia and increase anti-GD2 efficacy in animal models

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

High levels of polyamines are found in many human cancers including neuroblastoma. Elevated polyamines contribute significantly to tumor immunosuppression and polyamine depletion can exert antitumor effects and promote immunity. In addition, polyamines can enhance neuronal sensitization, including development of allodynia and other forms of pathological pain. Anti-GD2 therapy with dinutuximab is effective in improving the survival of high-risk neuroblastoma patients in remission and after relapse. However, only slightly over half of the patients with advanced disease have a favorable outcome, with allodynia hindering its usage at higher doses.

Aims

The aims of this study were to determine whether polyamine depletion might be an effective anti-tumor, anti-allodynia and pro-immune system strategy when used in conjunction with immunotherapy.

Methods/Materials

Allodynia experiments were conducted in male Sprague-Dawley rats where tail vein injection of anti-GD2 (14G2a) causes a paw-touch withdrawal reflex that strongly mimics allodynia seen in children. Tumor formation experiments utilized A/J mice injected intravenously with NXS2 neuroblastoma cells and C57BL/6 mice injected subcutaneously with EL4 lymphoma cells. Animals were also allowed to drink regular water or water containing various concentrations of DFMO (0.25%, 0.5% or 1.0%) for 7 days prior to any injections. DFMO and polyamine levels were determined by HPLC and mass spectrometry. Splenocytes were analyzed by flow cytometry.

Results

Rats injected with both 1 and 2 mg/kg 14G2a developed significant allodynia with similar onsets of development (latency) of about 1 hour post-injection, with maximum allodynia occurring about 3-5h's post-injection ($p<0.001$, repeated measures ANOVA). In contrast, animals allowed to drink water-containing 1% DFMO displayed withdrawal thresholds that were not significantly different from pre-injection levels for the 1 mg/kg injected animals ($p>0.05$). In animals injected with 2 mg/kg 14G2a, 1% DFMO significantly attenuated but did not completely prevent pain behavior. Consistent with the known actions of DFMO, serum putrescine and spermidine levels were significantly reduced in the DFMO-treated animals.
In both mouse models, survival was significantly improved for animals drinking water containing 1% DFMO (Log rank test, $p<0.01$), but suboptimal 14G2a injections (100 µg i.p. once weekly, $p>0.05$) did not yield additional survival benefit. In contrast, when the suboptimal growth suppressor concentrations of 0.25% to 0.5% DFMO were used in combination with 14G2a (100 ug i.p.), a significant prolongation of survival was observed ($p<0.01$). A significant reduction in the percent of myeloid-derived suppressor cells (MDSCs), which can inhibit anti-tumor immune reactions and stimulate tumor growth and metastasis, was observed in splenocytes of the 0.25% DFMO-treated C57BL/6 mice ($p<0.05$), with an even more significant reduction in the DFMO + 14G2a treated animals ($p<0.01$).

Summary/Conclusions

Given its favorable toxicity profile and ability to reduce anti-GD2 -induced allodynia, increase survival and modulate the tumor microenvironment, DFMO may be an important adjunct to anti-GD2 immunotherapy in addition to a role as a potential anti-cancer therapeutic.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 130

Late disappearance of bone marrow minimal residual disease after KIR-ligand-mismatched allogeneic CBT suggests KIR-mismatched NK cell activity against neuroblastoma

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Topic Other

Background/Introduction

Killer cell immunoglobulin-like receptor (KIR)/HLA genotypes predictive of missing KIR ligands are reported to be associated with a better outcome after autologous hematopoietic stem cell transplantation in patients with high risk neuroblastoma (NB). We previously reported that KIR-ligand-mismatched allogeneic cord blood transplantation (CBT) as a consolidation therapy reduced relapse of bone marrow and improved survival in children with primary high-risk

metastatic neuroblastoma (Nishio et al. ANR 2018). We believe that the detection of bone marrow minimal residual disease (BM-MRD) in patients with NB can be used to monitor their response to therapy and predict relapse.

Aims

We evaluated BM-MRD to monitor the response to KIR-ligand-mismatched CBT.

Methods/Materials

Seven patients with refractory/relapsed metastatic NB whose BM-MRD remained positive before CBT were included in this study. To detect BM-MRD, PHOX2B and tyrosine hydroxylase (TH) levels were measured by real-time quantitative PCR in BM aspirates before and after CBT, as previously reported. The BM samples were collected from both sides of the posterior iliac crest. BM-MRD was determined to be positive when the level of either PHOX2B or TH was detectable.

Results

The median age at diagnosis was 4.1 years (range: 2.8–7.7 years). All patients were heavily treated with a median of 16 courses (range: 13–24 courses) of chemotherapy, and 5 patients previously received high-dose chemotherapy with peripheral blood stem cell rescue before CBT. The conditioning regimen for CBT was 180 mg/m^2 of melphalan and 12 Gy of TBI in 4 patients and 125 mg/m^2 of fludarabine, 140 mg/m^2 of melphalan, and 2 Gy of TBI in 3 patients. The degree of HLA compatibility by serological methods was 5/6 for 3 patients and 4/6 for 4 patients. Five patients had C1/C1 of HLA-Cw and received C1/C2 cord blood (KIR-mismatched donor). One patient received HLA-A11+ cord blood, and another patient received HLA-A11+ and Bw4+ cord blood (KIR-mismatched donor). The median follow-up period from CBT was 2.0 years (range: 1.2–3.7 years). Engraftment was achieved in all patients, and none of the patients developed either grade II–IV acute graft-versus-host disease (GVHD) or chronic GVHD. Surprisingly, BM-MRD became negative after CBT in 5 patients (71%) at 2, 3, 8, 10, and 12 months of CBT. Three of these patients were alive without relapse until the last follow-up at 18, 31, and 50 months. In the remaining 2 patients, BM-MRD did not disappear. One patient developed brain metastasis at 11 months after CBT, whereas another patient was still alive without disease progression, although BM-MRD was positive at the last follow-up 18 months after CBT.

Summary/Conclusions

Late disappearance of BM-MRD and prolonged relapse-free survival after KIR-ligand-mismatched CBT suggests KIR-mismatched NK cell activity against neuroblastoma.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 131

Metabolic switch between serine/glycine pathway and TCA cycle for neuroblastoma therapy

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

Neuroblastoma is the most common extracranial childhood tumor of the sympathetic nervous system. Clinical outcome remains poor in patients with high-risk neuroblastoma, therefore, novel treatment approaches are required. Repositioning existing drugs for new indications could deliver the productivity increases and reduce development risk because they have well-known safety and pharmacokinetic profiles.

Aims

In this study, we aim to discover targets and repositioning drugs for neuroblastoma therapy.

Methods/Materials

We performed an integrative transcriptome analysis of high-risk neuroblastoma to find out potential drug targets. We further systematically investigated small-molecule gene-expression signatures across multiple cell lines from the L1000 data of the Library of Integrated Network-based Cellular Signatures (LINCS) project. These perturbational signatures were indeed linked to cancer hallmark and drug sensitivity. The drug-protein interaction was predicted by docking simulation, and the isothermal titration calorimetry (ITC) assay was further used to verify the prediction. Additionally, we utilized MS/MS spectrometry to measure the metabolites in the serine/glycine pathway and tricarboxylic acid (TCA) cycle in drug-treated neuroblastoma cells. The anti-tumor impact of the drug is also dissected by *in vitro* and *in vivo* study.

Results

We uncovered phosphoglycerate dehydrogenase (PHGDH) as a disease-relevant gene. High expression of PHGDH was found to positively correlate with poor survival in patients with

neuroblastoma. Consistent with this finding, PHGDH knockdown significantly reduced cell viability and clonogenic growth in SK-N-DZ cells expressing high levels of endogenous PHGDH, whereas PHGDH overexpression conferred selective growth advantage to SK-N-AS cells harboring low endogenous PHGDH. This suggests a key role of PHGDH in cellular programming and tumor aggressiveness in neuroblastoma. From the LINCS analysis, we predicted that PHGDH expression can be strongly inhibited by homoharringtonine (HHT), a natural alkaloid used as a traditional Chinese medicine for treating acute myeloid leukemia (AML) and chronic myeloid leukemia (CML). Consistent with the prediction, treatment of neuroblastoma cells with HHT decreased PHGDH expression, enhanced cytotoxicity, and triggered apoptosis at nanomolar concentrations. Docking simulation and ITC assay further verified that HHT can bind to PHGDH and inhibit its enzyme activity by competing with the substrate NAD^+. Overexpression of PHGDH in SK-N-AS and SK-N-SH neuroblastoma cells conferred resistance to HHT, further supporting the drug–target relationship. Given that PHGDH catalyzes the first step in the *de novo* serine synthesis pathway (SSP) and is essential for turnover of amino acids including glycine, we evaluated the changes of some selected metabolites in neuroblastoma cells treated with HHT. We found that HHT treatment decreased SSP while activating TCA cycle, accompanied with an increase in reactive oxygen species (ROS). Lastly, we examined the *in vivo* efficacy of HHT in a mouse neuroblastoma xenograft model by subcutaneously injecting SK-N-BE(2)C cells. Consistent with *in vitro* results, HHT treatment significantly reduced tumor volume and increased mouse survival, without observable changes in body weight or damages to organs such as liver, spleen, and kidney.

Summary/Conclusions

Taken together, these findings suggest that metabolic switch between serine/glycine pathway and TCA cycle with a new PHGDH inhibitor is a powerful therapeutic strategy for neuroblastoma.

Type Basic

Presentation Preference Traditional poster

Abstract nr. 132

Levels of Neuroblastoma-specific mRNAs in children with stage 2/3 MYCN-Amplified NB are below the prognostic cut-points

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

High levels of adrenergic mRNAs in bone marrow aspirates (BM) and peripheral blood (PB) samples from children[1] and infants/toddlers[2] with stage 4 disease are associated with a worse outcome.

Aims

To determine the frequency of NB-specific mRNAs detection in samples from the small subset of high-risk children with localized *MYCN*-amplified NB tumors, and ascertain whether the levels were predictive of outcome.

Methods/Materials

Out of 121 children with localized *MYCN*-amplified NB enrolled in the SIOPEN/HR-NBL-1 trial, we collected 86 BM and 67 PB samples from 91 patients at diagnosis. Samples were also collected at end of induction (54 BM and 47 PB), pre-maintenance (35 BM and 29 PB) and at end of treatment (37 BM and 37 PB). Samples collected in PAXgene blood RNA tubes[TM] were processed and analysed by RTqPCR according to standardized procedures.

Results

At diagnosis, 59/86 (69%) and 63/83 (76%) BM samples had detectable TH and PHOX2B mRNAs, respectively. In PB samples TH and PHOX2B mRNAs were detected in 48/67 (72%) and 50/65 (77%) samples respectively. At end of induction, detectable TH and PHOX2B mRNAs were found in 20/54 (37%) and 9/51 (18%) BM, 15/45 (33%) and 5/47 (11%) PB, respectively. Pre-maintenance, 6/35 (17%) and 1/34 (3%) BM, 8/29 (28%) and 2/29 (7%) PB had detectable TH and PHOX2B mRNAs, respectively; whereas at end of treatment, detectable TH and PHOX2B mRNAs were found in 9/37 (24%) and 3/35 (9%) BM, 6/37 (16%) and 3/36 (8%) PB, respectively.
However, when cut-points associated with outcome in children with metastatic NB[1] were applied, the results indicated that 0/86 BM and 1/67 PB at diagnosis, 5/54 BM and 0/45 PB at end of induction, 1/35 BM and 0/29 PB pre-maintenance, 0/37 BM and 0/37 PB at end of treatment had levels of TH mRNAs above the specific cut-points. For PHOX2B mRNA, 48/83 BM and 1/65 PB at diagnosis, 2/51 BM and 4/47 PB at end of induction, 0/34 BM and 1/29 PB pre-maintenance, and 0/35 BM and 3/36 PB at end of treatment were above the specific cut-points.
These findings indicated that in children with localized *MYCN*-amplified NB the levels of NB-specific mRNAs at each time points were mostly below the prognostic cut-points, with the exception of PHOX2B mRNAs in BM samples at diagnosis. Survival analysis performed by dividing patients with levels of PHOX2B mRNAs above or below the BM cut-point failed to show association with EFS or OS, likely because levels in 24/48 (50%) positive samples were close to the established cut-point.

Summary/Conclusions

In the small subset of high-risk children with localized *MYCN*-amplified NB tumors, the levels of TH and PHOX2B mRNAs in BM and PB samples are mostly below the specific cut-points associated to worse outcome of children and infants/toddlers with metastatic NB, confirming that low levels of these mRNAs associated indeed with a more favorable outcome.

[1] Viprey et al, J Clin Oncol, 32, 1074-83, 2014; [2]Corrias et al, Pediatric Blood &Cancer 65(7):e27052, 2017

Type Clinical

Presentation Preference Rapid Fire Poster Presentation

Abstract nr. 133

APR-246, which Restores p53 Function, is Highly Active against Alternative Lengthening of Telomere (ALT) Neuroblastoma Cell Lines and PDXs

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

Most cancers proliferate by activating telomerase. Some cancers employ the non-telomerase ALT mechanism and about 20% of high-risk neuroblastomas are ALT+. ALT in neuroblastoma has been associated with resistance to DNA damaging agents, p53 loss-of-function (p53LOF), *ATRX* mutations, and very poor survival. We showed that ATM kinase, which activates functional p53, is constitutively activated in ALT neuroblastoma cell lines and PDXs. We hypothesized that constitutive activation of ATM would selectively sensitize ALT neuroblastomas to p53 reactivation by APR-246.

Aims

Methods/Materials

In vitro cytotoxicity was assayed by DIMSCAN digital imaging microscopy, ATM/ATR activation by immunofluorescence microscopy, and protein expression by western blotting. Subcutaneous patient-derived xenografts (PDXs) and cell line-derived xenografts (CDXs) in nu/nu mice were treated with APR-246 and irinotecan.

Results

ALT, p53LOF neuroblastoma cell lines were significantly ($p<0.0001$) less sensitive to DNA-damaging agents relative to telomerase+ p53LOF comparators. We observed higher ($p< 0.005$) phosphorylation of ATM/ATR kinases (involved in DNA-damage signaling at telomeres) in ALT relative to TERT+ cell lines. ALT, p53LOF neuroblastoma cell lines showed significantly higher cytotoxicity in response to the clinical-stage p53 reactivator APR-246 ($p<0.0001$) compared to telomerase+ p53LOF neuroblastoma cell lines, despite no significant difference in basal p53 levels

(p=0.48). Induction of telomere dysfunction in a TERT+, p53LOF neuroblastoma cell line using dominant-negative TRF2 (a shelterin protein that blocks ATM activation via phosphorylation at telomeres) activated ATM and sensitized the cells to APR-246 (>70% reduction in IC90, p<0.01). Chemical inhibition of ATM or ATR kinase antagonized APR-246 in ALT cell lines with a mean 2.3-fold increase in APR-246 IC50 (p<0.0001). Knockdown of ATM kinase (shRNA) similarly antagonized APR-246 cytotoxicity. ALT neuroblastoma cell lines treated with clinically-achievable levels of APR-246 showed p53 phosphorylation and induction of the downstream p53 targets p21 (involved in induction of cell senescence) and NOXA (promotes activation of caspases and apoptosis). APR-246 enhanced the cytotoxicity of irinotecan (as SN38) in ALT cell lines in vitro to a much higher degree than seen in TERT+, p53LOF cell lines (p<0.001). Single-agent APR-246 significantly (p<0.01) increased event-free survival (EFS) of three ALT neuroblastoma CDXs/PDXs relative to controls. APR-246 enhanced the activity of irinotecan in 2 neuroblastoma ALT+ CDX models with most mice (14/18) in maintained complete response at 100 days compared to no complete responses and progression at < 60 days in mice treated with only irinotecan (p<0.0001). APR-246 + irinotecan had no significant effect (p=0.4) on EFS in a TERT+, p53LOF neuroblastoma PDX relative to irinotecan alone (EFS < 40 days).

Summary/Conclusions

The constitutive activation of ATM/ATR kinases together with p53 LOF found in ALT neuroblastomas results in a high sensitivity to restoring p53 function with APR-246. APR-246 warrants clinical testing in patients with ALT+ neuroblastoma.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 134

The astatine-labeled PARP inhibitor [211At]MM4 induces complete and durable responses in neuroblastoma patient derived xenograft (PDX) models

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

The Poly (ADP-ribose) polymerase 1 (PARP1) enzyme is essential for initiating DNA damage repair. While generally overexpressed in cancer, we previously showed that PARP1 is expressed at higher levels in neuroblastoma than other human cancers, and expression level is associate with patient outcome. We recently showed that targeting PARP1 with an alpha-particle emitting radionuclide, astatine-211, conjugated to the PARP1 inhibitor MM4 ([^{211}At]MM4) was over 10^9 times more potent than non-radioactive analogue PARP1 inhibitor, and potently cytotoxic against neuroblastoma cell lines *in vitro* (J Nuc Med, 2019).

Aims

To define the tolerability and efficacy of [^{211}At]MM4 in PDX models of high-risk neuroblastoma.

Methods/Materials

Dose finding and toxicities studies were performed to determine maximal tolerated dose (MTD) in CB17 SCID mice. Bone marrow and hematological toxicities were directly assessed in C57B6 mice. Tissue biodistribution of ^{211}At-MM4 was measured by gamma counting at time points beteween 30 min - 6 hours. Efficacy of [^{211}At]MM4 was assessed in a panel of 11 neuroblastoma

PDX models with clinically relevant variability in PARP-1 expression and other molecular features at 325 µCi/kg, 650 µCi/kg and 975 µCi/kg intraperitoneally (IP) in four fractionated doses twice weekly, and animals were monitored for up to 100 days. [^{211}At]MM4 impact on both tumors and normal tissues was evaluated by histopathology.

Results

Weight loss and thrombocytopenia defined the MTD at 975 µCi/kg. Biodistribution of [^{211}At]MM4 showed a tumor to muscle ratio of 10 at 1-hour post-infusion. Normal organs with elevated uptake included thyroid, stomach, and spleen. Nine out of eleven models treated at the MTD showing a complete response, and the two with the lowest PARP1 expression showed partial responses, albeit >90% reduction in tumor volume. Long term progression free survival (PFS) revealed two distinct sub-groups, classified herein as exceptional responders and responders (mean PFS: 88 vs. 28 days;p-value <0.001). Exceptional responders (5 models) had a median PFS of 83 days and 70% showed no evidence of disease at 100 days. Responders (6 models) had a median PFS of 27 days. Relapsed tumors in 2 exceptional responder models were sensitive to retreatment. Histopathology of tumors showed effects after a single dose with increased DNA damage which corresponded to decreased tumor cellularity and mitotic index. Post-treatment dissagregated tumors assesed by flow cytometry showed upregulation of gH2AX, accumulation of a pronounced sub-G1 peak, and absence of Ki-67 staining.

Summary/Conclusions

[^{211}At]MM4 is a potently active targeted radiotherapeutic that will be prioritized for clinical development. Biomarkers predictive of durability of response are being explored and will be presented.

The astatine-labeled PARP inhibitor [^{211}At]MM4 induces complete and durable responses in neuroblastoma patient derived xenograft (PDX) models

Type Translational

Presentation Preference Oral presentation

Abstract nr. 135

A Phase II Trial of Hu14.18K322A in Combination with Induction Chemotherapy in Children with Newly Diagnosed High-Risk Neuroblastoma: An Update on Early Response and EFS.

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

Myelosuppression from chemotherapy given concurrently with anti-GD2 antibodies was thought to adversely affect the effector cells necessary for the anticancer effects of antibodies. However, preclinical studies in neuroblastoma models and clinical studies of several antibody/chemotherapy combinations in adult cancers demonstrated that concurrent chemotherapy with various monoclonal antibodies provides additive/synergistic benefits. We therefore postulated that the addition of an anti-GD2 antibody to induction chemotherapy for neuroblastoma would further improve outcomes.

Aims

We prospectively evaluated the combination of a humanized anti-disialoganglioside monoclonal antibody (hu14.18K322A) with induction chemotherapy in patients with newly diagnosed high-risk neuroblastoma. This report updates our earlier observations (Clin Cancer Res, 2019; 25:6320-8) in a larger number of patients treated at our center.

Methods/Materials

We conducted a prospective nonrandomized, single-arm, three-stage, phase II clinical trial (NCT01857934). Six courses of induction chemotherapy, identical to that described by Park (JAMA, 2019), were coadministered with hu14.18K322A (40 mg/m^2/d x 4) and followed with granulocyte/macrophage colony-stimulating factor (GM-CSF; 250 mcg/m^2/d) and low-dose interleukin-2 (IL-2; 1 MIU/ m^2/ qod x 6). Consolidation used a busulfan/melphalan preparative regimen. An additional course of hu14.18K322A was administered with parent-derived natural killer cells, when available, during consolidation. After the first 47 patients completed consolidation the study was amended to eliminate the additional course of hu14.18K322A with or without parental-NK cells. Following completion of external beam radiation therapy, Hu14.18K322A, GM-CSF, IL-2, and isotretinoin were then administered, identical to that described by Yu et al, NEJM, 2010, with the substitution of hu14.18K322A for dinutuximab. Secondary outcomes included reduced tumor volume after the first two courses of induction and semiquantitative ^{123}I-metaiodobenzylguanidine scoring [i.e., Curie scores (CS)] at the end of induction. The results of the first 43 patients enrolled have been previously reported (Furman et al, Clin Ca Res 2019; 25:6320-8). We update those results with all patients who have been enrolled.

Results

Sixty-four patients have been enrolled on the trial and fifty-four have completed all planned therapy. Therapy was well tolerated, with continuous-infusion of narcotics adjusted to need. Response assessment following the first two courses of chemoimmunotherapy resulted in partial responses (PR) or better in 43/63 evaluable patients (68%, 95% confidence interval (CI), 55%-79%; P = 0.0131 when compared to ANBL02P1 early responses (40%, 12/30)). This was accompanied by a median of -73% primary tumor volume reductions (n=60 tumors; range, -100% to 5%). Of 53 patients with stage 4 disease who have completed induction, 46 had end of induction CS of 2 or less. No patients experienced progression during induction. With the median follow up of 3.4 years (0.4 – 6.3 yrs.), two-year event-free survival (EFS) was 82.6% (95% CI, 70.1 – 90.3%).

Summary/Conclusions

Adding hu14.18K322A to induction chemotherapy produced early PR or better in most patients, resulted in no progression during induction, and improved CSs at the end of induction. These early responses have translated into a promising 2-year EFS and merit further investigation in a larger multi-institutional trial.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 136

Modeling and Deciphering COJEC Resistance Using Neuroblastoma PDX Models

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

Current intense multi-modal treatment against high-risk neuroblastoma has significantly increased overall survival, but resistance and relapses are common, and side effects are severe. One critical step is rapid COJEC induction therapy, consisting of cycled administration of Cisplatin, Vincristine, Etoposide, Cyclophosphamide and Carboplatin. COJEC has been used in the clinic for many years, but a comprehensive preclinical study investigating the mechanisms of resistance to COJEC has not been performed.

Aims

The aims of this project are: 1) to establish a clinically relevant COJEC protocol with neuroblastoma patient-derived xenograft (PDX) models; 2) to analyze the mechanisms of COJEC treatment resistance; and 3) to establish tumor organoids from COJEC-resistant PDX tumors.

Methods/Materials

High-risk *MYCN*-amplified neuroblastoma PDX models were established in nude and NSG mice. Mice were treated with a COJEC-like protocol for 6 weeks. Several dose protocols were tested, and treatment was personalized depending on tolerance. Mice that responded well were also subjected to surgical resection and observed for relapse. All tumors were analyzed using chromosomal copy number analysis, RNA sequencing and immunohistochemistry. Fresh PDX tumors were dissociated to establish COJEC-resistant tumor organoids.

Results

Neuroblastoma PDXs treated with an optimized COJEC schedule present responses comparable

to those seen in humans, with high rates of resistance or relapse after surgery. Multidrug induction has variable results in PDXs derived from different patients, ranging from significant increased survival to poor or no response. Considerable tumor volume reduction was observed for most responding tumors, allowing surgical removal with a 50% relapse rate.
Data from a responsive PDX model showed that treated tumors displayed a more differentiated histological profile, with higher levels of key extracellular matrix components and increased cell death. Surprisingly, substantial tumor cell proliferation was still observed during treatment, pointing to a possible population of resistant cells. Moreover, treated tumors presented an accumulation of chromosomal aberrations, with sub-clonal dynamics enhanced in relapsed tumors. Additionally, preliminary data from tumors that did not relapse showed a downregulation of genes associated with cell cycle and DNA replication/repair at the moment of surgical removal. This significant downregulation can also be observed in patients that did not relapse.
Finally, PDX tumors that were resistant or relapsed after surgery were dissociated and successfully cultured in stem cell medium as organoids, providing a useful tool to study resistance mechanisms to COJEC drugs.

Summary/Conclusions

We have established and optimized a highly clinically relevant COJEC-induction protocol using high-risk neuroblastoma PDX models. This protocol will be helpful for studying COJEC resistance and identify biomarkers that could be indicative of resistance and relapse. Additionally, our COJEC-resistant organoids represent a useful tool for testing novel therapeutics that can overcome drug resistance in high-risk neuroblastoma.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 137

MRI Native T1 mapping of neuroblastoma pathology - Non-invasive early detection of response to MYCN-targeted therapy in the Th-MYCN model

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

The integration of advanced mouse modelling to accelerate the evaluation of novel therapies against high-risk neuroblastoma provides a unique opportunity to evaluate non-invasive functional imaging biomarkers to characterize MYCN-driven tumour micro-environment and its response to therapy. The Th-*MYCN* mouse which spontaneously develops tumours which are stroma-poor, undifferentiated or poorly-differentiated with a high mitosis-karyorrhexis index, mirroring the histology of childhood high-risk tumours. Small molecule inhibitors targeting the stability of MYCN protein via the selective inhibition of Aurora A kinase or mTOR activity have shown promising results in genetically-engineered murine (GEM) models of neuroblastoma and are currently being evaluated in early-phase paediatric clinical trials.

Aims

This study aimed to demonstrate that native T_1-mapping, a functional MRI technique, is sensitive to neuroblastoma rich histopathological heterogeneity and how a reduction in native tumour spin-lattice relaxation time T_1 is a sensitive biomarker of response to treatment with Aurora A kinase inhibitor alisertib (MLN8237) and mTOR inhibitor vistusertib (AZD2014) in the Th-*MYCN* model.

Methods/Materials

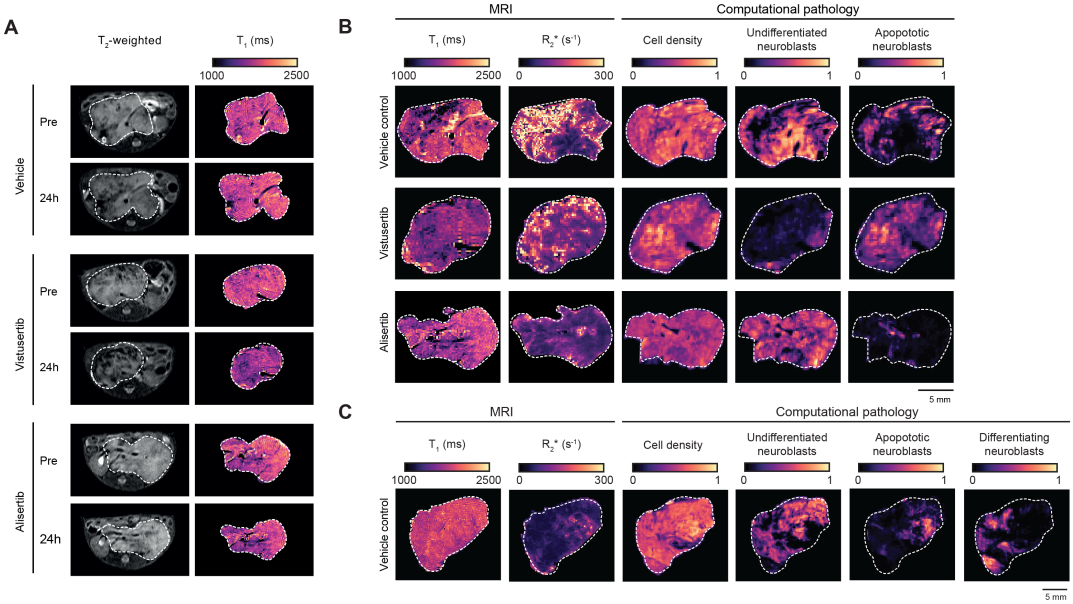
MRI was performed before (*Day 0*) and 24 hours after treatment started (*Day 2*). Th-*MYCN* mice were treated p.o. on *Day 1* with 30 mg/kg Alisertib, 25 mg/kg Vistusertib or vehicle. Tumour T_1 maps were compared with spatially-registered computed maps of undifferentiated, differentiated and apoptotic neuroblasts, generated from digitized H&E sections using a machine learning-based algorithm for the automatic detection and classification of neuroblasts.

Results

A significant reduction in tumour median T_1 value was observed 24h following treatment with Vistusertib (1712±25ms to 1553±25ms, p<0.0001) or Alisertib (1776±26ms to 1679±25ms, p<0.0001) (Fig.1A). Regions of high T_1 values spatially corresponded to regions of undifferentiated neuroblasts in treatment-naïve tumours, while lower T_1 values reflected erythrocyte content or extensive tissue damage (Fig.1B). The reduction in tumour T_1 observed upon treatment with Vistusertib was associated with a significant increase in apoptotic cells, i.e. a reduction of the fraction of undifferentiated neuroblasts, but not with Alisertib. Combining the MRI data from vistusertib- and alisertib- treated mice with matched histopathology revealed a significant negative correlation between the treatment-induced reduction in T_1 over 24h and the proportion of apoptotic neuroblasts present in the tumour at the study endpoint (r= -0.55, P=0.04). Overall combining the MRI data from vehicle control-, vistusertib- and alisertib-treated tumours with matched histopathology showed a positive correlation between median native T_1 and the ratio of undifferentiated neuroblasts (r= 0.70, P<0.0001) and a negative correlation with the fraction of apoptotic cells (r=-0.63, P=0.006). Interestingly, in three tumours exhibiting a significant amount of differentiating neuroblasts, regional differences in native tumour T_1 visually and spatially corresponded to differences in undifferentiated neuroblast density, with regions of lower T_1 values corresponding to hotspots of differentiating neuroblasts (Fig.1C).

Summary/Conclusions

This study demonstrates the sensitivity of MRI T_1-mapping for the rich pathology of neuroblastoma and its therapeutic modulation, which warrants further evaluation of its utility in the clinic, for diagnostic, guiding both surgical biopsies and resection and in clinical trials of novel therapies against neuroblastoma.



The spin lattice T1 is sensitive to neuroblastoma rich pathology and its modulation by MYCN-targeted therapeutics in the Th-MYCN model

Type Translational

Presentation Preference Oral presentation

Abstract nr. 138

Estimation of life expectancy and the expected years of life lost with Neuroblastoma in children of Taiwan

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Topic Other

Background/Introduction

Neuroblastoma is a childhood cancer of the peripheral sympathetic nervous system and it ranks as the second most common extracranial solid tumor of children and adolescents in Taiwan. Most patients present with disseminated disease, which confers a poor prognosis and is associated with a high mortality rate.

Aims

This study aimed to quantify the lifetime risks, life expectancy (LE) and the expected years of life lost (EYLL) after the diagnosis of neuroblastoma using a semiparametric method.

Methods/Materials

A total of 218 patients with neuroblastoma were registered with the Taiwan Cancer Registry during 2007 to 2015. They were linked to the National Mortality Registry through the end of 2017. We estimated the cumulative incidences rate for 0-19 years ($CIR_{0\text{-}19}$). The survival is estimated by Kaplan-Meier method up to the end of follow-up and extrapolated the survival function to lifetime by assuming a “constant excess hazard” for these cancer patients compared with age-, sex-, and calendar year matched referents generated from vital statistics. In this way, the LE of the neuroblastoma in childhood cancer cohort (with extrapolation up to 240 months or 960 months) after diagnosis was estimated. The EYLL was calculated by subtracting the LE from that of age- and sex- matched referents.

Results

Most patients with neuroblastoma were males (62.8%), and 98% were less than 10 years old, with incidence rates of 3.4, 1.4, 0.3, <0.1, and <0.1 per 100,000 person-year, respectively, for age

strata of 0-1.4, 1.5-4, 5-9, 10-14, and 15-19. The average EYLL was 29.6 years after extrapolation up to 80 years. Diagnosis at younger than 1.5 years old seemed to improve survival with a LE of 66.1 years in contrast to those diagnosed during 1.5-19 years with an LE of 27.80 years. EYLL can adjust for different age, sex, and calendar year of diagnosis, which also showed a smaller loss for those diagnosed before 1.5 years of age.

Summary/Conclusions

We concluded that early diagnosis of patients with neuroblastoma before age of 1.5 years appeared to be the key for a better outcome and deserve attention.

Type Basic

Presentation Preference Traditional poster

Abstract nr. 139

Modifying methylation-sensitive binding of MYCN by targeted DNA demethylation using CRISPR/Cas9-TET1 fusion proteins

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Topic Gene regulatory networks and epigenetics: from basic science to therapy

Background/Introduction

Amplified *MYCN* is found in 25% of neuroblastoma (NB) cases and is associated with high-risk disease and poor outcome. DNA methylation based-clustering identifies patient subgroups strongly associated with NB key clinical and biological features. The group of *MYCN*-amplified NBs is characterized by aberrant methylation of regulatory DNA elements being in line with an impact of MYCN on DNA methylation patterns. This aberrant methylation leads to pronounced transcriptomic and chromatin landscape changes that mediate tumor de-differentiation. Further, recent data suggest MYCN-binding itself to be highly responsive to DNA methylation creating an image of MYCN guiding its own binding profile via methylation.

Aims

Elucidate how methylation-sensitive binding of MYCN shapes MYCN-regulated gene expression programs. With an integrated approach analyzing DNA methylation, gene expression and MYCN-binding we plan to considerably contribute to the understanding of epigenetic patterns in high-risk NB and potentially identify candidate genes deregulated by aberrant MYCN E-Box methylation.

Methods/Materials

We used drug-mediated demethylation of SK-N-Be(2)C cells and globally assessed MYCN-binding via ChIP-sequencing analysis. High resolution DNA methylation changes were assessed by whole-genome bisulfite sequencing. The consequences of DNA methylation-associated MYCN redistribution were monitored by RNA-sequencing and chromatin mark ChiP-sequencing.

In order to validate candidate elements controlled by MYCN, we established a system for targeted epigenetic editing using CRISPR/Cas9-TET1 fusion proteins allowing us to specifically demethylate regions of interest.

Results

We could show methylation-sensitive binding of MYCN leading to thousands of new MYCN binding sites upon demethylation. MYCN binding interestingly occurred independently of a classical E-box preference, which is the only E-Box harboring a central CpG, or accumulation at distinct genomic regions. In accordance with other published MYCN profiles, MYCN binding was only associated with changes in gene expression in the minority of cases.
The bulk of newly occurring MYCN binding sites harbored CpGs that were already hypomethylated before pointing towards a hierarchy of additional regulatory mechanisms including chromatin compaction and/or methylation-dependent binding partners that guide MYCN to its target sequences.
New MYCN binding sites that also correlated with differential gene expression were predominantly found at distal elements, whereas promoter-associated binding sites seemed to be already established before and less affected by demethylation.
We could successfully de-repress MYCN-regulated genes by dCas9-TET1 mediated targeted demethylation of priori identified methylation-sensitive binding sites to further study MYCN-regulated networks.

Summary/Conclusions

With this integrated analysis we considerably contribute to the understanding of epigenetic patterns in high-risk NB by showing methylation-sensitive binding of MYCN in NB cell lines. Further, we could potentially identify candidate genes involved in pro-differentiation programs deregulated by aberrant methylation having significant clinical relevance for high-risk NB patients.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 140

Combined targeting of GD2 ganglioside and BET proteins in MYCN-amplified neuroblastoma cell lines - studies involving 3D models

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

Treatment of high-risk neuroblastoma patients still remains challenging. This stimulates a quest for new combined treatment modalities. GD2 ganglioside (GD2) is one of the pivotal targets for treatment of neuroblastoma patients. Our previous research has shown that some neuroblastoma cell lines treated with the anti-GD2 14G2a monoclonal antibody (mAb) alter their morphology, exhibit decreased cell viability accompanied by changes in transcriptome and in signaling networks (Horwacik and Rokita. Apoptosis. 2015; 20(5):679-688). *MYCN*-amplified neuroblastoma cell lines are sensitive to BET (bromodomain and extra-terminal) protein inhibitors (Puissant *et al.* Cancer Discov. 2013; 3:309-323). The agents are currently tested in clinical trials against various cancers.

Aims

We aimed to further characterize effects of the 14G2a mAb on selected signaling pathways and to verify outcome of combined targeting of *MYCN*-amplified neuroblastoma cells with the mAb and BET protein inhibitor CPI203.

Methods/Materials

In addition to standard cultures (2D), our research involved cultures of *MYCN*-amplified neuroblastoma cells (IMR-32, CHP-134, CHP-126, KELLY) on low binding surfaces (3D models). This can better approximate conditions in tumors, as cells interact both with each other and extra cellular matrix in 3 dimensions, which is important for testing compounds that are potential drugs. It should be stressed that the recently crystallized 14G2a mAb has the same antigen binding region as the ch14.18 mAb (Horwacik *et al.* Mol Cell Proteomics. 2015; 14(10):2577-2590). Effects of CPI203, OTX015, I-BET762 inhibitors (Sigma-Aldrich) were tested using cell viability assays. Molecular techniques (e.g., RT-qPCR, protein arrays from RayBiotech, Inc.) were applied to investigate proteome and transcriptome changes.

Results

Using intra-cellular ATP level measurements in our 2D and 3D models, we confirmed, for IMR-32 cells, that the cytotoxic effect of the 14G2a mAb is observed for both types of cultures. Based on microarrays data, we already reported transcriptome changes after the 14G2a mAb treatment of IMR-32 cells (Horwacik *et al*. Acta Biochim Pol. 2015; 62(3):423-433). Here, RT-qPCR was applied to check levels of transcripts of selected genes involved in RNA metabolism in 14G2a-treated IMR-32 cells in 3D cultures. We measured increased levels of *MSX1* and *JUN* transcripts and decreased levels of *ID1* and *TLX2*.

GD2 accumulates in the outer cell membrane and affects the cancer cell phenotype. We applied RTK phosphorylation antibody arrays to evaluate phosphorylation levels of 71 tyrosine kinases in the 14G2a-treated IMR-32 cells in 3D cultures. Interestingly, we showed a decrease in the tyrosine phosphorylation level of an oncogenic non-receptor kinase ACK1 and as a result the protein was selected for further research. Finally, we showed that IMR-32 cells are sensitive to selected inhibitors of BET proteins and further focused on testing efficacy of CPI203 alone or in combination with the 14G2a mAb in 3D models.

Summary/Conclusions

Treatment of high-risk neuroblastoma children requires multi-modal approach. We focused our experiments on combined targeting of GD2 and BET proteins in *MYCN*-amplified neuroblastoma cells, which extends knowledge on neuroblastoma biology and cell fate. Our preclinical data, gathered in 3D models, add to efforts to improve survival of the neuroblastoma patients.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 141

Hypoxia predicts poor prognosis and associates with the ability of the tumors to evade immune surveillance and gain immortality in neuroblastoma

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Neuroblastoma is one of the most deadly pediatric cancers despite treatments. Hypoxia, a condition of low oxygen tension occurring in aberrant vascularized tissue areas, has profound effects on tumor metastasization, metabolism, and resistance to radio-, immuno-, and chemo-therapy.

Aims

Our aims are to investigate the contribution of hypoxia to neuroblastoma prognosis in a large cohort of patients and to uncover the molecular mechanisms deregulated within the hypoxic microenvironment in neuroblastoma.

Methods/Materials

We collected the gene expression profile of 1620 neuroblastoma tumor specimens covering the entire spectrum of the disease included into three publicly available data sets. We used machine learning methods to build an accurate predictor of tumor hypoxia. We assessed the functional enrichment of gene sets by STRING-DB database. We used the Microenvironment Cell Populations-counter (MCP-counter) method to quantify the absolute abundance of eight immune and two stromal cell populations from the transcriptomic data set.

Results

We identified a small set of 7 genes (NB-hop) involved in the cellular response to hypoxia. We

integrated the gene expression profiles of the 1620 patients into one data set using bioinformatic techniques to achieve a large-scale genomic data analysis. We filtered out 834 patients from the data set for improving reliability, independence, and homogeneity of the data. The expression profiles of 236 randomly selected tumors out of 786 (30%) served to build a classifier and the profiles of the remaining 550 tumors (70%) were used to test its prognostic value in a validation data set. NB-hop classifier predicted 414 out of 550 patients (75%) with favorable prognosis (F) and 136 out of 550 patients (25%) with unfavorable prognosis (UF) and was able to stratify patients into subgroups that had a significantly different overall survival (OS) and event-free survival (EFS) (OS: HR 5.2 95% confidence interval (CI) 7.3-16.3 and EFS: HR 3.3 95%CI 3.8-7.5, both $p<0.0001$). NB-hop classifier maintained a significant prognostic effect in the model adjusted for these clinical covariates in multivariate analysis (OS: HR 1.8 95%CI 1.2-2.6, $p=0.004$ and EFS: HR 1.7 95%CI 1.2-2.5, $p=0.001$).
The molecular mechanisms altered by hypoxia in neuroblastoma primary tumors remain still unknown to date. Hence, we compared the transcriptome of the two populations of patients whose prognosis was predicted according to NB-hop in the validation set and identified a 2377 differentially regulated genes (fold change≥1.5 or fold change≤-1.5 and Benjamini-Hochberg $p<0.05$). Pathway analysis evidenced the significant deregulation of fundamental biological processes including the response to hypoxia, telomere maintenance, immune and inflammation response, and cell differentiation (Benjamini-Hochberg $p<0.05$). MCPcounter analysis evidenced a global immunosuppressed microenvironment associated with NB-hop unfavorable respect to NB-hop favorable prognosis.

Summary/Conclusions

Hypoxia is an unfavorable condition of the tumor microenvironment, which is prognostic for NB patient and might play a fundamental role on the ability of the most aggressive tumors to suppress immune response, to evade immune surveillance and to progress.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 142

Analyzing the influence of KDM1A on neuroblastoma-specific CAR T cell therapy

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

Neuroblastoma is the most common malignant extracranial solid tumor in children, arising from the sympathoadrenal lineage of the neural crest. Treating refractory or relapsed neuroblastoma remains a great challenge in pediatric oncology. An innovative immunotherapeutic approach uses chimeric antigen receptor (CAR) T cells to target antigens specifically and strongly expressed on neuroblastoma cells, such as L1CAM. However, a major challenge is the escape of antigen-negative tumor cells that cause tumor recurrence. Enzymes involved in epigenetic gene regulation, such as the histone demethylating enzyme, lysine-specific demethylase 1 (KDM1A, previously known as LSD1) are known to suppress the expression of different anti-tumor gene networks. The re-expression of these genes via KDM1A inhibition, might boost the efficacy of a CAR T cell therapy. Therefore, combining KDM1A inhibition with CAR T cell therapy may provide a new strategy to better fight this malignancy.

Aims

In this study, we aimed to identify changes in gene expression in established neuroblastoma cell lines caused by KDM1A suppression that could be exploited to improve CAR T cell therapy against neuroblastoma.

Methods/Materials

First, we determined the level of KDM1A expression in 8 neuroblastoma cell lines as well as primary T cells by western blot and RT-PCR. Neuroblastoma cell lines with different levels of

KDM1A expression were subjected to titrated amounts of KDM1A inhibitors (SP-2509 and SP-2577) and IC50 values were determined by analyzing cell survival. Next, we determined expression changes of cell surface molecules by flow cytometry of neuroblastoma cells treated for 1, 3 or 6 days with their respective IC50 KDM1A inhibitor concentrations.

Results

We could show that KDM1A was highly expressed in all 8 neuroblastoma cell lines analyzed. In contrast, KDM1A expression was significant lower expressed in primary T cells, but still detectable. We chose 3 neuroblastoma cell lines for further experiments, which were then treated with two different KDM1A inhibitors (SP-2509, SP-2577). The different neuroblastoma cell lines showed a differential response to the KDM1A inhibitors. While 50% of neuroblastoma cell line IMR5/75 died upon treatment with 3 µM of both inhibitors, cell line SK-N-BE(2) required higher concentration of both inhibitors. Importantly, our CAR T cells are more sensitive to the KDM1A inhibitor treatment compared to the tumor cells, indicating the need for a sequential rather than simultaneous treatment strategy for experiments in which CAR T cells and inhibitors will be combined. When we treated our 3 selected neuroblastoma cell lines with SP-2509 or SP-2577 at their respective IC50 concentrations, we found an upregulation of FAS (CD95), a death receptor critical for T cell-mediated killing.

Summary/Conclusions

KDM1A is highly expressed in neuroblastoma cell lines and we could demonstrate that all analyzed cell lines were susceptible to KDM1A inhibition therapy. KDM1A inhibition resulted in upregulation of Fas on the cell surface of neuroblastoma cells and might be exploited to enable antigen-independent lysis of the tumor cells via CAR T cells.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 143

Long-term outcome and role of biology within risk-adapted treatment strategies: The Austrian neuroblastoma trial A-NB94

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Topic Other

Background/Introduction

The Austrian neuroblastoma trial A-NB94 was initiated in 1994 to apply risk-adapted treatment intensities based on then recognized factors of tumor stage by the International Neuroblastoma Staging System (INSS), age at diagnosis, and *MYCN* oncogene amplification status. Based on encouraging results of the LMCE2 trial using tandem high-dose chemotherapy (HDT) followed by autologous stem cell rescue (ASCR), this study was one of the first to adopt a similar approach to high-risk patients with incomplete response to induction therapy.

Aims

To evaluate the long-term outcomes and association with genomic profiles in the Austrian Neuroblastoma Trial A-NB94 applying a risk-adapted strategy of treatment (RAST).

Methods/Materials

RAST depended on stage by INSS (International Neuroblastoma Staging System), age, and *MYCN* amplification (MNA) status and ranged from surgery only to intensity-adjusted chemotherapy, single or multiple courses of high-dose chemotherapy (HDT) followed by autologous stem cell rescue (ASCR), and irradiation to the primary tumor site. Segmental chromosomal alterations (SCAs) were investigated retrospectively using multi- and pan-genomic techniques. The median follow-up time was 12 years.

Results

The A-NB94 trial enrolled 163 patients. The proportion of patients with localized disease was high (n=109; ≤12 months n=41, >12 months n=68), likely related to the Austrian screening program. Their ten-year event free survival (10y-EFS) and overall survival (10y-OS) was excellent as apart from one unrelated death all patients became long-term survivors. Stage 4S infants (n=10) had a 10y-EFS and OS of 80±13% and 90±9%, respectively, and infants with non-MNA stage 4 (n=6) 83±15% each. High-risk patients (stage 4, either >12 months or MNA, n=38), receiving intensified induction (response rate 74%) and, depending on response, one or multiple courses of HDT (response rate 85%), had a 10y-EFS of 45±8% and 10y-OS of 47±8%. SCAs were present in increasing frequencies according to stage and age: in 29% of localized tumors but in 92% of stage 4 tumors ($p<0.001$), and in 39% of patients ≤12 months but in 63% of patients >12 months ($p<0.001$).

Summary/Conclusions

RAST achieved remarkable long-term results, reducing exposure to chemotherapy in low- and intermediate-risk patients successfully. Notably, the intensified HDT approach for high-risk patients showed very good response rates and markedly improved long-term outcomes along with acceptable toxicity profiles and morbidity. SCAs were significantly correlated with stage and age.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 144

The landscape of circular RNAs in neuroblastoma

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Circular RNAs (circRNAs) are co-transcriptional variants of cognate mRNAs produced by backsplicing events that result in a characteristic RNase-resistant loop structure. Not every gene produces circRNAs and their overall expression profile is highly tissue-specific. CircRNAs have diverse functional roles involving coding for proteins, acting as decoys or transporters of proteins and microRNAs, or acting as protein-scaffolds. Neuroblastoma arises from embryonic neural-crest cells and is stratified into distinct risk-groups reflecting disease prognosis. Here, we systematically investigated the circRNA expression profiles of 104 primary neuroblastomas stratified by risk-group and as a whole when compared to healthy human brain tissue and various cancers.

Aims

We aimed to comprehensively profile all neuroblastoma circRNAs and particularly those with functional relevance to the disease.

Methods/Materials

Whole transcriptome paired-end Illumina sequencing RNA libraries of 104 neuroblastomas were generated and analyzed.

Results

Originating from 2,290 genes, we identified 5,171 unique circRNAs whose acceptor and donor sites did not vary more than one nucleotide from annotated exon boundaries and were expressed

in at least 25%, or had strong support in at least three, of the 104 neuroblastoma tumours. The median number of unique circRNAs across tumours was 2,730. More than half of the 2,290 genes produced only a single circRNA isoform and circRNA and cognate mRNA expression was overall uncorrelated. High-risk non-MYCN-amplified tumours showed a higher number of unique circRNAs expressed compared to the rest of the risk-groups and had overall higher circRNA/mRNA expression ratios. The lowest number of unique circRNAs expressed with also the lowest circRNA/mRNA expression ratios were observed in the high-risk MYCN-amplified tumours. In this risk-group, we found also the majority of human splicing factors to be upregulated and that 540 genes were differentially spliced. These findings were independently reproduced in a Tetracycline-inducible MYCN-overexpression system based on the SKNAS neuroblastoma cell line, suggesting that MYCN drives this global circRNA repression and differential splicing. We found enrichment of RNA-binding-protein (RBP) motifs in both the flanking introns and exon sequences of the identified circRNAs, suggesting that RBPs might play regulatory roles beyond the known aspects of circRNA biogenesis. Finally, we identified 26 neuroblastoma-specific circRNAs with higher expression in neuroblastoma compared to healthy human brain tissue and various cancers. The RNaseR-resistance of 10 of them was validated in neuroblastoma cell lines.

Summary/Conclusions

We globally profiled for the first time in the field of neuroblastoma research the expression of circRNAs in a cohort of 104 primary tumours. Our results indicate that MYCN plays a crucial role in the global repression of circRNAs, whereas in non-MYCN amplified high-risk tumors circRNAs seem to proliferate. We also identified circRNAs that were significantly higher expressed in neuroblastoma when compared to healthy human brain tissue and various other cancers. We are currently in the process of assaying the functional roles of a selected subset of these neuroblastoma-specific circRNAs.

Type Basic

Presentation Preference Traditional poster

Abstract nr. 145

Nucleolin is a prognostic tumorigenic factor and a novel cell surface protein for targeted therapy in neuroblastoma

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

The use of targeted anti-cancer therapies is severely limited by the lack of specific receptors. Nucleolin (NCL), a multifunctional nucleolar protein involved in many biological processes, is overexpressed in adult tumors, where is also shuttled to the cell surface of both tumor cells and tumor endothelial cells. Little is reported about pediatric tumors, including neuroblastoma (NB).

Aims

To investigate NCL expression and function in NB.

Methods/Materials

The prognostic value of NCL was assessed in a cohort of 709 NB patients (whose gene expression of the primary tumor was profiled by Agilent customized 4x44k oligonucleotide microarray; ArrayExpress: E-MTAB-1781) and on 20 stage 4, NB patients, alive or dead, at 10 years follow-up. NCL expression was evaluated by immunohistochemistry (IHC) analysis in Schwannian-stroma poor NB patients, and by Flow Cytometry (FC) analysis in tumor fragments derived from relapsed NB patients and in bone marrow-infiltrating NB cells from patients, at either onset or relapse. In a translational setting, NCL expression was evaluated on *h*uman NB cell lines in culture and after injection in mice, by Imaging FC (IFC) and IHC analyses, respectively. The NCL-targeting peptide F3 was used to decorate liposomes (L) to test NB cell association and internalization. The cytotoxic potential of doxorubicin (DXR)-loaded, F3-decorated L (NCL-L[DXR]) was evaluated in terms of inhibition of NB cell proliferation and cell death induction *in vitro*, and by increasing life span in orthotopic and metastatic animal models of NB, mimicking the growth and spread of NB in patients, respectively.

Results

E-MTAB-1781 assay demonstrates that NCL mRNA expression is significantly higher in amplified versus (*vs*) not-amplified *MYCN* tumors ($p<0.0001$), and in INSS stage 4 *vs* INSS stage 1, 2, 3, and 4s tumors ($p<0.002$). *Kaplan-Meier and multivariate analyses of overall survival show a significant worse survival for patients with high NCL expression. This result was confirmed in stage 4 patients with a* 10 years of follow-up*, where a significantly higher expression of NCL corresponds to death events (*$p=0.015$, death *vs* alive) *and worst survival (*$p=0.0215$, high *vs* low NCL expression)*. A strong NCL protein expression in* NB tumors, also shuttled to the cell surface of almost all the examined patients, *was observed. Moreover, NCL is expressed at different extent on the surface of all NB cell lines analyzed. Notably, NCL staining is evident on both tumor and endothelial tumor cells in well-established NB xenografts. Moreover, F3-decorated rhodamine-labeled liposomes strongly associate to NB cells compared to untargeted formulations, exhibiting a co-localization with cell surface NCL. Finally, NCL-L[DXR] result significantly more effective, in terms of inhibition of cell proliferation and reduction of cell viability in vitro, and of delay of tumor growth in vivo, compared to* untargeted L[DXR] .

Summary/Conclusions

Our findings *indicate that NCL is an* unfavorable *independent prognostic factor for NB patients and* demonstrate that cell surface NCL might represent a novel protein to be targeted by drug-loaded nanoparticles as innovative therapeutic strategy against NB.

Type Translational

Presentation Preference Traditional poster

Abstract nr. 146

Exploiting DNA damage response defects with ATR and PARP inhibitors for the treatment of high risk neuroblastoma

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

DNA damage response (DDR) defects are frequently observed in high-risk neuroblastoma (HR-NB) including allelic loss and loss of function mutations in key DDR genes on chromosome 11q, oncogene induced replication stress (RS) and cell cycle checkpoint dysfunction. Around 50% of HR-NB tumours have amplification of the *MYCN* oncogene that promotes rapid DNA replication and cell cycle dysfunction, leading to errors and RS. Defects in the p53 pathway, a key tumour suppressor pathway, are frequently observed at NB relapse, including mutation in *TP53* (gene coding for p53). Cancer cells with defective cell cycle checkpoint signalling and/or increased oncogene-driven RS are acutely dependent on the DNA damage sensor kinase ATR, which orchestrates DNA repair by homologous recombination (HRR). PARP inhibition results in unrepaired single strand DNA breaks progressing to replication, further increasing RS. We hypothesise that combining PARP and ATR inhibition will lead to greater cytotoxicity due to increased RS.

Aims

To determine the molecular features conferring sensitivity to ATR inhibition in cell lines derived from HR-NB tumours.
To assess synergy of PARP and ATR inhibition including RS and cytotoxicity in HR-NB cell lines.

Methods/Materials

The effects of the ATR inhibitors, VE-821 and AZD6738, and the PARP inhibitor, olaparib were investigated in a panel of 10 NB cell lines with varying *MYCN*, 11q and *TP53* status. All inhibitors

were purchased from Stratech (Cambridge, UK). Growth inhibition following exposure to increasing concentrations of VE-821 or olaparib for 72 hours was assessed by XTT assay (Roche) in all 10 NB cell lines. The effect of ATR inhibition on olaparib-induced growth inhibition in 4 cell lines: SHSY5Y (non-*MYCN* amplified (MNA), *TP53* wt), SKNAS (non-MNA, *TP53* mut), NGP (MNA, *TP53* wt), and N20_R1 (MNA, *TP53* mut). Basal expression of DDR proteins including ATM and ATR was assessed using Western blotting. CHK1^{S345} and H2AX^{S129} phosphorylation was assessed using Western blotting to determine ATR activity and RS respectively. RS and HRR activity was also measured by gH2AX and Rad51 foci formation using immunofluorescent microscopy.

Results

Sensitivity to VE-821 was associated with *MYCN* amplification and/or low protein ATM expression (p<0.05) but not p53 status or 11q status. ATR inhibition by VE-821 or AZD6738 was synergistic with olaparib (CI value 0.04-0.89) independent of *MYCN* or ATM status. Olaparib increased H2AX^{S129} phosphorylation which was further increased by VE-821. Rad51 foci formation in response to olaparib was reduced by VE-821 suggesting inhibition of HRR.

Summary/Conclusions

MYCN amplification and low ATM protein expression are determinants of ATRi sensitivity in NB cell lines and could be useful as predictive biomarkers. ATR inhibition is synergistically cytotoxic with PARP inhibition. This synergy is likely a result of an increase in PARPi-induced RS and inhibition of HRR by ATR inhibition. These findings suggest a potential novel therapeutic strategy for the treatment of HR-NB.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 147

Establishment of a preclinical 3D in vitro model simulating the neuroblastoma microenvironment enabling investigation of tumor-stroma drug targeting

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

Cancer related inflammation is a complex tumor-promoting interaction between malignant cancer cells and benign stromal cells. We previously reported that the neuroblastoma microenvironment contains infiltrating cancer-associated fibroblasts (CAFs) expressing microsomal prostaglandin E synthase-1 (mPGES-1), responsible for prostaglandin E_2 (PGE_2) synthesis. The bioactive lipid PGE_2 promotes immunosuppression, angiogenesis, tumor growth and therapy resistance. Conventional therapies used in the clinic to treat cancer, i.e. chemotherapy and irradiation, induce apoptosis and cell death, processes that leads to increased inflammation and PGE_2 production creating a niche for tumor cell repopulation.

Aims

To investigate the impact of mPGES-1/PGE_2 inhibition on cells in the tumor microenvironment in neuroblastoma with the purpose to increase efficiency of treatment and host immunity. We propose that targeting the CAF derived PGE_2 production in combination with cytotoxic drugs will abrogate tumor recovery and improve treatment outcome.

Methods/Materials

To evaluate mPGES-1 inhibition in combination with conventional cancer therapies *in vitro* we established a multicellular tumor spheroid (MCTS) model, simulating the neuroblastoma microenvironment by co-culturing neuroblastoma cells and fibroblasts. MCTS were treated with doxorubicin or vincristine in combination with small molecule mPGES-1 inhibitors.

Results

The MCTS model successfully recapitulated the neuroblastoma microenvironment where fibroblasts express mPGES-1 and several markers for CAFs. Combination treatment of MCTS with mPGES-1 inhibitors CIII and 934 revealed an enhanced cytotoxic effect of the chemotherapeutic drugs doxorubicin and vincristine. The combination treatment also reduced post treatment MCTS recovery. The *in vitro* findings were translational to an *in vivo* neuroblastoma mouse model where mPGES-1 inhibition with CIII enhanced the effect of vincristine in reducing tumor growth.

Summary/Conclusions

The conventional therapies used in the clinic today mainly target the cancer cells overlooking the contribution of tumor-promoting inflammation to tumor progression, and therapy resistance. In the present work, we established an *in vitro* 3D neuroblastoma model, enabling screening for stroma-targeting therapies in combination with established tumor cells targeting therapies. MCTS were treated with a combination of mPGES-1 inhibitor and vincristine, and inhibition of mPGES-1 was found to enhance the cytotoxic effect of vincristine, also translated to an enhanced effect *in vivo*.

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Type Translational

Presentation Preference Oral presentation

Abstract nr. 148

Inhibition of Tenuerin 4 Suppresses Growth and Induces Differentiation in Neuroblastoma

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Neuritogenesis is the process during embryonic development when neural crest cells migrate and differentiate into multiple cell types. Neuroblastoma originates from neural crest progenitor cells of the sympathoadrenal lineage. The teneurin proteins (TEN) are highly expressed during the development and differentiation of the central nervous system in the drosophila fly, mouse and chicken embryos. Genetic mutations of teneurins (*TENM)* in humans have shown to play a role in different diseases affecting the nervous system, such as eye anomalies, schizophrenia and tremor, suggesting a link between the nervous system and the teneurin family of proteins. Several studies have shown disruption of the teneurin family of proteins in tumors, where somatic mutations and structural aberrations of the teneurin proteins have been found in for example neuroblastoma. However, investigations of a deeper cellular and functional understanding of this family of proteins in cancer is missing.

Aims

The aim of this study is to investigate the teneurin family of proteins, and in particular teneurin 4, to determine the role and function of these proteins in neuroblastoma tumor development, progression and metastasis.

Methods/Materials

Tumor samples from 53 Swedish neuroblastoma patients were collected with matched germline DNA and were whole genome or exome sequenced. In addition, gene expressions were analyzed in the publicly available R2 database, correlating gene expressions with overall survival of patients. To further explore the role of the teneurins, functional studies in four neuroblastoma cell lines were conducted. We used silencing RNA (siRNA) to study the effects of teneurin 1 to 4

knock-down on proliferation by measuring real-time impedance. Furthermore, we also performed RNA sequencing and pathway analysis after siRNA mediated knock down of *TENM4*. Two different CRISPR/Cas9 cell line models of the cell line SK-N-BE(2)C have since been created; a TENM4 knockout cell line, and an inducible *TENM4* knockdown by a tetracycline promoter that are currently used for further investigation.

Results

From the whole genome sequencing of the 53 neuroblastoma tumor samples, we found that five (9.5%) of the patient samples contained a somatic mutation or a structural aberration in the protein family. When investigated in publicly available and validated neuroblastoma cohorts of the R2 database, low expression levels of *TENM1*, *TENM2* and *TENM3* were associated with poor overall survival, while low *TENM4* expression correlated with high overall survival. Using transient siRNA against *TENM1*, *TENM2*, *TENM3* and *TENM4*, we observed that *TENM4* consistently and significantly inhibited proliferation in all cell lines knocked down, by at least 50% reduction in proliferation. However, knockdown of *TENM1*, *TENM2* and *TENM3* lead to varied results regarding effect on tumor growth in the four cell lines investigated. Interestingly, RNA sequencing of *TENM4* knockdown cells showed an upregulation in important pathways in neural differentiation and neuritogenesis, while among other pathways mTOR signaling was significantly downregulated.

Summary/Conclusions

Our data suggests that teneurin 4 plays a role in neuroblastoma growth and differentiation, and that manipulation could offer a possible therapy for neuroblastoma. Ongoing studies will further explore the specific functions of teneurins in neuroblastoma.

Thale Kristin Olsen

Women's and Children's Health

Women's and Children's Health

Women's and Children's Health

Type Basic

Presentation Preference Oral presentation

Abstract nr. 149

The role of histone deacetylases and lysosomes in neuroblastoma chemoresistance

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

The course and clinical outcome of neuroblastoma is highly heterogeneous, ranging from relatively benign disease to highly aggressive and often fatal disease in high-risk neuroblastomas. Lysosomes, acidic vesicular organelles that mainly function as a degradation center for cellular macromolecules as well as aged organelles, promote tumor multidrug resistance by at least three distinct mechanisms. Firstly, they increase tumor cell stress resistance by promoting the turnover of damaged organelles (macroautophagy). Secondly, they trap weakly basic chemotherapeutics, hindering them from reaching their cellular target. Thirdly, they promote secretion of drugs by fusion with the cell membrane (lysosomal exocytosis).

Aims

We aim for a better understanding of the underlying resistance mechanisms in these tumors to open up possibilities for novel treatment approaches.

Methods/Materials

Several cell models (human neuroblastoma cell lines; non-malignant proliferating human fibroblasts; HDAC10-knockout HAP1 cells) were used. The lysosomal phenotype was characterized by LysoTracker staining (FACS and confocal microscopy) and surface LAMP-1 versus total LAMP-1 expression (FACS and Western Blot). Intracellular doxorubicin levels were measured via flow cytometry and visualized by fluorescent confocal microscopy. Cell viability was

determined by trypan blue exclusion and ATP metabolic activity assays.

Results

Our studies demonstrate that all three mechanisms contribute to chemoresistance in a panel of highly aggressive neuroblastoma cell lines, suggesting that resistance critically depends on lysosomal function. Specifically, we have demonstrated that the histone deacetylase member HDAC10 promotes fusion of doxorubicin-filled lysosomes with the plasma membrane (lysosomal exocytosis) and thereby clearance of doxorubicin from neuroblastoma cells. Consequently, HDAC10 depletion or inhibition causes intracellular accumulation of doxorubicin and sensitizes highly resistant neuroblastoma cells to chemotherapeutic treatment. To systematically analyze the contribution of lysosomes to therapy resistance in neuroblastoma, we are currently using both established cell lines, as well as patient-derived short-term cultures. In particular, we investigate the correlation between cellular lysosomal content and resistance to a set of 75 FDA/EMA approved anti-cancer therapeutics. We further investigate if specific anti-cancer drugs commonly induce lysosomal adaptation mechanisms during treatment and how such adaptation mechanisms correlate to treatment resistance.

Summary/Conclusions

The available information on the full molecular characterization of all our cellular models, including primary patient material, allows us to identify gene expression patterns or genetic aberrations (point mutations, chromosomal rearrangements) that predict lysosomal adaptation mechanisms and thus possibly treatment resistance. In the future, the identification of lysosomal dependencies could enable the combination of targeted therapies with lysosome-targeting approaches to revert these resistance mechanisms.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 150

Therapeutic vulnerabilities in the DNA damage response for the treatment of ATRX mutant neuroblastoma

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

In neuroblastoma, genetic alterations in *ATRX,* define a distinct poor outcome patient subgroup with older age at diagnosis, poor response to standard therapies and a chronic slowly progressive disease course. Despite the clear need for new effective treatments for this subgroup, there is a dearth of research in the field, partly due to the lack of available pre-clinical models.

Aims

To generate isogenic models of *ATRX* loss of function (LoF) neuroblastoma and then to utilise these and other available models to identify synthetic lethal vulnerabilities associated with *ATRX* LoF, which can be therapeutically exploited.

Methods/Materials

We designed CRISPR-Cas9 guide RNA probes to target the helicase domain of *ATRX*, and using the SK-N-SH neuroblastoma cell line, generated three single cell clones that were devoid of ATRX expression and had differing frame-shift alterations in the targeted region of *ATRX*. We then utilised the isogenic cell lines to evaluate the impact of *ATRX* LoF in neuroblastoma, specifically focusing on DNA damage response (DDR) signalling pathways and replication fork processivity, in view of the known functions of *ATRX*. For an unbiased assessment of the relative influence of phenotypic changes on therapeutic sensitivity we then performed a high-throughput compound screen of >400 clinically available compounds using the isogenic cell lines. Finally, findings in the isogenic cell lines were confirmed using the native *ATRX* deleted neuroblastoma cell line: CHLA-90 and the *ATRX* deleted patient derived xenograft (PDX) AMC-772.

Results

We found that inactivation of ATRX results in increased DNA damage, homologous recombination repair (HRR) defects and impairment of replication fork processivity and protection. Accordingly, *ATRX* mutant cells showed selective sensitivity to multiple PARP inhibitors (olaparib, rucaparib and talazoparib) as well as to the small molecule ATM inhibitor KU60019. Consistent with a DNA repair defective phenotype, *ATRX* mutant cells also showed selective sensitivity to the DNA damaging agent, sapacitabine and the topoisomerase 1 poison, irinotecan. In confirmatory studies, genetic knockdown of PARP-1 also resulted in significantly greater cytotoxicity in *ATRX* mutant cells.
HRR deficiency was also seen in the *ATRX* deleted CHLA-90 cell line, and significant *in-vitro* synergy was demonstrated to irinotecan and olaparib combination therapy in both the isogenic *ATRX* mutant cell lines and CHLA-90. Furthermore, we identified significant *in-vivo* sensitivity to olaparib and irinotecan combination therapy in *ATRX* mutant neuroblastoma xenografts, but not in the isogenic *ATRX* wild-type counterparts. Finally, in the *ATRX* deleted neuroblastoma PDX AMC-772 we show sustained remissions after only one cycle of olaparib and irinotecan therapy.

Summary/Conclusions

ATRX LoF results in specific DNA damage repair defects that can be therapeutically exploited. We demonstrate a synthetic lethal relationship between ATRX and PARP and preclinical sensitivity to combination therapy with olaparib and irinotecan. This represents a therapeutic strategy that can be rapidly translated into clinical trials for this difficult to treat group of patients.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 151

CD146 is a potential therapeutic target for neuroblastoma

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

Neuroblastoma (NB) is the most common extracranial solid tumor of the childhood, which is thought to arise from neural crest-derived immature cells. Since the prognosis of patients with high-risk or recurrent/refractory NB remains quite poor despite the intensive multimodality therapy, novel therapeutic interventions are required. CD146 (also known as MCAM), a cell adhesion molecule expressed by neural crest and its derivatives, has been reported to be closely associated with tumor progression and metastasis in various types of malignancies.

Aims

The objective of the current study was to examine the expression of CD146 in NB cell lines and clinical samples, and to evaluate the anti-tumor effect of CD146-targeting treatment (shRNA knockdown of CD146 or anti-CD146 polyclonal antibody) for NB cells *in vitro* and *in vivo.*

Methods/Materials

The expression level of CD146 in NB cell lines and clinical samples was investigated by flow cytometric and immunohistochemical analysis. The effect of blocking CD146 was analyzed by our developed rabbit anti-CD146 neutralizing antibody and by shRNA knockdown of CD146 both *in vivo* and *in vitro*. The major pathways downstream of CD146 signaling was examined by western blotting assays in conditional CD146 knockdown NB cells.

Results

The current study demonstrated that CD146 was expressed by all six NB cell lines and the majority of any stage NB primary tumors. Blocking of CD146 pathway, either by treatment with an anti-CD146 polyclonal antibody or by shRNA knockdown, effectively suppressed tumor growth of NB cells both *in vitro* proliferation and *in vivo* tumor formation, principally due to increased apoptosis by focal adhesion kinase/nuclear factor kappa B signaling pathway.

Summary/Conclusions

These results suggest that CD146 represents a promising therapeutic target for NB.

Type Basic

Presentation Preference Traditional poster

Abstract nr. 152

Naxitamab, an antibody with distinct complementary determining regions and high binding affinity to disialoganglioside GD2

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Monoclonal antibodies (mAbs) that target disialoganglioside 2 (GD2) have shown clinical efficacy in the treatment of GD2 expressing tumours and are being used as part of the new standard of care for the treatment of high risk neuroblastoma. Two chimeric antibodies already have received regulatory approval for pediatric neuroblastoma: dinutuximab (Unituxin) and dinutuximab beta (Qarziba). Here we present detailed structural characterizations of naxitamab, a humanized form of mu3F8 antibody targeting GD2, which has been in clinical development since 2011.

Aims

The three-dimensional structural models of the GD2-targeting complementary determining regions (CDRs) of dinutuximab beta and naxitamab were compared and their *in vitro* GD2 binding kinetics and affinity were evaluated side-by-side. The fragment crystallizable region (Fc) structure and function of the two mAbs were also compared in terms of their N-glycan profiles and *in vitro* antibody-dependent cellular phagocytosis (ADCP) and complement-dependent cytotoxicity (CDC) activities.

Methods/Materials

The position of the CDRs in naxitamab and dinutuximab beta were determined using Kabat numbering and visualized in 3D using the known X-ray structures of m14G2a, m14G2a in complex with GD2, and m3F8. Surface plasmon resonance (SPR) and enzyme-linked immunosorbent assay (ELISA) were used to characterize GD2 binding. ADCP activity was assessed using an in-vitro cell-based reporter assay based on a modified human Jurkat cell line transfected to express CD32 receptors. CDC activity was measured using an in vitro cell-based assay in which binding of the mAb Fc region to C1q activates the complement cascade and induces killing of GD2-expressing target cells. The N-glycan profiles of the mAbs were characterized by releasing the N-glycans from the mAbs, labelling with a fluorescent marker and quantification using high-pressure

liquid chromatography with fluorescence detection (HPLC-FLD).

Results

Clear differences in the CDR regions of naxitamab and dinutuximab beta were observed with only 23% similarity. The significance of this is substantiated by the structural analysis showing that the three-dimensional structure of the GD2-targeting CDRs of dinutuximab beta and naxitamab are distinct. Furthermore, a difference in GD2 binding properties resulting in an approximately ten-fold higher affinity for naxitamab to GD2 compared to dinutuximab beta was demonstrated. The higher affinity predominantly was due to its slower off-rate.

Summary/Conclusions

Naxitamab is a humanized anti-GD2 antibody which has been in clinical development for more than 8 years. The prominent differences in the sequence and three-dimensional structure of the GD2-targeting CDRs indicate a differentiated binding capability. This is supported by an approximate 10-fold higher affinity for GD2, predominantly due to a slower off-rate. These properties together with optimized clinical dosing schedules suggest that naxitamab could represent a new approach to target GD2 in paediatric oncology.

Type Basic

Presentation Preference Traditional poster

Abstract nr. 153

Pentraxin 3 (PTX3) in the neuroblastoma microenvironment

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Topic Other

Background/Introduction

The tumor microenvironment (TME) is a complex interplay between tumor cells, supporting immune and stromal cells, and non-cellular factors. Cancer-related inflammation is an important modulator of the tumor microenvironment with potent tumor promoting and tumor suppressing capabilities. Persistent inflammatory processes can contribute to all stages of tumorigenesis and various inflammatory mediators and pathways have been identified as promising therapeutic targets. Pentraxin 3 (PTX3) is a humoral pattern recognition molecule with functions in innate immunity and inflammation. In cancer, a complex role of PTX3 is emerging as both tumor promoting and tumor suppressing functions have been described. Moreover, PTX3 has been identified as a prognostic biomarker in a variety of cancers. Despite advances in diagnosis and therapy, 50% of high-risk neuroblastoma (NB) patients are refractory to treatment or relapse within two years. Several inflammatory mediators and pathways have to date been identified that can contribute to NB tumorigenesis. However, further work is warranted for an in-depth understanding of the NB microenvironment, which may lead to the identification of novel therapeutic approaches and biomarkers.

Aims

To evaluate the function of PTX3 in the NB microenvironment and its' potential as a biomarker and therapeutic target.

Methods/Materials

Gene expression analysis was performed using the publically available R2:Genomics Analysis and Visualization Platform (http://r2.amc.nl). The expression and secretion of PTX3 in neuroblastoma cell lines was determined using RT-PCR, ICC, ELISA, and Western blot. Furthermore, ELISAs were used to investigate the effect of the pro-inflammatory cytokines TNFα,

IL-1β, and IL-6 on the secretion of PTX3 from NB cell lines.

Results

Examination of two publically available gene expression datasets indicated a correlation between high expression of PTX3 and a reduced overall survival probability in NB. Screening of NB cell lines demonstrated that *PTX3* mRNA was present in a variety of NB cell lines. Moreover, using Western blots, PTX3 was detected in the protein precipitates from cell supernatants of half the NB cell lines examined. In contrast, no significant levels of PTX3 protein were observed in the cell lysates. ELISAs confirmed the presence of PTX3 in the cell supernatants of certain NB cell lines. Furthermore, TNFα and IL-1β promoted the secretion of PTX3 from SK-N-AS and SK-N-FI cells. Of note, the highest levels of PTX3 were detected following stimulation with a combination of TNFα and IL-1β. In contrast, no effect on PTX3 secretion was observed in response to IL-6. While a rapid increase in PTX3 secretion from SK-N-AS cells, 3h post stimulation, was detected, SK-N-FI cells reacted more slowly and increased levels of PTX3 were observed after 24h of stimulation.

Summary/Conclusions

Our findings demonstrate that inflammatory cytokines can promote the secretion of PTX3 from certain NB cell lines. Further studies will investigate the specific functions of PTX3 in the NB microenvironment and its potential as a biomarker and therapeutic target.

Type Basic

Presentation Preference Traditional poster

Abstract nr. 154

DHODH impact on Neuroblastoma biology

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Despite intensive therapy, the cure rate for children diagnosed with high-risk neuroblastoma is still too low, accentuating the need for more effective therapies. Inhibition of DHODH is effective as differentiation therapy in myeloid leukemia (Sykes et al., Cell, 2016), but the mechanisms on how DHODH inhibition causes differentiation in cancer is unknown. DHODH is an enzyme that converts dihydroorotate (DHO) to orotate (OA) in the only redox step in the de novo synthesis of pyrimidines.

Aims

We analyzed the potential therapeutic role of DHODH in high-risk neuroblastoma and evaluate the mechanism through which DHODH inhibition promotes differentiation.

Methods/Materials

Survival and gene expression analyses (Kaplan-Meier analysis and multivariate Cox regression) were performed using available TARGET and SEQC-498 datasets (n=647). The activity of DHODH inhibition using brequinar was studied in neuroblastoma cell lines, transgenic TH-MYCN mice and the human neuroblastoma cell lines SK-N-AS and SK-N-BE(2). . Th-MYCN tumors, SK-N-AS and SK-N-BE(2) xenografts were analyzed using RNA-seq and/or ChIP-seq. Mechanistic insights were based on RNA-sequencing and ChIP-sequencing analysis of transgenic

neuroblastoma tumors treated *in vivo* with brequinar or vehicle control.

Results

Data from two independent neuroblastoma patient cohorts demonstrated that *DHODH* expression is an independent risk factor for aggressive disease; higher *DHODH* levels were correlated to worse overall and event-free survival. Strikingly, we identified a subset of high-risk patients in Stage 4 disease with very high expression of *DHODH* that correlated with a dismal prognosis (5 year survival less than 10%). Furthermore, increased *DHODH* expression was significantly correlated to advanced stages of neuroblastoma disease and *MYCN* amplification.
We show *in vitro* that several human neuroblastoma cell lines are sensitive to DHODH inhibition (IC_{50} values ~ 5 nM), and that brequinar sensitivity is positively correlated to *MYCN* gene expression. In TH-MYCN mice, we demonstrate a significant reduction in tumor size after only 72 hours of brequinar treatment and a dramatic prolongation of survival (mean survival of controls: 39 days; mean survival of brequinar-treated: 173 days). Following discontinuation of brequinar after 120 days of treatment, mice relapsed with the reappearance of tumors (median survival 12 days). Brequinar significantly reduced the growth of established human neuroblastoma xenografts (SK-N-BE (2) in NMRI nu/nu mice.). ChIP and/or RNA-seq analysis of mouse tumors showed a shift towards a more mesenchymal phenotype after brequinar treatment, whereas tumors at relapse had re-gained their adrenergic phenotype.

Summary/Conclusions

We conclude that DHODH is a critical metabolic mediator of neuroblastoma growth and that specific DHODH inhibition is a highly effective therapy in neuroblastoma. Targeting DHODH as a cancer-specific metabolic dependency may benefit high-risk patients, in particular the MYCN-amplified subset, and should be further tested in clinical studies.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 155

Detecting alternative lengthening of telomeres in personalized oncology

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Telomere maintenance, which is achieved either by MYCN/MYC activation resulting in telomerase activation or alternative lengthening of telomeres (ALT), is associated with poor clinical outcome in neuroblastoma (NB). Clinically and biologically, ALT-positive NBs were found to form a distinct subgroup with different disease progression and potentially different response to standard therapies. Thus, ALT is an important marker in NB risk assessment.

Aims

In the presented study, we aimed to assess ALT frequency in an unbiased relapse cohort of the registry trial INFORM. INFORM profiles the genetic features of individual tumors in order to provide a personalized treatment. We intended to identify ALT-activity using only the omics data generated in the INFORM pipeline without using classical ALT markers like C-Circles or APBs to

enable ALT scoring in the routine analysis.

Methods/Materials

Using exome sequencing, low coverage whole genome sequencing, methylation array and RNA sequencing data generated via the INFORM workflow from 99 relapsed NBs, the presence of an active TMM (ALT, TERT or MYCN activation) was analyzed.

Results

Criteria to detect ALT activity were a higher telomere content in the tumor compared to the control, increased TERRA expression and the presence of a 1q42.2-1qter deletion. Due to the almost mutual exclusivity of ALT, amplified *MYCN* and *TERT* activation, absence of amplified *MYCN* and lack of *TERT* expression were used as additional parameters. Our preliminary dataset of 40 relapsed tumors with known C-Circle status was used as a training set (n=40) for the applied criteria and subsequently the classification was done for 59 additional relapsed NBs.
In total, ALT activity was identified in about 40% of the relapsed NB cases indicating an enrichment of ALT in relapsed disease.
Taken together, for the majority of cases it was possible to detect ALT activity or to identify another TMM being MYCN or TERT activation. However, for about 10% of the tumors this was not possible using the available data. The lack of available RNA sequencing data for some cases was thereby an important limitation. Detection of *ATRX* mutations, which are found in about half of the ALT-positive cases based on our previous data, was very challenging using the INFORM pipeline due to the lack of high coverage whole genome sequencing data.

Summary/Conclusions

In summary, the assessment of ALT activation is possible using the routine INFORM pipeline and can be used for accurate risk assessment. However, targeted therapies for this subgroup of patients being enriched in the relapse are still missing, but are urgently needed and should therefore be an extensive area of research.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 156

Single-cell RNA sequencing of human neuroblastoma reveals Schwann cell precursors as putative cancer stem cells

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Topic Neuroblastoma as developmental disease

Background/Introduction

Cell identities and heterogeneity underlying initiation and recurrence of neuroblastoma still remain elusive. Neuroblastoma is a neural crest derived tumor and recent evidence suggests that neuroblastoma cells display different phenotypes with distinct super-enhancer and gene expression patterns, including malignant adrenergic and mesenchymal cells. However, the precursors for these phenotypes and their interplay within intact tumor tissues have not yet been investigated.

Aims

To determine the identity and composition of cells within the neuroblastoma microenvironment we performed single-cell RNA sequencing (scRNA-seq) of human samples from children with tumors of all genetic subsets.

Methods/Materials

Fresh tumor samples were collected at diagnosis and/or relapse through needle biopsy or surgical sampling. Tumor cell content was confirmed and SNP array CGH was performed on all samples. After enzymatic tissue dissociation, FACS was performed to enrich for all viable, non-erythrocytic cells. Libraries were prepared using the 10x Chromium Single-Cell 3' v2 protocol and sequenced on the Illumina NextSeq platform. Initial alignment and gene expression quantification was performed using 10x *cellranger* software. Downstream QC/filtering and bioinformatic analyses were performed using the *seurat, pagoda2* and *conos* R packages. Sample-wise cross-comparison of SNP array CGH and scRNA-seq data was used to identify cells with tumor-specific copy number variations (CNVs). Our findings were validated with protein, RNA and DNA immunofluorescence stainings.

Results

We sequenced 17 samples from 15 children. The patient cohort was heterogeneous, including all genetic subsets and various age and risk groups. After pre-processing and filtering we obtained

72,704 single transcriptomes of high quality. Using unbiased clustering, we identified 13 main cell types spanning the immune, mesenchymal-stromal and neural crest-derived compartments. Neural crest-derived cells included adrenergic and Schwann-like populations. Schwann-like cells were observed in 16 of 17 samples and formed a transcriptional “bridge” between fibroblast-like mesenchymal cells and adrenergic cells. RNA velocity analysis revealed bi-directional movement of Schwann-like cells towards both the adrenergic and fibroblast-like mesenchymal cells. Subsets of adrenergic, fibroblast-like mesenchymal, and Schwann-like “bridge” cells all displayed tumor-corresponding CNVs, suggesting a malignant nature. Bridge cells harboring CNVs were seen in 13 of 17 samples and in all risk groups. When comparing our dataset to scRNA-seq data from human fetal adrenal gland, bridge cells aligned to Schwann cell precursors (SCPs). We observed that the vast majority of Schwann-like cells were located in close proximity to neurofilament and that they did not express myelin protein zero (MPZ), which is a marker of myelinating glial cells.

Summary/Conclusions

We confirmed the adrenergic and mesenchymal phenotypes of human neuroblastoma at single-cell level. In addition, we identified malignant Schwann-like cells with a bi-directional differentiation capacity into mesenchymal and adrenergic phenotypes. These were detected in tumors from all risk groups and genetic subtypes and closely resemble SCPs, which have previously been shown to represent precursors of adrenergic and mesenchymal cell types during neural crest development. In our proposed model, SCPs acquire cancer-promoting genomic aberrations and subsequently fuel the bulk tumor populations. Our findings carry important clinical implications regarding therapeutic resistance and tumor recurrence.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 157

Telomere maintenance versus gene expression-based classification for risk assessment of neuroblastoma patients

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Gene expression-based classification is a promising strategy for risk stratification of neuroblastoma patients. Previous studies showed improved outcome prediction when a gene expression-based classifier (SVM-th10) was integrated into current risk stratification systems. Recent studies, however, suggested that the individual risk of neuroblastoma patients may be also precisely assessed by a mechanistic classification that takes telomere maintenance (TM) mechanisms into account, including activation of telomerase and alternative lengthening of telomeres (ALT).

Aims

We here aimed to evaluate and compare the performance of two distinct molecular risk assessment systems, i.e., gene expression-based classification vs. telomere maintenance, for outcome prediction of neuroblastoma patients.

Methods/Materials

Microarray-based gene expression profiles and TM were determined in diagnostic tumor samples of 201 neuroblastoma patients covering the entire spectrum of the disease. Patients were classified into favorable or unfavorable subgroups using the previously described SVM-th10 gene expression classifier. Tumor samples were considered as TM-positive if they harbored *TERT* rearrangements or *MYCN* amplification (as determined by FISH), elevated *TERT* expression (as determined by microarrays), or ALT-associated PML nuclear bodies (as determined by combined immunofluorescence/FISH).

Results

TM was found in 99/201 tumors (49.3%), and unfavorable gene expression profiles in 82 patients (40.8%). Unfavorable gene expression was associated with TM. 75/99 patients (75.8%) with TM and 7/102 patients without TM had an unfavorable gene expression classification ($p<0.001$). Both TM and unfavorable classification were associated with stage 4, *MYCN* amplification, age ≥18 months and high-risk disease ($p<0.001$ each). Both TM and unfavorable classification were also associated with poor outcome: Three-year event-free survival (EFS) of patients with TM-positive tumors was 0.43 ± 0.05 compared to 0.91 ± 0.03 for patients with TM-negative tumors ($p<0.001$; 3-year overall survival (OS), 0.72 ± 0.05 vs. 1.0, $p<0.001$). Three-year EFS of patients with unfavorable gene expression classification was 0.47 ± 0.06 compared to 0.81 ± 0.04 for patients with favorable classification ($p<0.001$; 3-year OS, 0.74 ± 0.05 vs. 0.95 ± 0.02; $p<0.001$). For the whole cohort, multivariable analysis for EFS revealed TM (hazard ratio: 15.6, CI 7.0 – 34.6, $p<0.001$), but not unfavorable gene expression profile ($p=0.71$) as significant. Similar results were found for non-high-risk patients (n=119; TM: hazard ratio: 14.1, CI 6.2 – 31.8, $p<0.001$; SVM-th10: $p=0.325$). Separate multivariable analysis for high-risk patients was not possible because only one high-risk patient was TM negative.

Summary/Conclusions

Assessment of TM appears to be superior to gene expression-based classification in outcome predication of neuroblastoma patients. The accuracy of TM in neuroblastoma risk assessment should thus be evaluated in large prospective studies.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 158

Impact on genomics of extracellular matrix biotensegrity using 3D bioprinted and orthotopic xenograft mice models of neuroblastoma

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Tumor cells are submitted to mechanical forces, tensional networks or biotensegrity, some of them related to the surrounding extracellular matrix (ECM). Our previous results about tumor microenvironment of neuroblastoma (NB) noted an increase of ECM stiffness in the most aggressive tumors, defining a group of ultra-high risk patients and an abundant amount of intra-pericellular vitronectin (VN) expression in genetically instable tumors. However, it is still unknown how ECM biotensegrity affects the genomics.

Aims

To find out the genomic consequences of growing two NB cell-lines in variable ECM stiffness scaffolding models.

Methods/Materials

NB cell-lines were cultured independently in three platforms: a) 2D traditional *in vitro* culture; b) 3D bioprinting with a variable *in vitro* growing time (2 to 10 weeks) composed of hydrogel with different stiffness depending on methacrylated alginate concentration (0 to 2%) plus tumor cells (SK-N-BE-(2) NB cells with *MYCN* amplification (MNA), or SH-SY5Y NB cells with *ALK* mutated,

with and without SW10 Schwann cells); and c) intra-adrenal gland serial xenograft VN knock-out mice ($RAG1^{-/-}VN^{-/-}$) and control mice ($RAG1^{-/-}VN^{+/+}$). Serial tumor passages were carried out up to, at least, three generations of xenografted mice with unaltered high quantity of tumor VN-rich ECM. We have performed single nucleotide polymorphism array (aSNP, Affymetrix) analysis to identify whole genome copy number aberrations (CNAs).

Results

We identified variable patterns of genomic aberrations in the SK-N-BE-(2) cells, mainly affecting to chromosomes 1, 9 and 21, related to the used models and its time of *in vitro* growth/mice passages. The cells cultured in 2D, all the 3D hydrogels with less stiffness, short time stiffest hydrogels and initial passages control mice, had similar CNAs, unlike the cells cultured in long time stiffest hydrogels and $RAG1^{-/-}VN^{-/-}$ mice that promoted selection of the same CNAs that were minority present in 2D culture. Special interest had been the KANK gene deletion located in 9p which protein encoded inhibits actin fiber formation and cell migration. No genomic differences in SH-SY5Y cell-line between any of the assayed conditions were found.

Summary/Conclusions

Evidence of clonal selection of SK-N-BE-(2) cells with the same CNAs could be related to mechanosensing of ECM stiffness. Large Genetic studies of other cell-lines and human tumors should be done to confirm the CNAs changes related to VN-rich ECM stiffness.

Type Translational

Presentation Preference Traditional poster

Abstract nr. 159

Analysis of MYCN amplification, ALK alterations and RASSF1a methylation in cfDNA: Utility for the therapy of neuroblastoma patients

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Introduction

The aim of this study was to compare for the same patient the *MYCN* status obtained from circulating free DNA (cfDNA) analyzed at diagnosis to the one observed by analysis of the primary tumor and/or metastasis within a prospective study IC2007-09/PHRCNB07 (ClinicalTrials.org, NCT02864563) including all patients with neuroblastic tumors treated in the centers of the SFCE (Société Française des Cancers de l'Enfant). Moreover, using cfDNA, *MYCN* amplification was studied at different timepoints during treatment and follow-up and compared to other known

biomarkers such as *ALK* mutation and/or amplification and *RASSF1a* methylation.

Aims

Methods/Materials

Methods:

MYCN status from the tumor and/or metastasis samples was evaluated by FISH analysis and/or aCGH. *MYCN* amplification in cfDNA obtained after extraction from plasma samples was analyzed by qPCR from 2007 to 2014 and after this date by ddPCR. An amplification was defined by a ratio of gene of interest /reference gene >3. The other biomarkers were analyzed by ddPCR. The presence of more than 6 mutated or methylated events identified a mutation or a methylation.

Results

Results:

Among the 580 patients included in this study, cfDNA was studied at diagnosis for 348 patients. 70 showed presence of *MYCN* amplification, 275 showed absence of *MYCN* amplification, and a borderline result was observed in 3 cases.
Among the 275 cfDNA samples without *MYCN* amplification, concordance with the results obtained by tumor samples analysis was observed in 246 cases. For 26 cases, *MYCN* amplification was never analyzed in tumor samples because of very low tumor cell percentage (22 cases) or they were not received (4 cases). For 3 cases; there was a discrepancy between cfDNA and tumor sample result since a *MYCN* amplification was detected in tumors but not in cfDNA.
Among the 70 cfDNA samples with *MYCN* amplification, concordance with tumor samples was observed in 66 cases (48 stage 4, 14 stage 3, 2 stage 2, 2 stage 4s). For 1 case, the tumor sample was not received and consequently MYCN had never analyzed and for 3 cases the tumor samples were not contributive since the percentage of tumor cells was very low.
Using cfDNA, *MYCN* amplification was studied at different timepoints of the disease in 30 patients showing *MYCN* amplification. Furthermore *RASSF1a* methylation could be analysed sequentially in 10 patients and *ALK* mutation and/or amplification in 7 patients with known *ALK* alterations. All three biomarkers decreased under treatment and increased at relapse. For one patient showing *MYCN* amplification and *ALK* amplification, both biomarkers were detected for each analyzed timepoint between diagnosis and relapse.

Summary/Conclusions

Conclusion

The data obtained from this prospective study confirmed that *MYCN* amplification and *ALK* alterations could be detected at diagnosis in cfDNA, contributing to clinical decision-making in particular in cases when primary tumor analysis was not contributive. The analysis of these biomarkers and *RASSF1a* methylation at different timepoints of the disease could also help to define minimal residual disease and response to treatment. The analysis of *RASSF1a* methylation should be of interest especially for the patients without genomic alterations.

Type Translational

Presentation Preference Traditional poster

Abstract nr. 160

EXPLORATORY ANALYSIS OF GENOMIC INSTABILITY IN HIGH-RISK NEUROBLASTOMA

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Survival of high-risk neuroblastoma (HR-NB) patients remains poor despite multimodal treatment. Identification of ultra-high-risk (UHR) patients (if death occurred before 18 months from diagnosis) is pivotal to offer them therapeutic alternatives and to gain insight into the biology of NB. For some types of cancer driven by copy number aberrations (CNAs), it has been demonstrated that the length of the altered genome encompassed by CNAs, that is the CNA burden, is related to biological features and prognostic. The impact of the CNA burden and other measurements of segmental chromosomal aberrations (SCAs) to define genomic instability (GI) in HR-NB remains unknown.

Aims

To explore the GI derived from SCAs in primary HR-NB tumors in relation to biological features and survival in order to gain insight into the identification of UHR patients.

Methods/Materials

We reviewed primary tumor profiles previously analyzed by SNP array for clinical diagnosis from 127 HR-NB patients. For each sample we annotated the length of all SCAs (>2mb) and calculated 3 GI indexes: i) the CNA burden ($\%gains_{length}+\%losses_{length}$), ii) the Copy Number Load (CNL), based on the predominance of gained or lost chromosomal material ($\%gains_{length}-\%losses_{length}$) and iii) the Net Genomic Load (NGL), considering the number of copies gained or lost for each SCA ($\%gains_{amount}-\%losses_{amount}$). Tumors were classified according to a negative CNL (nCNL) or positive CNL (pCNL) and decreased or increased NGL. Statistical analyses were

performed to determine the relation of GI indexes to age, stage, histology, ploidy, *MYCN* status (69 non-amplified, 43 homogeneous amplified and 15 heterogeneous amplified), 11q loss, number and type of SCAs, overall survival (OS) and event-free survival (EFS).

Results

The CNA burden and the NGL were not related to survival. Combinations of CNL genomic type and *MYCN* status allowed us to identify patients with worse prognostic. We found that, within the nCNL group (71/127), the subgroups of patients with inferior outcome (5-year OS<20%, OS time<5 years) were 24 patients with homogeneous *MYCN* amplification and 17 patients with non-amplified tumors plus high nCNL values (>2.2%, above the mean). However, we did not find any difference in the survival within the pCNL group (56/127). For patients with heterogeneous amplification, the pCNL group (8/15) was related to a worse OS than the nCNL (5-year OS=22% vs 67%). Overall, a classification based on the CNL genomic type distinguished that the 39% of our cohort had an especially poor prognostic. Differences between CNL genomic types were related to number of SCAs, loss of 1p, 4p and 14q, and gain of 1q. We highlight that the frequency of 1p loss in non-amplified nCNL tumors was higher than expected (48% vs 14% for pCNL tumors).

Summary/Conclusions

We propose that CNL can be useful to better characterize UHR-NB patients. Ongoing studies are needed to validate the results in a higher cohort.

Type Translational

Presentation Preference Traditional poster

Abstract nr. 161

EGFR inhibitors are effective against chemoresistant neuroblastoma models

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

Patients with high-risk neuroblastoma (HR NB) are treated with multimodal therapy including intensive chemotherapy, radiation, surgery and immunotherapy. Approximately 60% of HR NB patients that initially respond to chemotherapy will eventually suffer disease relapse and/or develop resistance to conventional treatments. Recent studies have shown that molecular profiles of relapsed NB tumors are significantly different from those at diagnosis mainly because of genetic aberrations induced and/or selected by chemoradiotherapy. In most cancers, including HR NB, epidermal growth factor receptor (EGFR) expression increases in therapy-induced chemoresistant cancer cells. Preclinical studies have provided evidence that treatment with EGFR-specific tyrosine kinase (RTKs) inhibitors results in suppression of tumor growth and increases the sensitivity to diverse cytotoxic drugs in chemoresistant cancer cell lines.

Aims

We established *in vitro* and *in vivo* chemoresistant NB models to explore the activity of EGFR inhibitors and provide insights into the biology of the chemorefractory disease.

Methods/Materials

We generated five NB cell line models with induced resistance to doxorubicin, cisplatin and vincristine by long-term exposure to increasing drug concentrations. Drug sensitivity was measured overtime using the half maximal inhibitory concentration (IC_{50}) value for each cytotoxic drug. Molecular markers associated to drug resistance were evaluated by RTqPCR in chemoresistant cells as well as *in vitro* functional assays. Genome-wide expression profiles were obtained for paired chemoresistant and non-chemoresitant cell lines to explore the effect of drugs. Drug sensitivity and proliferation assays using an EGFR inhibitor were performed in chemoresistant cells. The *in vivo* efficacy of EGFR inhibitor treatment was evaluated by measuring

tumor growth in paired chemoresistant and non-chemoresistant HR NB patient-derived xenograft (PDX) models.

Results

Induced chemoresistant NB cell lines showed a marked increase of the IC_{50} values and significant changes in cell morphology as compared to the non-treated counterparts. Increased relative expression of ABC transporters, stemness, EMT and DNA damage markers were found in chemoresistant cells. Transcriptome analysis showed differential gene expression profiles associated to drug resistance mechanisms that differed depending on the cytotoxic drug used in the distinct cell line models. *In vitro* results revealed the irreversible EGFR inhibitor canertinib as the most sensitive RTK tested in NB cells. Combination of the EGFR inhibitor with cytotoxic drugs was significantly more toxic than treatment with single drugs. *In vivo* treatment with canertinib showed a significant decrease in the tumor growth for the chemoresistant PDX model.

Summary/Conclusions

Canertinib in combination with chemotherapy represents a promising therapeutic strategy for chemoresistant HR NB. Ongoing experimental work will also provide further insights into the molecular mechanisms and signaling pathways involved in drug resistance in NB.

Type Translational

Presentation Preference Traditional poster

Abstract nr. 162

Metformin can enhance the anti-neuroblastoma effect of ketogenic diet

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

Neuroblastoma (NB), like other solid tumors, is characterized by a high dependency on glycolysis and low but still functional oxidative phosphorylation (OXPHOS), a biochemical phenomenon also known as Warburg effect. Our previous studies showed that a high-fat, low carbohydrate diet (ketogenic diet; KD) can successfully target NB. The glucose dependency combined with a decreased capacity to utilize alternative substrates by OXPHOS sensitizes NB cells to KD. In addition, owing to the KD's low-carbohydrate content, the KD not only reduces the circulating glucose, but consequently also lowers insulin and IGF-1 levels. Recent studies suggested that the inhibition of residual OXPHOS activity can be beneficial in cancer treatment. Metformin (MET), an anti-diabetic drug, reduces the blood glucose levels and targets complex I of the OXPHOS. In agreement, Kumar et al. have reported that MET inhibits NB tumor growth *in vivo* (Kumar et al. Oncotarget, 2014).

Aims

The aim of this study was to elucidate whether MET can enhance the anti-NB effects of a KD.

Methods/Materials

The NB cell lines SKNBE (2) (*MYCN*-amplified) and SH-SY5Y (non-*MYCN*-amplified) were treated for 3 days with different concentrations of MET and cell viability was determined by a cell

viability assay using 3-(4, 5-dimethylthiazol-2-yl)-5-(3- carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTT). NB xenografts were established in CD-1 nude mice with SKNBE (2) cells. The NB bearing mice were fed with a control diet (CTRL) and a KD (ketogenic ratio 8:1, supplemented with medium-chain triglycerides) with/without MET (oral gavage, 100 mg/kg body weight). Tumor growth, body weight, blood glucose, and ketone body concentration, were monitored every fourth day. Plasma and tumor samples were collected for molecular analysis.

Results

In vitro, treatment of SKNBE(2) and SH-SY5Y cells with MET resulted in a dose dependent decrease of cancer cell proliferation. In contrast to the literature, MET alone did not reduce tumor growth *in vivo*. However, MET enhanced the anti-NB effects of KD and improved the survival rate of SKNBE(2) xenograft bearing mice. The MET concentration in plasma was not influenced by the administration of KD. Only the combination of KD and MET reduced blood glucose levels although after 13 days of therapy they returned to the levels of the CTRL diet group**. As expected, KD led to higher levels of ketosis in the KD and KD + MET groups, whereas MET alone had no effect on blood ketone body levels.** Moreover, CD31 staining revealed a significant reduction of tumor vessel density in the KD+MET group compared to the CTRL group.

Summary/Conclusions

In contrast to the study of Kumar et al. (Oncotarget, 2014), MET alone had no effect on NB growth. However, our data suggest that MET could be used to enhance the anti-NB effect of KD and therefore, icould represent a new adjuvant multi modal metabolic therapy.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 163

A comprehensive genetic analysis of paired diagnosis and relapse neuroblastoma cell lines

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Around 50-60% of all high risk neuroblastoma patients relapse, resulting in incurable disease. An improved understanding of relapsed neuroblastoma biology is required to develop new treatments. Copy number aberrations and other single nucleotide variant (SNV) genetic abnormalities are linked to poor patient outcome. Known genetic abnormalities associated with relapsed neuroblastoma include an increased frequency of anaplastic lymphoma kinase (ALK), RAS/MAPK and p53 pathway gene mutations.

Aims

To perform a comprehensive genetic analysis of paired neuroblastoma cell lines using whole exome sequencing (WES), high-density single nucleotide polymorphism (SNP) array data and targeted sequencing to identify genetic abnormalities involved in neuroblastoma at diagnosis and relapse.

Methods/Materials

DNA from *MYCN* amplified NBLW, NLBW-R, PER-106, PER-107, PER-108, SKNBE1n (BE1n) and SKNBE2c (BE2c) paired neuroblastoma cell lines established at diagnosis, relapse or further relapse from three different patients were analysed by whole exome sequencing (WES) (Illumina Truseq Rapid Exome Library Prep Kit) to a read depth of 100x. Sequence reads were analysed using a GATK pipeline. SNP arrays were generated using Illumina CytoSNP-850k arrays and data

analysed using Nexus copy number software. Targeted next generation sequencing was performed using a panel of 38 genes frequently mutated in neuroblastoma on NBLW, NBLW and BE2c cell line DNA with an Illumina Truseq custom amplicon kit and sequenced on an Illumina NextSeq 550.

Results

An increase in copy number abnormalities was observed in all relapsed cell lines compared to the matched diagnostic one (median = 5). Segmental chromosomal aberrations -1p, +1q and +17q often seen in neuroblastoma were observed in all of the cell lines assessed with the exception of -1p in NBLW cells. Aberrations -4q and +20q developed in two or more cell lines at relapse. A loss of +2q and +3q was also observed between the BE1n and BE2c cell lines. No additional amplicons except for *MYCN* were observed at diagnosis or relapse. WES identified an increased mutational burden at relapse in two out of the three paired cell lines (median increase = 25.5 mutations). Genetic mutations identified by WES and targeted sequencing included those previously reported for *ALK* (NBLW and NBLW-R), *TP53* (BE2c), *NF1* (NBLW), *MDM4* (NBLW), *PTPRD* (NBLW-R) and *TERT* (BE2c). Those mutations detected by targeted sequencing but not WES were *MDM4*, *PTPRD*, *TERT* and *NF1* (BE2c only). Interestingly an *ALK* mutation was detected in the diagnostic BE1n cell line (F1174S), but was lost in the matched relapse cell line, BE2c. Previously unreported mutations in *CREBBP* and *ERBB3* in BE2c cells, as well as *CHEK2* in PER-106 cells were also identified in this study.

Summary/Conclusions

Paired neuroblastoma cell lines established from patients at diagnosis and relapse treated with combination chemotherapies currently used in the clinic are valuable preclinical models for studying disease progression. Identification of novel variants at relapse as well as loss of pathogenic variants from clonal selection will improve our understanding of neuroblastoma evolution and aids selection of cell lines for future research testing novel targeted therapies.

Type Basic

Presentation Preference Oral presentation

Advances in Neuroblastoma Research (ANR) Meeting
ANR 2020
Amsterdam
11-14
May
www.anr2020.org

Abstract nr. 164

Role of GOLPH3 and TPX2 in neuroblastoma DNA damage response and cell resistance to chemotherapy

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Golgi phosphoprotein 3 (GOLPH3) has been reported to be involved in the development and in DNA damage response of various human cancers. Golgi dispersal is a common feature of DNA damage response in mammalian cells. It has been demonstrated that DNA damage triggers dramatic reorganization of the Golgi shape, resulting in its dispersal throughout the cytoplasm via Golgi protein GOLPH3. Several studies have paid attention to the relationship between TPX2 and DNA damage response. TPX2 is a protein involved in spindle apparatus assembly and specifically associated to cell microtubules. *TPX2* gene is part of the signature of chromosomal instability from specific genes whose expression was consistently correlated with clinical outcome in multiple human cancers, and it has been identified as a driving oncogene in different kinds of neoplasm.

Aims

Because little was known regarding the effects of DNA damage on the Golgi, and about the Golgi shape in neuroblastic tumors, we focused on the underlying molecular mechanism of GOPLH3 in neuroblastoma cells. Understanding how neuroblastoma cells react to DNA damage is essential in order to recognize the systems used to escape from elimination.

Methods/Materials

To investigate the mechanism of resistance of neuroblastoma cells to elimination induced by DNA damaging agents, we used two inducible neuroblastoma cell models: IMR-32 cell line with amplification of *MYCN* oncogene, and SH-SY5Y cell line with *MYCN* single copy but with high level of c-MYC expression. We induced DNA damage in these neuroblastoma cell lines with curcumin. To investigate the relationship between GOLPH3 expression and DNA damage after treatment with curcumin for 48 hrs, we analyzed the expression of GOLPH3 and of other proteins such as MYO18A, γH2AX, TPX2, MYCN, MYC, MAP-kinases, Akt, PARP, Beclin-1 and LC3-II in treated and untreated neuroblastoma cell lines by western blotting assay or immunofluorescence staining. Then, we examined GOLPH3, γH2AX, and TPX2 protein expression by immunofluorescence in primary neuroblastoma human tissue coming from pre-chemotherapy and

post-chemotherapy surgical resection. We evaluated the association of *TPX2* gene expression with neuroblastoma patient outcome using the public R2 Platform.

Results

Exposure of neuroblastoma cells to curcumin induced: 1) up-regulation of GOLPH3^{+} cells; 2) augmentation of double-strand-DNA breaks; 3) Golgi fragmentation and dispersal throughout the cytoplasm; 4) increase of apoptosis and autophagy; 5) increased expression of TPX2 oncoprotein, able to repair DNA damage. Primary neuroblastoma samples analysis confirmed these observations.

Summary/Conclusions

Over-expression of GOLPH3 and TPX2 caused by DNA damage induced by curcumin seems to confer a survival advantage to neuroblastoma cells. We have observed that cells drastically stop growing or die when incubated with curcumin, but, if surviving, they rapidly recover after curcumin depletion, starting growing again very rapidly, in spite of a so detrimental treatment. The GOLPH3 activation together with Golgi dispersal throughout the cytoplasm were co-localized with autophagy markers expression, that could be the process through which these cells better resist and survive. Our findings suggest that GOLPH3 and TPX2 expression levels may represent the clinical markers of neuroblastoma patients' responsiveness to DNA damaging therapies, and of possible resistance to them.

Type Translational

Presentation Preference Traditional poster

Abstract nr. 165

Targeting MIF/CXCR4 axis reduces neuroblastoma cell viability, invasion and chemoresistance in the bone marrow niche.

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

The receptor CXCR4 has a pivotal role for bone marrow (BM) homeostasis and BM metastasis in several cancers. In neuroblastoma (NB), CXCR4 is expressed in tumor cells; however, the oncogenic role of its ligand CXCL12 has shown contradictory results.

Aims

We investigated the role of Macrophage Inhibitory Factor (MIF), an alternative CXCR4 ligand, in BM environment and metastasis of NB. Using an *in vitro* model that recapitulates low oxygen levels and chemokine signaling, we explored whether MIF/CXCR4 signaling is critical for NB survival and proliferation in the BM niche. We also evaluated MIF inhibition as a therapeutic option for NB.

Methods/Materials

To develop a BM-based *in vitro* model, NB cells were cultured in different conditioned media (CMs) derived from supernatants of BM primary cells cultured alone (CM-BM), or in the presence of NB cells (CM-NB/BM; 1:1 NB:BM ratio), supernatant of NB cells cultured alone (CM-NB), or standard media (CM-CNT). To mimic BM oxygen levels, NB cells were cultured under hypoxia (1% O_2), as compared to cell culture normoxia (21% O_2). Cellular and chemokine characterization of BM cultures and CMs was performed using flow cytometry, cytokine array and ELISAs. *In vitro* functional studies were performed using MTS reagent, Transwell assays, flow cytometry and

western blot with or without the covalent MIF inhibitor 4-IPP.

Results

Expanded BM cultures used to generate CMs contained a predominant cell population positive for mesenchymal stromal markers (49.2% $CD90^+$, 29.9% $CD105^+$). Cytokine arrays and ELISA assays of CMs revealed MIF as the highest released cytokine, whereas CXCL12 was not detected. CMs were used to perform functional cell viability, invasion, drug resistance and cell signaling studies. Under hypoxia, CM-BM and CM-NB/BM enhanced LAN-1 cell viability at 48, 72 and 96 h ($P<0.01$). This effect was reversed by adding sub-lethal concentrations of the MIF inhibitor 4-IPP. Using Matrigel-coated Transwells, CM-NB/BM chemoattracted and enhanced invasion of LAN-1 cells cultured under hypoxic conditions ($P<0.01$). Chemotaxis and invasion was reversed by preconditioning cells with 4-IPP ($P<0.05$). We also explored whether CMs affected response to chemotherapy. After treatment with doxorubicin and etoposide at IC50 values (30 and 415 nM), LAN-1 cell viability increased 22% and 18% in CM-NB/BM compared to CM-CNT ($P<0.05$). In both cases, chemo-sensitivity was restored when 4-IPP was added to CM-NB/BM. We also evaluated whether CMs and hypoxia could increase expression of CXCR4 and CD74 (alternative MIF receptor), and activate intracellular signaling pathways. Under hypoxia, CM-BM and CM-BM/NB increased CXCR4 but not CD74 expression in LAN-1 cells by flow cytometry, and increased phosphorylated levels of AKT more than ERK measured by western blot.

Summary/Conclusions

We have generated an *in vitro* model of the BM microenvironment that recapitulates features such as hypoxia and chemokine signaling, which proved to enhance the pro-metastatic properties of NB cells. This BM-model enabled us to identify MIF/CXCR4 as a critical axis in NB chemotaxis and invasion, and demonstrate the *in vitro* efficacy of MIF inhibitors. Ongoing studies are being performed to elucidate the activity of MIF inhibitors *in vivo.*

Type Basic

Presentation Preference Traditional poster

Abstract nr. 166

G-CSF as a suitable alternative cytokine to boost neutrophil-mediated antibody immunotherapy of neuroblastoma

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

The current immunotherapy for high-risk neuroblastoma patients targets GD2 ganglioside with the therapeutic antibody dinutuximab. Opsonized tumor cells are killed by antibody-dependent cellular cytotoxicity (ADCC), a process mediated by various immune cells, including neutrophils. The killing capacity of neutrophils to kill dinutuximab-opsonized tumor cells is further enhanced by granulocyte-macrophage colony stimulating factor (GM-CSF), which has been shown in the past to improve clinical response to anti-GD2 antibody therapy. However, GM-CSF is not available for clinical use in Europe, creating a high clinical need for an alternative method to stimulate dinutuximab responsiveness. In this study, we propose to use clinically well-established granulocyte-colony stimulating factor (G-CSF) as a potential replacement for unavailable GM-CSF in the dinutuximab immunotherapy regimen of European neuroblastoma patients.

Aims

To show that granulocyte-colony stimulating factor (G-CSF) is an efficacious and safe alternative to GM-CSF to boost neutrophil-mediated antibody therapy.

Methods/Materials

We tested the capacity of either fresh (unstimulated) or *in vitro* G-CSF or GM-CSF stimulated neutrophils isolated from healthy donors to kill dinutuximab-opsonized GD2 positive neuroblastoma cell lines. Blocking experiments with F(ab')2 fragments of antibodies inhibiting either respective FcγR or a major neutrophil integrin CD11b/CD18 were used to decipher a mechanism by which the cytokines affect ability of neutrophils to perform ADCC. Live cell imaging microscopy was used to visualize neutrophil mediated tumor killing. To assess the effect of G-CSF on tumor cells, we cultured neuroblastoma cell lines in the presence or absence of the cytokine in the culture medium for various time points. We analyzed tumor cell proliferation rate, susceptibility for neutrophil-mediated ADCC, and markers of epithelial-to-mesenchymal transition (EMT) by qRT-PCR.

Results

We observed that G-CSF was as powerful as GM-CSF in enhancing destruction of dinutuximab-opsonized tumor cells in all studied cell lines. This effect was consistent regardless of dinutuximab concentration, amount of cytokine used, or tumor:neutrophil ratio. Blocking experiments of the three main Fc receptors that could be expressed by neutrophils (FcγRI, FcγRIIa, and FcγRIIIb) revealed that it was FcγRIIa that mediated ADCC in neuroblastoma cell lines, and this was consistent for both stimulation conditions. Neutrophils stimulated either with G-CSF or GM-CSF showed an activated CD11b/CD18 integrin that turned out to be indispensable for efficient ADCC. Live cell imaging experiments showed trogocytosis to be the main killing mechanism by which differentially stimulated neutrophils destroyed antibody-opsonized tumor cells. Importantly, although neuroblastoma cells express the receptor for G-CSF, in our hands their proliferation rate, as well as their susceptibility to neutrophil-mediated ADCC was unaffected upon exposure to the cytokine. Also, no signs of EMT were observed at any analyzed time point.

Summary/Conclusions

We demonstrated that G-CSF potentiates neutrophils to kill dinutuximab-opsonized neuroblastoma cells equally well as GM-CSF and exposure to the proposed cytokine did not change the phenotype of tumor cells. Altogether, our data supports the use of G-CSF as an alternative to GM-CSF and warrants further testing of G-CSF in a clinical setting for the improvement of immunotherapy of neuroblastoma.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 167

Identification of novel vulnerabilities of therapy resistant Neuroblastomas

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

Relapse, often accompanied by treatment resistance, is a common clinical problem in high-risk childhood malignancies. The INFORM registry “**IN**dividualized Therapy **FO**r **R**elapsed **M** alignancies in Childhood” addresses this by molecular-biologically characterizing tumor samples from pediatric patients with relapsed or refractory high-risk disease. The ERBB family of receptor tyrosine kinases is well known for its role in cancer development and progression. However, the role of ERBB4 in relapsed neuroblastoma has not been studied widely. Aberrant activation of ERBB4 can lead to the activation of various cancer-relevant pathways, including the PI3K/AKT-, SRC- and MAPK-pathways.

Aims

We aim to identify and target novel vulnerabilities in relapsed neuroblastoma to induce efficient cell death of the resistant cells. Furthermore, we elucidate which subpopulation of relapsed neuroblastomas can be targeted by a certain vulnerability. As there are many different mechanisms of programmed cell death we further intent to unravel which type is achieved by the novel treatment combination.

Methods/Materials

To identify novel vulnerabilities, gene expression data of relapsed INFORM tumors was compared to publicly available gene expression datasets from primary tumors using a rank based analysis. The identified lists of differentially expressed genes between primary and relapsed tumors were then tested for enrichment of molecular pathways. We validated ERBB4 expression in susceptible and resistant neuroblastoma cell lines on RNA and protein levels. The effect of treatment

combination of vincristine (VCR) and the ERBB4 inhibitor afatinib on cell viability was controlled by trypan blue exclusion. Influence on the downstream pathways was monitored by western blot. To differentiate between different types of programmed cell death, we established a toolbox to capture several characteristic features: e.g. caspase activity as marker for apoptosis or lipid peroxidation as sign of ferroptosis.

Results

Differentially expressed genes identified between primary and relapsed tumors were enriched in pathways involving the extracellular matrix (ECM) and ERBB signaling, especially ERBB4. Combining afatinib with VCR overcame resistance in VCR resistant BE(2)-C cells. Inhibition of the individual downstream pathways (PI3K, MAPK, SRC) of ERBB4 signaling was less efficient in breaking VCR resistance. Moreover, analysis of downstream signaling revealed no effect of afatinib treatment on the PI3K/AKT- and the MAPK-pathways. SRC phosphorylation in resistant BE(2)-C was significantly decreased compared to the control cells. For the elucidation of cell death mechanism, so far, a panel of high-risk neuroblastoma cell lines has been tested for apoptosis, which shows significant differences in caspase activation between different cell lines.

Summary/Conclusions

We identified pathways of the ECM and ERBB4 signaling to be associated with relapsed, therapy resistant neuroblastoma. Inhibition of ERBB4 with afatinib breaks resistance in VCR resistant BE(2)-C cells, which involves the SRC pathway. Thus, we identified novel vulnerabilities in relapsed pediatric tumors, which might enable the efficient elimination of treatment-resistant cells through pathway targeted therapy.

Type Translational

Presentation Preference Traditional poster

Abstract nr. 168

Analyzing circulating exosomal DNA in high-risk neuroblastoma

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Tumors release into the blood the exosomes, small vesicles that reflect the state of tumor cells and contain double-stranded DNA (exo-DNA) that can be used as a biomarker of disease, and for the detection of resistant clones leading to relapse occurrence. However, the ability of the exo-DNA to reflect mutational status of the parental tumor cells in neuroblastoma patients is largely unknown.

Aims

We focused on exo-DNA from neuroblastoma exosomes, and its potential as specimen for screening somatic mutations present in the parental tumor cells. Characterization of mutations of the exo-DNA from high-risk neuroblastoma patients at diagnosis, during treatment and possibly at relapse could serve as a promising noninvasive biomarker for clinical genomic treatment. Exo-DNA analysis will allow us: 1) to identify the genetic mutations associated with high-risk neuroblastoma, and 2) to choose the most suitable therapy in consideration of the dozens of FDA-approved oncology drugs that base the indications on the genomic profile of the tumor cells.

Methods/Materials

We isolated exosomes from plasma samples of 23 high-risk neuroblastoma patients including onset and relapse phases. Plasma was stored at -80°C or used immediately for exosomes isolation. Exosomes were isolated with the Exo-RNeasy serum/plasma midi kit (QIAGEN). Exosomes were lysed with Triton X-100 (4%), SDS (10%) and Buffer AL, and exo-DNA was extracted using the QIAamp MinElute ccfDNA kit (QIAGEN). Exo-DNA quantity and quality were assessed with 1x dsDNA HS assay on Qubit 4.0 fluorometer and with high sensitivity DNA assay on Agilent Bioanalyzer, respectively. Illumina libraries were prepared with KAPA Hyper Prep and

captured with Twist Human Core Exome + RefSeq 2. Whole exome sequencing was performed using an Illumina NovaSeq 6000 system: 300x theoretical coverage, in 150PE (33-52 M fragments per sample). All sequenced fragments were mapped to the reference genome GRCh38 using BWA-mem. After duplicates removal, Mutect2 was used for somatic variant calling using the peripheral blood sample as germline control. Annotation and prioritization of the identified variants was performed using Varseq (GoldenHelix).

Results

The novel finding is consistent with the presence of double-stranded DNA in the exosomes from neuroblastoma cells. The results of whole-exome sequencing demonstrated that the exo-DNA shared the same mutations in the most frequently mutated genes present in the parental neuroblastoma cells. Our results revealed that the entire exome is covered by the exo-DNA in an unbiased manner.

Summary/Conclusions

We showed that the plasma derived exo-DNA in high-risk neuroblastoma patients was highly consistent with the paired tumor genome profile, and covered all chromosomes. Our findings provide the first evidence of the presence of circulating exosomes than reflect the mutation status of neuroblastoma cells. The circulating neuroblastoma exo-DNA can be used as a noninvasive somatic mutation screens for the diagnosis when tissue biopsy is not feasible, for the detection of resistant clones leading to relapse occurrence, and for the set-up of a personalized treatment.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 169

Apoptosis, but not necroptosis is induced by specific aurora A kinase inhibitors in IMR-32 and CHP-134 neuroblastoma cells.

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

Aurora A kinase is highly expressed in multiple human tumors, including neuroblastoma. Previously we have shown that the specific, small-molecule inhibitor of aurora A kinase, MK-5108, combined with the GD2 ganglioside-specific 14G2a mAb significantly enhances cytotoxic effect against IMR-32 and CHP-134 cells [Horwacik et al., 2013, Durbas et al., 2018]. However, molecular mechanism of aurora A kinase inhibition by MK-5108 in neuroblastoma cells needs to be better characterised.

Aims

We investigated the cytotoxic potential of MK-5108 in the regulation of apoptosis or necroptosis in neuroblastoma cells.

Methods/Materials

To assess the response of neuroblastoma cells to small-molecule inhibitors we measured expression and phosphorylation of various proteins by western blot and cellular ATP levels by luminescence assays.

Results

MK-5108 treatment induced the expression of crucial apoptosis markers *i.e.,* cleaved caspase-3 and cleaved-PARP proteins at 72 h in IMR-32 and CHP-134 cells, as assessed by western blot. Additionally, the activities of caspase 3 and 7 were increased in MK-5108-treated IMR-32 and CHP-134 cells, confirming the induction of apoptosis in these neuroblastoma cultures. To investigate the involvement of necroptosis in MK-5108 treatment, we assessed the expression and

phosphorylation of key necroptosis-associated proteins *i.e.,* receptor-interacting serine/threonine-protein kinases (RIPs) and mixed lineage kinase domain-like pseudokinase (MLKL). We observed the elevated phosphorylation of RIP3 and significantly increased phosphorylation levels of MLKL in IMR-32 cells. Although, in CHP-134, the phosphorylation level of RIP1 and RIP3 were markedly increased, there was no increase of MLKL phosphorylated form. The expression level of unphosphorylated RIP1 and RIP3 kinases remained unchanged both in IMR-32 and CHP-134. To verify the involvement of necroptosis in MK-5108-stimulated cell death, we used specific inhibitors of RIP1 and RIP3 kinases *i.e.*, necrostatin-1 and GSK'872, respectively. No recovery of cytotoxic effect, as assessed by ATP level, was observed in MK-5108 and necroptosis inhibitors-co-treated cells, as compared to MK-5108 used alone, proving a lack of contribution of necroptosis in the observed cytotoxic effect. Interestingly, inhibition of RIP1 and RIP3 kinases combined with using MK-5108 inhibitor decreased ATP level, advancing the observed cytotoxic effect, as compared to monotherapy with MK-5108 inhibitor. Moreover, the combination of MK-5108 inhibitor and necrosatin-1/GSK'872 significantly increased the activities of caspase 3 and 7, proving better efficacy of the proposed combination therapy. We report that similar results were also obtained for another aurora A kinase inhibitor, MK-8745, which caused increased expression of key apoptosis-associated molecules and increased phosphorylation of RIP3 in both cell lines.

Summary/Conclusions

These studies demonstrate that pharmacological inhibition of aurora A kinase by two small molecule inhibitors tested by us, MK-5108 and MK-8745, causes cell death by apoptosis, but not necroptosis. Additionaly, inhibitors of RIP kinases might potentiate apoptosis evoked by aurora A kinase inhibitors.

Type Basic

Presentation Preference Traditional poster

Abstract nr. 170

Contribution of TWIST1 in the aggressiveness of neuroblastoma by modulation of the tumor-stroma crosstalk

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Reprogrammed stromal cells play key role in regulating several hallmarks of cancer, notably tumor growth, invasion, epithelial-to-mesenchymal transition and metastasis. Reactivation of the embryonic transcription factor TWIST1 is frequent in a wide range of tumors, where it acts as a multifunctional oncogene. Previous data from our lab highlighted an elevated expression of TWIST1 in neuroblastoma (NB) of poor prognosis as well as in metastasis.

Aims

Our aim was to investigate the contribution of TWIST1 on NB tumor formation and dissemination.

Methods/Materials

TWIST1 knock-out (KO) in the SK-N-Be2C cell line was performed by CRISPR/Cas9 technology. An *in vivo* model was obtained by orthotopic implantations of NB cells in the adrenal glands of athymic Swiss nude mice. Transcriptomic analyses were carried out by RNA-seq (Illumina). Histological analyses were conducted on tumors sections following Hematoxylin-Eosin, Gomori and Masson's Trichrome stains. Lungs metastases were detected through anti-mCherry

immunohistochemistry. Secretome analysis was performed by LC-MS/MS and data processed by the Perseus module of the MaxQuant software.

Results

Upon orthotopic injection of SK-N-Be2C TWIST1-control or TWIST1-KO, a delay in both tumor take (22.7days) and tumor growth (13.25 days) was observed in the TWIST1-KO group. Besides, a significant increase in the number of pulmonary macro-metastases was detected in the TWIST1-positive group of mice. Histological tumor analyses highlighted a phenotype of aggressive and invasive tumors in the TWIST1-positive group, characterized by: spindle-shaped cells with fascicular organization; more immune cells infiltration; a major disruption of the Extra Cellular Matrix (ECM) reticulin mesh; as well as reduced cell cohesion and differentiation. Gene ontology enrichment analysis of tumors revealed that, among the pathways mainly deregulated, several were linked to the ECM organization, stroma remodeling, cell adhesion and migration. This result was also confirmed *in vitro* at the protein level by the analyses of cells secretome profile. The tumor transcriptome displayed a subset of differentially expressed genes (chemokines, cytokines, growth factors, interleukins and integrins). We referred to them as “TWIST1-associated paracrine signature” for they are known to be involved in the paracrine communication between cells and in the activation of resident stromal cells. This signature was also partially confirmed in the cell secretome of TWIST1-positive cells (e.g. TGFB1, HGF, EGFL7, VGF and CXCL12). Furthermore, tumor stroma RNA-sequencing revealed in the TWIST1-postive group an enriched cluster of genes related to muscle tissue. We speculated that such gene signature could be linked to the presence of myofibroblasts, stromal cells becoming activated and specialized for ECM secretion and contraction. Finally, STRING analyses of interactions between the two aforementioned gene signatures showed a significant enrichment (PPI enrichment p-value: < 1.0e-16), indicative of their functional interconnection. Of note, despite its higher level of protein expression in the TWIST1-KO tumors, MYCN protein alone was not able to sustain an activated tumor micro environment phenotype and ECM remodeling.

Summary/Conclusions

Taken together, our results suggest a TWIST1-mediated enhancement of tumor growth and pulmonary macro-metastasis development, as well as the activation of a transcriptional program enabling the tumor cells vs tumor stroma cross-talk.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 171

Use of the genetic basis of Beckwith-Wiedemann to reveal the genetic variation associated with the development of sporadic neuroblastoma

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

Beckwith–Wiedemann syndrome (BWS) is an overgrowth disorder characterized by perinatal overgrowth, macroglossia, exomphalos, hemihyperplasia, and postnatal hypoglycemia, and associated with an increased risk to develop embryonic tumors like neuroblastoma (NB). Patients with BWS who develop NB, in 2,8% of cases have mutations in *CDKN1C*, a well-known tumor suppressor. Thus we speculated that common genetic variants affecting *CDKN1C* could be associated with risk of NB development.

Aims

Our aim was to identify single nucleotide polymorphisms (SNPs) in *CDKN1C* predisposing to NB.

Methods/Materials

We performed genotype imputation of the *CDKN1C* locus using GWAS data from 2101 NB cases and 4202 controls of European ancestry. The variants were filtered based on the allelic frequency (MAF> 5% classified as "high-frequency (HF)"; 1%> MAF >= 5% classified as "low-frequency (LF)") and the statistical significance ($p<0.05$). A replication study was conducted on 455 cases and 969 controls of Italian origin.

Results

After imputation, we identified 313 SNPs in the *CDKN1C* gene locus. We selected 231 HF SNPs and 66 LF SNPs. Between these, only 38 HF variants and 3 LF variants have $p<0.05$. Among HF and LF SNPs, rs12293538 and rs143992355 were the most significant, respectively ($p = 1.77 \times 10^{-4}$, OR: 1.16; $p = 0.02$, OR: 1.28). We have validated the association of rs12293538 in the Italian

population (p = 0.077, OR: 1.16). Using public ChIP-seq data, we observed that the SNP rs12293538 falls in a region characterized by H3K27Ac in the SKNBE cell lines while the SNP rs143992355 falls in a region characterized by both H3K27Ac and H3K4me3 in the SKNBE2C. The analysis of gene expression microarrays showed that *CDKN1C* gene seems to function as a tumor suppressor; in fact, its expression is higher in 18 NB cell lines when compared to 6 healthy tissues ($p = 7.15x10^{-15}$),in 46 relapsed NBs when compared to 56 non-relapsed NBs (p = 0.02). Moreover, Kaplan-Maier analysis with patients grouped by the optimal cut-off (calculated using R2 web tool) of *CDKN1C* expression for overall survival shows that higher *CDKN1C* expression is significantly associated with higher probability of survival in NB patients in different datasets.

Summary/Conclusions

Using the genetic predisposition of BWS patients to develop NB, we identified rare and common variants in the *CDKN1C* tumor suppressor gene, located in DNA regulatory region and associated with the risk of developing NB. Future objectives include the functional study of these variants and the investigation of biological role of *CDKN1C* in NB development.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 172

Targeting transcription-replication conflicts in MYCN-driven neuroblastoma

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

Targeting MYC proteins or their function remains one of the major challenges in tumor biology.MYC proteins are transcription factors that bind globally to active promoters and promote transcriptional elongation by RNA polymerase II (RNAPII). In most cancers MYC proteins are dysregulated suggesting that cancer cells need mechanism to avoid transcriptional stress. To identify effector proteins that mediate this function, we performed mass spectrometry on MYCN complexes in neuroblastoma cells. We identified several new interactors of MYCN and showed that Aurora-A competes with binding of coactivators to MYCN during S phase. This complex switch is blocking MYCN-dependent release of RNAPII from the promoter resulting in a global repression of transcriptional elongation during S phase. Inhibition of Aurora-A activates ATR, indicating that dysregulation of transcription during S phase impairs replication fork progression. We concluded that interaction of MYCN with Aurora-A is important to avoid transcription-replication conflicts opening the possibility for new treatment options (Büchel et al., 2017).

Aims

We further want to investigate the interaction of Aurora-A with MYCN and propose that there is a synergism between Aurora-A and ATR inhibition in MYCN-driven neuroblastomas. We aim to decipher the molecular mechanism underlying the combination therapy and use this information to further improve strategies for targeting *MYCN*-amplified neuroblastoma.

Methods/Materials

Results

Association of Aurora-A with MYCN activates its kinase activity. By inhibiting Aurora-A with MLN8237 H3S10 phosphorylation, a marker for histone compaction, is reduced in S and early G2 phase. In parallel we saw an increase of R-loop formation. R-loop formation delays pause release of RNAPII resulting in ATR activation. Combining MLN8237 with AZD6738, an ATR inhibitor, lead to elevated phosphorylation of g-H2AX and of KAP1. From this experiments we concluded that inhibition of Aurora-A enhances the dependence of MYCN-amplified cells on the ATR kinase. Using the combination of an Aurora-A and an ATR inhibitor in vivo showed a robust tumor regression. Two out of eight TH-MYCN mice treated remained tumor-free until the end of the trial. Already 24 hours after starting the therapy tumors presented with architectural disintegration, expansion of hemorrhage and an increase of tumor cells with fragmented nuclei. We saw a decrease in pH3S10 spots as well as an increase in R-loops and in phosphorylation of g-H2AX and of KAP1. As a consequence we detected more apoptosis marked by cleaved caspase 3.
To test the therapy in human tumor samples, we treated patient-derived xenografts (PDX) of neuroblastoma. While treatment responses partly mirrored the response of TH-MYCN mice the reaction was much weaker suggesting an involvement of the immune system. Indeed GSEA showed that the downregulation of MYCN target genes was paralleled by an induction of multiple genes involved in cytokine signaling. Additionally immunohistochemistry of tumors from TH-MYCN model documented the activation of cGAS/STING in parallel with infiltration of immune cells.

Summary/Conclusions

Our data show that Aurora-A builds complexes with MYCN to prevent conflicts of transcription with DNA replication forks. Treatments of different mouse models show that a combination treatment of Aurora-A inhibitors and ATR inhibitors is beneficial. Concluding that targeting this mechanism is an effective therapy for MYCN driven neuroblastoma.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 173

Quantitative and semiquantitative assessment of 123I-MIBG uptake in prediction of unfavorable histopathology in patients with neuroblastic tumors

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

^{123}I-metaiodobenzylguanidine (^{123}I-MIBG) scintigraphy is widely used for the detection and staging of neuroblastoma. The intensity of ^{123}I-MIBG uptake in the primary neuroblastic tumors can vary significantly in same types of neuroblastic tumors as well as in different histological variants. Only visual assessment of ^{123}I-MIBG uptake can be qualitative, but not quantitative. Fortunately, with the development of SPECT/CT and new software in recent years the semiquantitative (TLCRR – time to liver count rate ratio) and quantitative (SUV_{max} – maximum standardized uptake value) assessment of ^{123}I-MIBG uptake in primary tumors is possible.

Aims

Quantitative and semiquantitative assessment of ^{123}I-MIBG uptake in prediction of unfavorable histopathology in patients with neuroblastic tumors.

Methods/Materials

105 ^{123}I-MIBG-possitive patients were included in this retrospective study. ^{123}I-MIBG-scintigraphy including whole body planar images and SPECT/CT were performed for all patients before any type of treatment. Semiquantitative (TLCRR) and quantitative (SUV_{max}) assessment of ^{123}I-MIBG uptake were calculated and analyzed for all patients.

Results

Out of 105 patients: 60 patients were with neuroblastoma (NB), 28 with ganglioneuroblastoma

(GNB) and 17 with ganglioneuroma (GN). The values of SUVmax and TLCRR were significantly higher in neuroblastoma group than ganglioneuroma. The average values of SUVmax and TLCRR for NB were 7.5 and 5.6, for GNB - 3.1 and 2.58 and for GN - 1.7 and 1.3, respectively. Multiple statistical analysis were used including ROC curve for quality assessment of different models. AUC values for SUV_{max}, TLCRR and NSE were 0.923, 0.895, 0.858 in NB patients; 0.826, 0.735, 0.762 in GNB patients and 0.926, 0.898, 0.924 in GN patients respectively.

Summary/Conclusions

SPECT/CT in combination with modern software allows quantitative assessment of ^{123}I-MIBG uptake in neuroblastic tumor. The SUV_{max} and TLCRR can be used separately as well as in combination with NSE for prediction of histological variant. Our study demonstrates that quantitative assessment (SUV_{max}) of ^{123}I-MIBG uptake alone as well as in combination with other parameters is more specific. Moreover, the quantitative assessment (SUV_{max}) of ^{123}I-MIBG uptake is more simple method and it minimizes the possible error in patients with diffuse liver involvement.

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Type Clinical

Presentation Preference Oral presentation

Abstract nr. 174

MYCN mRNA as a post-transcriptional regulator of RNA transcripts via microRNAs in High-Risk Neuroblastoma

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Topic Gene regulatory networks and epigenetics: from basic science to therapy

Background/Introduction

MicroRNAs (miRNAs) are small RNA sequences which downregulate gene expression through targeting complementary sequences in mRNA 3'-untranslated region (UTR), known as miRNA response elements (MRE). miRNAs can regulate multiple mRNAs; whereas a single mRNA transcript can be regulated by multiple miRNAs. Competition for shared miRNAs between protein-coding and noncoding transcripts, known as "competing endogenous RNA" (ceRNA), represents a mechanism of gene regulation. ceRNAs act as a miRNA sponge or decoy for common miRNAs. By altering the pool of miRNAs, ceRNAs affect expression levels of other mRNAs. Aberrant expression of specific miRNAs have been reported to actively contribute to neuroblastoma (NB) pathogenesis.

In NB, the amplification of the oncogene *MYCN* is associated with high-risk disease. Interestingly, *MYCN* mRNA 3′-UTR contains MRE highly conserved among vertebrates, suggesting strong functional relevance. *MYCN* mRNA can thus function as a sponge that regulates other mRNAs *via* shared miRNAs, acting as ceRNA. In *MYCN* amplified tumors, the increase of mRNA abundance may affect ceRNA networks and consequently alter the transcriptional output of key genes. The effect of concomitant deregulated levels of multiple miRNAs on gene regulatory processes in NB has not been studied.

Aims

This study aimed to explore *MYCN* mRNA as a post-transcriptional regulator of distinct RNA transcripts *via* shared pool of miRNAs, and characterize the ceRNA network associated with *MYCN* amplification.

Methods/Materials

We generated and analyzed genome-wide miRNA and gene expression microarray data (Affymetrix GeneChip miRNA4.0 and Affymetrix U219; respectively) from twenty primary NB tumors. *MYCN* status was characterized using qPCR or/and SNP arrays (Affymetrix CytoscanHD). Clinical and biological data was available for all NB included in the study. Bioinformatic tools and publicly available databases such as TargetScan were used to examine mRNA/miRNA interactions. NB cell lines, patient-derived xenografts (PDXs) and primary NB tumors were used to validate the ceRNA network by real-time qPCR.

Results

Unsupervised analysis of the miRNA microarray data revealed differential profiles among NB clinical risk groups. These miRNA expression profiles were found to be significantly associated with *MYCN* amplification status. We developed and applied an R-based bioinformatic tool that performs correlation expression analyses between miRNA and RNA transcripts. The analysis identified a potential ceRNA network associated with *MYCN* amplification. After data filtering, the most promising *MYCN* targets and their associated miRNAs were validated in NB cell lines, in PDX and in a set of independent primary NB tumors. The results enabled us to identify a network of ceRNAs that affect biological pathways that contribute to the aggressive phenotype of the disease.

Summary/Conclusions

High levels of *MYCN* mRNA act as a miRNA sponge deregulating the expression of several ceRNAs that compete for common miRNAs. These findings could provide a potential functional explanation of how genes with no previously known direct interactions are potentially interlinked and identify interactions between relevant signaling pathways.

Type Basic

Presentation Preference Traditional poster

Abstract nr. 175

Identification of a minimal region of loss on chromosome 6q27 associated with poor survival of high-risk neuroblastoma patients

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Patients with high-risk neuroblastoma initially respond to therapy, but afterwards up to 60% of these become resistant and disease recurred. Neuroblastoma is the example of how specific chromosome rearrangements can influence the trend of the disease. Currently, whole-genome analysis revealed large genomic DNA copy-number alterations (loss of chromosomes 1p, 3p, 4p, 11q and gain of 1q, 2p, 17q) that are of prognostic significance for newly diagnosed neuroblastoma patients. Unfortunately, it does not exist one or more specific chromosome defect associated with relapse or refractory high-risk neuroblastoma.

Aims

Previous studies have delineated a 7 Mb region at distal 6q27 loss in high-risk neuroblastoma patients with extremely poor survival outcome. A complete genomic analysis of the distal 6q region would help identifying the putative neuroblastoma tumor suppressor gene(s).

Methods/Materials

We recovered 15 primary untreated high-risk neuroblastoma patients diagnosed by the Italian Association of Italian Pediatric Hematology and Oncology (AIEOP). These patients harbored tumors with chromosome 6q loss. We determined both size and genetic content of these losses by high resolution array-CGH using Human Genome CGH Microarray 4x180K Kit (Agilent Technologies, CA, USA). We inquired for any association between *T*, *SFT2D1*, *RP56KA2*, *FGFR1OP* and *UNC93A* gene expression and high-risk neuroblastoma patients survival using two large neuroblastoma tumor datasets with available survival endpoints via the R2 Genomic Analysis and Visualization Platform (http://r2.amc.nl; SEQC n=498 and TARGET-Asgharzadeh n=249).

Results

Array-CGH disclosed multiple segmental chromosome gains or losses in the genomic profile of 15 neuroblastoma samples analyzed. Partial chromosome losses were most often detected for chromosome arms 1p, 3p, 4p, 11q, and partial chromosome gains for 1q, 2p, 7q, 17q, these abnormalities were associated with grim patients survival outcome. All neuroblastoma samples harbored 6q loss: 7 cases showed distal loss, 6 had interstitial loss, and 2 carried both distal and interstitial losses. Of interest, we defined a minimal common region of genomic loss in distal 6q by a sample displaying a small loss spanning an area of 4.68 Mb. The 6q27 region was deleted from oligomer A_14_P103168 (166.203.071 bp) (first deleted) to oligomer A_14_P120743 (170.890.108 bp) (last deleted). Among the cases carried distal 6q loss one sample showed a gain adjacent to the 6q loss. In particular, this sample carried in 6q27 region a focal gain from oligomer A_14_P200779 (168303033 bp) (first gained) to oligomer A_14_P120743 (170890108 bp) (last gained). So, by subtraction we can define a critical Smallest Region of Overlap (SRO) on chromosome 6q27 spanning an area of 2.09 Mb. The minimal common region of loss harbored the five genes *T*, *SFT2D1*, *RPS6KA2*, *FGFR1OP*, and *UNC93A*. We found that low *SFT2D1*, *RPS6KA2* and *FGFR1OP* gene expression predicted poor outcome in high-risk neuroblastoma patients.

Summary/Conclusions

We hypothesize that the genes contained in the commonly loss region are dosage-sensitive, and that the haploinsufficiency of one or more of these genes is associated with extremely poor survival outcome of high-risk neuroblastoma.

Type Translational

Presentation Preference Traditional poster

Abstract nr. 176

Aurora-A regulates phosphorylation of Histone 3 S10 in S phase to prevent Transcription-Replication conflicts.

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Members of the Aurora kinase family have important function in cell division and in the maintenance of genome stability. The functions of Aurora kinases are particularly well understood in mitosis (Marumoto et al., 2005). Aurora-A is known to stabilize MYCN in neuroblastoma cells and thereby preventing proteasomal degradation. Aurora-A is known to stabilize MYCN in neuroblastoma cells and thereby preventing proteasomal degradation (Otto et al., 2009). MYCN, which belongs to the proto-oncogene MYC family of transcription factors, is often amplified in neuroblastoma. *MYCN* amplification represent a marker of poor prognosis and less overall survival in neuroblastoma patients.
Recently, our laboratory could show that MYCN and Aurora-A mainly interact during S phase of the cell cycle (Büchel et al., 2017) but the function of this complex remains open.

Aims

Methods/Materials

Results

For our approach cells were synchronized in S phase and fractionated. Hereby, we identified that MYCN as well as Aurora-A are mainly bound to chromatin. We were able to show that the association of Aurora-A with chromatin is cell cycle dependent and its association in S phase is MYCN dependent.
It is known that Aurora-A is a kinase for Histone 3 Ser10 (H3S10) phosphorylation (Hsu et al., 2000). We identified that H3S10 is phosphorylated by Aurora-A only in S and to a lesser degree in G2 phase. Chromatin compaction marked by phosphorylation of H3S10 counteracts the formation of transcription-dependent R-loops (Castellano-Pozo et al., 2013). Consistently, inhibition of Aurora-A, by using MLN8237, led to a large increase in formation of RNA/DNA-hybrids.

Aurora-A prevents global stalling of elongating RNA Polymerase II (RNAPII) at the pause site and at exon/intron boundaries and promotes mRNA splicing. Additionally, we show that an extensive number of splicing related phosphosites are indirectly phosphorylated by Aurora-A in S phase. Our data suggest that inhibition of Aurora-A increases transcription-replication conflicts.
Those conflicts lead to the activation of the Ataxia telangiectasia and Rad3 related (ATR) kinase and stalling of RNAPII impede replication fork progression. Simultaneous blockade of Aurora-A and ATR kinases induces double-strand break formation and apoptosis.

Summary/Conclusions

We were able to show that Aurora-A is bound to chromatin in S phase where it is required to phosphorylate H3S10 to prevent transcription-replication conflicts.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 177

BILATERAL ADRENAL NEUROBLASTOMA: CLINICAL AND BIOLOGICAL CHARACTERISTICS

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

In 30-40% of cases neuroblastoma (NB) is localized in the adrenal gland. Bilateral adrenal involvement is extremely rare.

Aims

We aimed to compare clinical and biological characteristics of unilateral and bilateral adrenal NB.

Methods/Materials

The study included 285 patients with adrenal NB, ganglioneuroblastoma (GNB) and ganglioneuroma (GN) who received the treatment at Dmitry Rogachev NMRC PHOI from 01.2012 to 04.2019. The diagnosis was established based on the international criteria. The bilateral

adrenal involvement was confirmed by different imaging modalities. Bilateral adrenal biopsy was done in selected cases. Patients were stratified and therapy was carried out according to the modified German NB2004 protocol.

Results

Of the 285 patients with peripheral neuroblastic adrenal tumors, 253 (88.8%) had unilateral and 32 (11.2%) had bilateral lesions. Patients with bilateral adrenal tumors were younger (1.7 months versus 17.6 months, $p<0.001$). Nine of 253 (3.6%) patients with unilateral adrenal gland lesions were diagnosed GN. The analysis of patients with malignant peripheral neuroblastic tumors (NB and GNB) showed that metastatic forms of the disease (4 and 4S stages) occurred more often in bilateral cases (27/32 (84%) vs. 113/244 (46%) in unilateral cases ($p=0.001$). Stage 4S were diagnosed more frequently in bilateral NB (19/32 (59.4%) vs. 34/244 (13.9%) in unilateral, $p=0,00001$). Metastatic pattern correlated with the laterality of the adrenal involvement. Bone (62/113 (54.9%), $p=0.0007$) and distant lymph node metastases (34/113 (30.1%), $p=0.01$) were significantly more often observed in unilateral diseases. Opposite, skin (3/27 (11.1%), $p=0.02$) and liver (26/27 (96.3%), $p= 0.0001$) metastases were more common in bilateral cases. There was no statistical difference in the incidence of *MYCN* amplification and 1p deletion. Also, there was no statistical difference in the distribution to the risk groups between unilateral and bilateral cases. 3-year event-free survival (EFS) of patients with bilateral adrenal NB was 84% (95% confidence interval (CI) 71.8-97.0), compared with 69% (95% CI 63.0-75.6) of patients with unilateral adrenal NB ($p=0.12$). 3-year overall survival (OS) of patients with bilateral adrenal NB was 96% (95% CI 90.8-100.0), compared with 81% (95% CI 75.4-86.5) of patients with unilateral adrenal NB ($p=0.03$).

Summary/Conclusions

Bilateral adrenal NB is more common in children during the first months of life. There were no cases with GN and bilateral adrenal involvement. Bilateral adrenal NB more likely to have distant metastasis corresponding to stage 4S disease. Lack of the differences in the frequency of occurrence of unfavorable cytogenetic markers was most likely due to the small number of patients with bilateral NB. OS was higher in the group of patients with bilateral adrenal NB in comparison to unilateral cases.

Type Clinical

Presentation Preference Rapid Fire Poster Presentation

Abstract nr. 178

A detailed look into high-risk neuroblastoma tumor heterogeneity using single-cell RNA sequencing

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

Recent studies in various cancer entities have revealed remarkable genetic heterogeneity in primary tumors. In neuroblastoma, minor *ALK* mutated subclones were observed at diagnosis that were present in all tumor cells at relapse. Furthermore. evidence is emerging that neuroblastoma cells can switch gene regulatory circuits to alter cell identity from adrenergic to neural crest-like under therapeutic pressure. A better understanding of the cellular heterogeneity of aggressive neuroblastoma tumors would provide deeper insights into the emergence of therapy resistance in neuroblastoma patients with aggressive disease and guide novel therapeutic interventions towards reducing relapse risks.

Aims

We aimed to develop and implement an analytical and bioinformatic pipeline for accurate single-cell RNA sequencing of neuroblastoma tumors in order to gain deeper insights into the cellular heterogeneity present in high-risk neuroblastoma tumors. As long-term aim, we anticipate to use single cell data to dissect the complex tumor tissues into specific cell states with their own regulatory networks and to study the dynamics of gene regulation during therapy induced cell state transitions (e.g. adrenergic to neural-crest like mesenchymal cell states).

Methods/Materials

We developed a protocol for live cell cryo-preservation of Tru-Cut biopsies and surgically dissected neuroblastoma tumors for single cell RNA-seq. In addition, we have set up a pipeline for life-dead cell staining and sorting of unthawed samples and subsequent single cell transcriptome profiling using the 10X Genomics technology.

Results

We have successfully generated single-cell suspensions and single cell RNA-sequencing libraries from 5 high-stage neuroblastoma tumors (1 *MYCN* single copy and 4 *MYCN* amplified cases). Our initial data analyses point at the validity of the generated data sets to shed further light on the heterogeneity of neuroblastoma tumors. For example, for the first sample, we generated RNA sequencing data of more than 8500 cells for 2000 genes per cell on average (26K reads/cell). Projection of cell-type specific genes on cells clustered using UMAP and/or tSNE reveals the presence of fibroblasts as well as infiltrating immune-cells (e.g. CD8+ T-cells). Among the *MYCN* positive cells, we recognize several cell clusters including a small cluster of cells that are highly positive for embryonal stem cell and DNA repair signature genes marked by high *FOXM1*, *RRM2* and *MYBL2* expression and high *MKI-67* expression indicative for a highly proliferative subpopulation of cells. We are currently also comparing the human single cell RNA sequencing tumor data with those obtained from zebrafish *MYCN* driven tumors in a cross-species data mining effort.

Summary/Conclusions

We have successfully introduced a protocol for sample preparation for single-cell RNA sequencing of primary neuroblastoma tumor samples of high-risk patients and observed first insights into tumor heterogeneity and microenvironment.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 179

Diagnostic significance of SPECT/CT in addition to planar 123I-MIBG-scintigraphy in patients with neuroblastoma

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Topic Other

Background/Introduction

Neuroblastoma is the most common extracranial solid tumor in children, which accounts for 8% of all pediatric malignancies. Metaiodobenzylguanidine radiolabeled with ^{123}I (^{123}I-MIBG) offers high-quality images and is widely used in the evaluation of patients with neuroblastoma. Unfortunately, the assessment of conventional planar ^{123}I-MIBG images presents some difficulties due to the limited resolution of planar images and the lack of anatomical localization, which leads to the appearance of both false-negative as well as false-positive results. In recent years, hybrid imaging has played an important role in radionuclide imaging. Single photon emission tomography/computed tomography (SPECT/CT) provides both functional and anatomical information. SPECT/CT in addition to planar scintigraphy increases the imaging time which may lead to increased sedation in most of the patients.

Aims

To determine the diagnostic significance of the SPECT/CT protocol in addition to planar ^{123}I-MIBG-scintigraphy in patients with neuroblastoma.

Methods/Materials

251 patients (118 boys, 133 girls, aged from 0 to 17 years) were included in this retrospective study. ^{123}I-MIBG-scintigraphy including whole body planner images and SPECT/CT of region of interest were performed for all patients using standard protocol. Out of the 251 patients 204 were primary without any type of treatment and 47 - after surgical removal of the primary tumor but without any type of chemotherapy.

Results

Out of 204 primary patients, 149 results of planar images and SPECT/CT were congruent, however, in 55 cases SPECT/CT played an important role to rule out either false-negative (N=42) or false-positive results (N=13).
Out of 47 postoperative cases the results of planar images and SPECT/CT were congruent in 32 cases, but in 15 cases SPECT/CT ruled out either false-negative (N=3) or false-positive results (N=12).

Summary/Conclusions

Our study clearly demonstrates that SPECT/CT protocol play an important role in ^{123}I-MIBG-scintigraphy and could significantly impact the patient's management. SPECT/CT provided additional diagnostic information in more than 26% of cases at the time of initial study and more than 30% in postoperative cases.

Diagnostic significance of SPECT/CT in addition to planar ^{123}I-MIBG-scintigraphy in patients with neuroblastoma

Shamanskaya

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Type Clinical

Presentation Preference Oral presentation

Abstract nr. 180

The antibody-drug conjugate D3-GPC2-PBD induces immunogenic cell death and triggers antitumor immune responses in preclinical neuroblastoma models

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

GPC2 is a cell surface MYCN-driven immunotherapeutic target in high-risk neuroblastoma (NB). We have previously demonstrated potent antitumor effects of a GPC2-directed antibody drug conjugate (ADC) bearing PBD dimers (D3-GPC2-PBD) in neuroblastoma patient-derived xenografts; however, their tumor immunomodulatory properties remain undefined.

Aims

We aim to study whether the D3-GPC2-PBD ADC induces immunogenic cell death (ICD) in NB cells and triggers an antitumor immune response in immunocompetent NB murine models.

Methods/Materials

Induction of ICD was evaluated by measuring calreticulin (CALR) membrane translocation by flow cytometry and high mobility group box 1 (HMGB1) secretion by ELISA in 2 human NB cell lines (SMS-SAN and NB-EBC1), 2 Gpc2 isogenic murine NB cell lines (NXS2-Gpc2 and 9464D-Gpc2) and a Gpc2 isogenic model melanoma cell line (B78-Gpc2) treated with different concentrations of D3-GPC2-PBD (1.8-200 pM). Tumor cell phagocytosis was evaluated by flow cytometry after co-culturing human macrophages with GFP-labeled, ADC-treated NB-EBC1 cells with and without dinutuximab (1 µg/mL). *In vivo* tumor growth and antitumor immune responses were studied after vaccination of A/J mice with ADC-pretreated NXS2-Gpc2 cells followed by tumor re-challenge with naïve NXS2-Gpc2 cells.

Results

D3-GPC2-PBD ADC treatment of murine Gpc2 isogenic cell lines induced potent cytotoxicity comparable to that observed in human neuroblastoma cell lines (n=3; mean IC_{50} = 115 pM). D3-GPC2-PBD treatment induced CALR membrane translocation in a concentration-dependent manner (maximum CRT fold-increase of 2.2-20 at 96 hours after treatment with 200 pM of D3-GPC2-PBD ADC). HMGB1 secretion also increased after D3-GPC2-PBD treatment in a dose-dependent manner, similarly achieving maximum secretion levels of 37-65 ng/mL at 96 h post 200 pM D3-GPC2-PBD ADC treatment. Across the murine NB Gpc2 isogenic cell lines, NXS2-Gpc2 cells showed the most robust biomarkers of ICD, which correlated with their prior reported tumor mutational burden (13.9 vs. 1.5 mutations per MB in the 9464D cell line). The percentage of macrophage engulfed NB cells was higher in ADC pretreated NB-EBC1 cells, a phenomenon that was further enhanced upon combination with dinutuximab treatment. Prophylactic subcutaneous vaccination with $2x10^6$ ADC pre-treated NXS2-Gpc2 cells prevented subcutaneous tumor re-challenge with $2x10^6$ viable NXS2-Gpc2 cells in 72% of animals, and significantly increased murine survival compared to control animals vaccinated with freeze-thawed cells (p=0.01). The NXS2-Gpc2 tumors that grew after vaccination showed increased $PD1^+$ T cell tumor infiltration compared to controls (n=3, p=0.04), postulating T cell exhaustion as a potential mechanism of tumor recurrence. Systemic isolated $CD8^+$ T cells from vaccinated animals underwent potent activation upon *ex-vivo* co-culture with native NXS2-Gpc2 cells. Percentage of $CD137^+$ and $CD69^+$ cytotoxic T cells increased 1.3 and 2.4-fold in ADC-vaccinated animals compared to controls, further supporting a vaccination-induced T-cell mediated antitumor immune response. Ongoing experiments are further exploring whether ADC-mediated vaccination also prevents disseminated tumor re-challenge in a NXS2-Gpc2 metastatic model.

Summary/Conclusions

The GPC2-directed antibody drug-conjugate D3-GPC2-PBD induces immunogenic cell death, enhances tumor cell phagocytosis and stimulates antitumor immune responses *in vivo* mediated by cytotoxic T cell activation. Ongoing studies are focused on elucidating the synergistic antitumor activity of the D3-GPC2-PBD ADC combined with anti-GD2 antibodies and/or immune checkpoint inhibitors.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 181

DNA methylation profiling of High Risk Neuroblastoma: potential epigenetic biomarkers for treatment response assessment.

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

High risk NB (HR-NB) represents a heterogeneous group of tumors, whereby patients can display response to treatment and long-term outcome or develop early progressive, chemoresistant disease with poor outcome (ultra-high risk; UHR-NB). However, most patients with HR-NB are treated uniformly without further stratification. Genetics underlying this aggressive subgroup is still greatly unknown and risk-stratification within HR-NB is challenging.

Aims

We explored DNA methylation (DNAme) changes present in HR-NB tumors at diagnosis, and identify (epi)genetic alterations potentially related with prognosis of patients and response to treatment. Thereby, identify risk-stratification biomarkers and potential therapeutic targets.

Methods/Materials

We analyzed previously published DNAme (Infinium HumanMethylation450 BeadChip) and gene expression (GE) datasets of 100 and 428 HR-NB samples, respectively. The study was restricted to patients aged >18 months at diagnosis with metastatic disease or/and *MYCN* amplified NB tumors. Median follow-up was of 5 years. DNAme data was annotated according to gene location, CpG islands and chromatin state categories. Discovery of potential biomarkers was performed using Cox regression models. Survival curves were estimated by Kaplan-Meier method and compared among groups by log-rank test. Pathway analysis was performed using KEGG Pathway Database. Bisulfite sequencing, pyrosequencing, quantitative RT-PCR and immunohistochemistry were used for validation purposes.

Results

Unsupervised analysis of DNAme data of HR-NB tumors revealed two distinct profiles with differences in clinical evolution. Survival analysis identified CpG sites strongly associated with outcome, that permitted to differentiate a subgroup of rapidly progressing, chemo-refractory tumors, identified as UHR-NB (median overall survival (OS) 33.23 months), and a subgroup with long-term survival (median OS 63.55 months). There was no statistical evidence of *MYCN* amplification enrichment associated to any of the two subgroups. Functional genomic analysis of the HR-NB subgroups unveiled CpG hypermethylation enrichment in UHR-NB tumors both at 5'-UTR and 3'-UTR regions and associated to CpG islands. These DNA methylation changes were located in regions flanking transcription start sites, polycomb repressed and enhancer chromatin state regions. Differentially methylated genes were associated with developmental pathways and metabolism processes.
Next, we performed a correlation analysis between differentially methylated and expressed genes comparing the two HR-NB subgroups using matching patient data. This analysis revealed differences in pathways related to metabolism and purine biosynthesis. Results were validated using an independent cohort of 329 NB samples.

Summary/Conclusions

Our findings show that the heterogeneous clinical behavior of HR-NB is associated with specific DNA methylation changes significantly associated with clinical evolution of tumors. We have identified a reduced set of methylation-based biomarkers that can identify UHR-NB tumors and thus enhance risk-stratification. This has enabled us to identify aberrantly activated biological pathways that could be relevant for risk-adapted therapeutic strategies.

Type Basic

Presentation Preference Traditional poster

Abstract nr. 182

41BB or CD28 driven disialoganglioside (GD2)-specific CAR-T, but not T-cell engaging bispecific antibody, induces fatal neurotoxicity in mice

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

Immunotherapy has become a powerful weapon in the fight to cure cancer. As a result of its high potency, however, safety has become a major focus. Ganglioside GD2 (GD2) is an established tumor associated antigen in neuroblastoma, melanoma, osteosarcoma and many soft tissue sarcomas, but is also be expressed on normal tissues such as the brain and peripheral nerves. Through advances in protein engineering T-cells can now be retargeted toward GD2 using bispecific antibodies (BsAb) or chimeric antigen-receptors (CAR). Several recent preclinical studies looking at GD2-specific CAR-T cells have observed strong and fatal neurotoxicity, warranting evaluation of how GD2-specific BsAb therapy compares.

Aims

The aim of this study were to evaluate the safety of a GD2-specific humanized IgG-[L]-scFv BsAb in comparison to two GD2-specific CAR modifications of human T cells (either CD28 or 41BB domains for costimulation). Specifically we aimed to compare how each of these T-cell retargeting strategies facilitate infiltration, activation and cytotoxicity in the central nervous system.

Methods/Materials

GD2-BsAb and GD2-CART cells based on hu3F8 (naxitamab) antibody sequences were

evaluated in multiple preclinical mouse models including immunodeficient BALB-*Rag2*$^{-/-}$ IL-2R-$\gamma c^{-/-}$ mice bearing human tumor xenografts and immunocompetent C57BL/6 mice bearing a huCD3 transgene. In addition, GD2-BsAb was evaluated in two separate GLP toxicity studies in preparation for its IND application (NCT03860207). CAR-T cells were prepared using lentivirus transduction and GD2-BsAb was prepared through GMP manufacturing.

Results

Both CD28 and 41BB GD2-specific CAR-T cells ($2x10^7$ iv per mouse 1x/week x3) displayed significant infiltration into brains and spines of mice bearing tumor or without tumor. This resulted in major late onset (28-35 days after treatment) neurotoxicities such as hind-limb paralysis, altered gait and death. In contrast, treatment with GD2-BsAb (10 ug/dose iv per mouse, 2x/week x 3) plus activated human T cells ($2x10^7$ iv per mouse 1x/week x 3) failed to elicit any neurotoxicity. In addition, no neurotoxicity was seen when mice were treated with therapeutic T-cells armed *ex vivo* with GD2-BsAb, or in control BsAb and control CAR-T treated mice. Treatment of huCD3-transgenic mice with GD2-BsAb (10 ug/dose iv per mouse, 2x/week x3) showed no signs of neurotoxicity. Extensive histological examination of murine CNS tissues in two GD2-BsAb GLP toxicity studies confirmed the absence of human or mouse T-cell infiltration into the into GD2(+) areas in brain or spine.

Summary/Conclusions

While T-cells are known to be able to cross the blood-brain barrier (BBB), our data suggests that there is an important difference between BsAb and CAR-T modalities when it comes to penetration into CNS. This study provides valuable insights for designing safe and potent immunotherapy drugs that may cross react with CNS tissues.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 183

Integrative analysis of genome-wide DNA methylation at single-base resolution identifies epigenetic subtypes of high-risk neuroblastoma

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Topic Gene regulatory networks and epigenetics: from basic science to therapy

Background/Introduction

Neuroblastoma is a pediatric tumor derived from precursors of the sympathetic nervous system. Half of the children diagnosed have high-risk disease, disproportionately contributing to overall childhood cancer-related deaths. Known genetic aberrations only partially explain this aggressive tumor behavior, and evidence is accumulating that epigenetic deregulation plays a prominent role in neuroblastoma pathogenesis.

Aims

Our aim is to analyze the regulatory and developmental role of DNA methylation on a genome-wide scale by an integrated approach covering genomes, transcriptomes and chromatin landscapes of primary neuroblastomas as well as transcriptional trajectories of the developing adrenal medulla.

Methods/Materials

Whole-genome bisulfite sequencing (WGBS) and RNAseq were performed in 51 primary neuroblastomas including *MYCN*-amplified, *TERT*-rearranged, ALT-positive and low-risk tumors. For 35 of these, H3k27ac ChIPseq was performed. Array-based DNA methylation estimation

complemented by RNAseq was done in an additional cohort of 180 neuroblastomas. Single cell RNAseq data from the developing mouse adrenal medulla were used for projection of epigenetically defined subtype-specific profiles. Targeted in vitro demethylation of candidate regions was done using a CRISPR-dCas9-TET1 construct.

Results

Dimension reduction approaches and unsupervised clustering on WGBS data identified three epigeneticially defined groups being characterized by low-risk disease, amplified *MYCN* or presence of telomere maintenance mechanisms (TMMs, including *TERT* and ALT), respectively. Differentially methylated regions, partially methylated regions and DNA methylation valleys specific for these subgroups were identified and integration of associated transcriptional programs revealed subgroup-specific regulatory networks. Targeted demethylation of candidate regulatory sites revealed direct transcriptional impact. H3K27ac integration identified enhancer and super enhancer methylation patterns as highly caracteristic of subtype identity, and array-based enhancer methylation analysis confirmed the relevance of the three epigenetic subtypes in a large tumor cohort. WGBS and RNAseq-based tracing of enhancer networks found subgroup-specific transcription factor/enhancer networks converging on a limited number of nodes. Projecting methylation-defined transcriptional profiles of the three subgroups onto developmental trajectories of mouse adrenal medulla precursors revealed divergent candidate cells of origin.

Summary/Conclusions

Our integrative investigation of DNA methylation at single nucleotide level identifies neuroblastoma subtypes, suggests their differential developmental origin and sheds light on their epigenetically manifested regulatory networks, which will aid identification of therapeutic intervention targets.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 184

Modelling neuroblastoma using patient-derived induced pluripotent stem cells

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

Neuroblastoma (NB) is a neuroendocrine tumor of the peripheral nervous system originating from neural crest cells (NCC) and usually manifesting in the adrenal gland, in the abdomen and chest. The origin of NB is thought to be Neural Crest (NC) cells and its derivative sympathoadrenal (SA) cells (that gives rise to sympathetic neurons and adrenal chromaffin cells). Although hereditary NB is rare, germ line mutations have been found in ALK, mimicking common sporadic ALK mutations in NB. *In vitro* modeling of complex diseases is now a possibility with the use of induced pluripotent stem cells (iPS) cells.

Aims

We aim to model neuroblastoma onset and progression using reprogrammed patient cells carrying familial driver mutations in ALK.

Methods/Materials

To study the contribution of ALK mutations in NB development, we reprogrammed non-cancerous fibroblasts patients carrying germline mutations in ALK (R1275Q) and healthy somatic cells (called as NB2 and Control, respectively) into iPS cells. Then, we have used a NCC differentiation protocol deriving trunk NCC from iPS cells grown geltrex. For further SA differentiation, trunk NCC were cultured in neurotrophic medium which yields a high percentage of sympathoadrenal progenitors and sympathetic neurons.

Results

The patient-specific iPS cells and iPS cells from healthy individuals have been characterized by morphology, expression of pluripotency markers, as well as differentiation to all three germ layers.

More importantly, we have established a feeder- and matrigel-free neural crest cell (NCC) differentiation protocol using iPS cells grown on geltrex and are able to generate NCC that express relevant markers such as p75, SOX10, SOX9, TFAP2A and PAX3. To examine the tumorigenic ability of these cells, we have orthotopically transplanted luciferase-labeled Ctrl or patient NCC into the adrenal medulla of immunocompromised (NOD-SCID-Fcg) mice and monitored tumor growth over time using the IVIS bioluminescence in vivo system. Luciferase activity could be detected in mice injected with patient NCC cells eight weeks after injection and luciferase signal intensities increased over time, while no luciferase activity was observed in mice injected with Ctrl NCC.

Summary/Conclusions

By using somatic cells carrying germ line mutation as the cell source, instead of using cancer cells derived from an established tumor that carry many more mutations, we will be able to follow the stepwise tumor development resulting from the original mutation. We believe our model will help to uncover new networks important for tumor initiation, discover potential early-stage biomarkers, and give us better models system in which therapeutics can be evaluated.

Type Basic

Presentation Preference Traditional poster

Abstract nr. 185

A possible role for EPAS1/HIF2a in promoting a noradrenergic cellular state in neuroblastoma

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

Neuroblastoma arises within the sympathetic nervous system and is the most frequent extracranial solid childhood cancer. It exhibits a high degree of clinical heterogeneity ranging from spontaneous regression to fatal progression. The hypoxia inducible factor, *EPAS1*/HIF2a, has been proposed to be NB oncogene. However, several studies challenge this concept and high expression levels of *EPAS1* is associated with increased survival of neuroblastoma patients. To address the functional role of *EPAS1*/HIF2a in neuroblastoma we have performed analysis of single cell sequenced neuroblastoma and initiated gain- and loss-of-function experiments in neuroblastoma cells. Our analysis of single cell sequenced human neuroblastoma tumors revealed that *EPAS1* is significantly enriched in cells from low-risk tumors characterized by high expression of sympathoadrenal markers such as *PHOX2B* and *TH*. This reflects the expression pattern of *Epas1* during development of the mouse sympathoadrenal lineage and suggests that *EPAS1*/HIF2a could play a role in promoting a differentiated sympathetic noradrenergic cellular state (NORAD), which have been shown to be more sensitive to chemotherapy. In contrast, neuroblastoma cells expressing markers defining the more chemoresistant mesenchymal cellular state (MES), e.g. *PRRX1* and *YAP1,* exhibit lower levels of *EPAS1*/HIF2a. Crispr/Cas9 depletion of *EPAS1* in SK-N-SH cells which harbor both MES and NORAD populations, resulted in rapid loss of PHOX2B and PHOX2A. Conversely, overexpression of *EPAS1* in SH-EP2 neuroblastoma cells that only harbor the MES population, resulted in reduced YAP1 expression and altered morphology. This implies that high *EPAS1*/HIF2a levels are required to maintain the NORAD cellular state and that transition to a MES cellular states is associated with reduced *EPAS1*/HIF2a levels. Thus, our preliminary data suggest a novel role for *EPAS1*/HIF2a which is not associated with the response to hypoxia but rather as a determining factor of neuroblastoma heterogeneity.

Aims

Methods/Materials

Results

Summary/Conclusions

Bedoya Reina

A possible role for *EPAS1*/HIF2α in promoting a noradrenergic cellular state in neuroblastoma

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Type Basic

Presentation Preference Traditional poster

Abstract nr. 186

Fusion transcripts impact patient outcome in neuroblastoma

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

Chromosomal rearrangements and fusion transcripts provide a mechanism for activation of oncogenes and inactivation of tumor suppressor in cancer. Because of a lack of suitable techniques, the prevalence and clinical impact of these alterations in neuroblastoma may have been underappreciated in the past.

Aims

We aimed to systematically determine the pattern of expressed fusion transcripts and their potential relevance for neuroblastoma pathogenesis and clinical outcome. We also addressed the question whether fusion transcripts originate from genomic alterations or during RNA processing

and investigated the association of fusion events with recurrent copy number alterations.

Methods/Materials

We analyzed 498 primary tumors by RNA-seq. Whole genome sequencing (WGS) data were available for 39/498 samples, and genomic copy number data for 239/498 samples. Fusion transcripts were detected by FusionHunter and TRUP, and were validated by dideoxynucleotide sequencing. Ectopic *FGFR2* expression and *FGFR2* knockdown was performed in JoMa1 neural crest progenitor cells and NBL-S neuroblastoma cells, respectively.

Results

We detected a total of 97 fusion transcripts in 78 tumors, involving 169 distinct genes. Fifty-two and 45 fusion transcripts were predicted to be potentially protein-coding and truncating, respectively. For 21/22 fusion transcripts, corresponding genomic rearrangements were identified by WGS, indicating that genomic events are the main cause for gene fusions. The spectrum of fusion transcripts was heterogeneous, with most fusions being private events. We observed, that chromosomes 2, 12, and 17 were significantly enriched for fusion partner genes. Furthermore, 58% of fusion breakpoints were located in close vicinity to a breakpoint of a recurrent segmental copy number alteration, such as 1p loss, 11q loss, 17p gain and *MYCN* amplification.The number of segmental copy number alterations in tumors having fusion transcripts was significantly higher than that of tumors lacking fusions ($p<0.001$), indicating a general genomic instability in these tumors. Fusion transcripts were significantly enriched for genes annotated in the COSMIC ($p=0.002$) or the Mitelman database ($p<0.001$). These genes included *FOXR1*, *HACE1*, *FGFR2* and *TERT*, which have been previously associated with neuroblastoma and/or other cancers. We evaluated the functional effect of *FGFR2*, the full-length coding sequence of which was involved in a *KLHL13-FGFR2* fusion. Ectopic expression of *FGFR2* resulted in augmented clonogenic growth, while *FGFR2* knock-down significantly impaired proliferation. Finally, we observed that the outcome of patients whose tumors harbored fusion transcripts was significantly worse than that of patients without fusion transcripts, both in the entire cohort and in subgroups, such as *MYCN*-amplified or high-risk patients. In a subset of 4 tumors, we found that 5 of 6 fusion transcripts were also detected in the corresponding relapse samples, indicating that such alterations are mostly stable genetic events during neuroblastoma development and progression.

Summary/Conclusions

We here demonstrate that fusion transcripts are regularly occurring events in primary neuroblastoma. Our data strongly suggest that the vast majority of fusion transcripts result from genomic alterations, and that they may represent a potential mechanism by which genomic alterations impact the clinical outcome of neuroblastoma patients.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 187

A novel MYCN-specific antigene oligonucleotide inhibits the broad N-Myc tumorigenic cell deregulation in neuroblastoma

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Roughly half of high-risk neuroblastoma (NB) is characterized by MYCN-amplification. N-Myc promotes tumor progression by inducing cell growth and inhibiting differentiation. Moreover, MYCN-overexpression is leading to a broad gene expression reprogramming, spanning from metabolism to chromosome re-organization. Indeed, not all the aspects of MYCN neuroblastoma relationship are well understood. While N-Myc is a known driver of the disease, it remains a target for which no therapeutic drug exists.

Aims

we aimed to investigate how N-Myc overexpression was altering the neuroblastoma cell biology and whether the specific inhibition of MYCN can affect it and subsequently inhibits tumor progression.

Methods/Materials

we developed a bioinformatic pipeline to analyze gene expression data from neuroblastoma patients in order to identify specific MYCN induced alterations. Here, we evaluated a novel MYCN-specific antigene PNA oligonucleotide (BGA002) in MYCN-amplified (MNA) or MYCN-expressing NB and investigated the mechanism of its anti-tumor activity. We confirm by *in vivo* BGA002 administration block of tumor progression by MYCN inhibition.

Results

we identified MYCN induced biological alterations in neuroblastoma and their mechanistic role in tumor progression. Indeed, we showed a previous unknown mechanism on how N-Myc is reprogramming mitochondria by repressing autophagy and by controlling reactive oxygen species (ROS). Furthermore, we also showed how N-Myc is acting on metabolism and differentiation through different mechanisms. In vivo BGA002 administration in a MYCN-amplified neuroblastoma xenograft mouse model leaded to tumor growth inhibition and improved survival.

Summary/Conclusions

we are presenting undescribed mechanisms about N-Myc impact on neuroblastoma biology deregulation. Thus, we are showing how a specific inhibitor of MYCN expression is affecting this pathogenic program leading to blocking tumor progression.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 188

Defining the mechanisms of lorlatinib efficacy against ALK-mutated neuroblastomas.

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

Gain-of-function germline and somatic mutations in the anaplastic lymphoma kinase (*ALK*) oncogene has positioned it as the major tractable oncogene for targeted therapy in patients with neuroblastoma. ALK mutations are found mostly in three kinase domain hotspots. Although the R1275* mutation shows sensitivity, F1174* and F1245* mutations confer *de novo* resistance to first and second generation ALK inhibitors including crizotinib. Lorlatinib, a third-generation ALK/ROS inhibitor, overcomes this *de novo* resistance, and is now in Phase 1 testing for patients with relapsed/refractory ALK-driven neuroblastoma.

Aims

To define the mechanisms underlying the superior efficacy of lorlatinib in ALK-mutated neuroblastomas in order to design rationale combination therapies designed to circumvent acquired resistance.

Methods/Materials

We employed Multiplexed Inhibitor Beads (MIBs) coupled with quantitative Mass Spectrometry (MS) to quantitatively measure kinase activity dynamics on a proteomic scale following ALK inhibition therapy. PDX models harboring F1174L (COG-N-453x), R1275Q (NB1643), R1245C (Felix) and amplified ALK (NB1) were treated with either crizotinib (100 mg/kg/day) or lorlatinib (10

mg/kg/day) for 2.5 days or 6.5 days prior to MIB/MS analysis. The results from MIB/MS analysis were validated by immunoblotting and qRTPCR. We performed RNA sequencing in COG-N-453 and NB1643 PDX models to study the transcriptomic changes following treatment with crizotinib or lorlatinib. We used ChIP seq and ChIP qPCR to study enrichment of MYCN and Pol II at promoters of genes of interest.

Results

MIB/MS data showed that lorlatinib inhibited ALK a thousand-fold more potently than crizotinib in all models studied. In addition to ALK, there was an enrichment of kinases involved in G2/M transition that were significantly inhibited by lorlatinib and not altered by crizotinib, verified by qRTPCR and immunoblotting in all four mouse models. Furthermore, lorlatinib also upregulated several tumor cell intrinsic and extrinsic kinases such as Csf1r, and Pdgrfb involved in innate immunity and Ripk2, Tie2, Tbk1 and Irak4 involved in inflammatory response. RNA sequencing in COG-N-453 and NB1643 PDX models showed downregulation of multiple MYCN target genes involved in cell cycle, DNA repair and metabolism following treatment with lorlatinib, accompanied by decreased expression of G2/M and mitotic genes including *PLK1, PLK4, CDK1, AURKA and AURKB*. Moreover, immunoblotting showed downregulation of MYCN in COG-N-453 and NB1643 cell lines upon treatment with lorlatinib for 6 hours. ChIP seq analysis of NB1643, Kelly, COG-N-415, LAN-5 and NGP cells showed enrichment of MYCN at the promoters of *PLK1, PLK4, CDK1, AURKA and AURKB* prior to ALK inhibition therapy, and ChIP qPCR showed displacement of MYCN and Pol II from promoters of these G2/M kinases following 6.5 day treatment with lorlatinib in the COG-N-453 PDX model.

Summary/Conclusions

Lorlatinib exhibits superior efficacy against ALK driven NB models by potent ALK inhibition, as well as direct downregulation of MYCN and multiple key G2/M cell cycle transition proteins. Whether and how MYCN is regulated by inhibition of ALK signaling and/or by inhibition of other kinases is currently under investigation.

Defining the mechanisms of lorlatinib efficacy against ALK-mutated neuroblastomas.

D'Aulerio

Biomedical and Health Informatics

Alessia

Type Translational

Presentation Preference Oral presentation

Abstract nr. 189

Screening of the embryonic sympathoadrenal secretome to identify microenvironmental triggers of neuroblastoma metastatic dissemination reveals key contribution of Noelin1/OLFM1

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Topic Neuroblastoma as developmental disease

Background/Introduction

Neuroblastoma (NB), the most common extracranial solid tumor in childhood, is a neural crest-derived malignancy that arises from the sympatho-adrenal lineage. NB aggressive forms are usually diagnosed with already established metastases, resulting in low survival rate and lack of understanding of early steps of the dissemination process. Our previous work conducted in a novel avian NB model revealed that the sympathetic embryonic environment profoundly impacts the gene program of NB cells, switching-off cell-cell cohesion pathways in the primary tumor to trigger the metastatic process (Delloye-Bourgeois *et al.*, Cancer Cell, 2017).

Aims

The first objective of our work was to characterize the impact of the embryonic sympathetic secretome on NB cell-cell cohesion and migratory behavior. Our second aim was to provide an exhaustive description of the sympathetic secretome with the underlying idea of identifying key molecular signals of the microenvironment that impact on NB cell gene program to trigger their detachment from the primary tumor and switch on their migratory potential.

Methods/Materials

We produced media conditioned by sympathetic ganglia (SG^{CM}) from chick embryos dissected out at different developmental stages and from mouse embryos, and assessed their functional properties on NB cell-cell cohesion, migratory and invasive behaviors. We analyzed by mass spectrometry the proteomic content of SG^{CM} and the transcriptomic profiles of SG^{CM}-treated NB

cells. We extracted a list of relevant candidates and further functionally characterized their effect on NB cell-cell cohesion and migration *in vitro* and *in vivo* in our avian model of metastatic NB.

Results

We observed that SG^{CM} profoundly affects NB cell-cell cohesion properties and increases their invasive behavior. Proteomic analysis of SG^{CM} and RNAseq data of NB cells exposed to SG^{CM} allowed to identify a molecular signature centered on the glycoprotein OLFM1 alias Noelin1, whose developmental role in neural crest migration has previously been described. Functional *in vitro* studies combined to in vivo experiments consisting in blocking endogenous OLFM1 in our avian tumoral model confirmed that OLFM1 plays a major contribution to the onset of NB metastasis.

Summary/Conclusions

Our study provides the first picture of the embryonic sympatho-adrenal microenvironment in which NB primary tumors develop and its impact on NB gene activity and cell behavior. The identification of OLFM1/Noelin1 as a key microenvironment-derived trigger of NB metastatic dissemination underlines the central implication of developmental signaling in the course of the disease and opens the avenue on novel therapeutic strategies for aggressive NB.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 190

Uncovering the molecular dialogs of neuroblastoma cells with their different embryonic microenvironments during primary tumorigenesis and metastatic disease progression

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Topic Neuroblastoma as developmental disease

Background/Introduction

Aggressive neuroblastoma (NB) is usually diagnosed with already established metastases, resulting in a low survival rate despite intensive treatments. This feature underlines a very efficient, early metastatic process occuring in a still developing organism, making it difficult to have a clinical access to the etiological course of the dissemination. We previously reported the setting of an avian model of NB, in which human NB cells are grafted back within their original micro-environment, the neural crest. In this embryonic context, NB cells are driven to form sympatho-adrenal primary tumors and to disseminate into the embryonic tissues, thus mimicking key steps of the NB disease. Comparative RNAseq analyses of pre-grafted NB cells and primary tumors revealed a profound impact of the sympathetic microenvironment, driving changes of NB gene program notably to release NB cell-cell cohesion to allow their secondary dissemination (Delloye-Bourgeois et al, 2017).

Aims

We first aimed at analyzing NB metastatic progression at the whole embryo scale using lightsheet microscopy to image the dissemination routes and the metastatic foci in the distal organs at different time points after the graft. Our second aim was to characterize the genome/epigenome states of the different cellular contingents composing NB tumors, when cells colonize primary tumor sites, migrate along distinct metastatic routes -ie, the peripheral nerves or the dorsal aorta-, and settle within distant secondary sites. The underlying objective was to identify genetic and

epigenetic signatures reflecting choices taken by NB cells during their metastatic progression and to correlate these features with the cellular and molecular heterogeneity of NBs.

Methods/Materials

We engrafted human NB cell lines within the chick embryonic neural crest and processed the embryos for clearing procedure and immunofluorescence prior to light sheet imaging. Next, we micro-dissected tumor foci twelve days post-graft within sympathetic ganglia and adrenal glands (primary tumors), migrating along peripheral nerves and within the dorsal aorta (en route towards secondary tumor sites), and within the bone marrow (metastatic target site). We performed single cell and bulk whole RNA sequencing of cells at each of these anatomic sites.

Results

First, we observed huge dissemination along the peripheral nerves and vessels, with sheets of NB cells reaching the thoracic cavity. Later on, NB cells were observed to colonize the skeletal bones and to settle within the bone marrow. Second, data from our RNAseq program were analyzed to extract signatures reflecting the dialogs of NB cells with the embryonic microenvironment.

Summary/Conclusions

Our approach, which makes use of a novel NB model, will provide an unprecedented and comprehensive view of NB metastatic process. The data will bring a novel basis for better understanding the processes of NB metastatic dissemination, as well as for determining to which extent the mechanisms of neural crest cell development have been retained and exploited by NB cells to migrate towards distal organs.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 191

89Zr-DFO-Dinutuximab-Beta to assess differential expression of surface GD2 in neuroblastoma.

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Topic Other

Background/Introduction

The GD2 ganglioside is highly expressed on the surface of neuroblastoma (NB) cells with minimal expression in healthy tissues; as a result it is a key target for immunotherapy in high-risk NB patients. Over 95% of primary NBs express GD2, nevertheless target expression level is heterogeneous both inter- and intra-tumourally. In addition, relapse is associated with a positive, yet low GD2 expression in tumour biopsies. Currently, immunotherapy is given regardless of the degree of positivity in tumour GD2 expression and prospective studies are still needed to accurately identify biomarkers of anti-GD2 response. Non-invasive GD2 immuno-PET (positron emission tomography) would enable real time evaluation of GD2 expression for response assessment; and function as a predictive biomarker for accurate patient selection into clinical trials.

Aims

In our studies, we investigated whether a zirconium-89 labelled anti-GD2 antibody could successfully delineate tumour volumes in NB xenograft mouse models and differentiate between tumours with low and high expression of GD2.

Methods/Materials

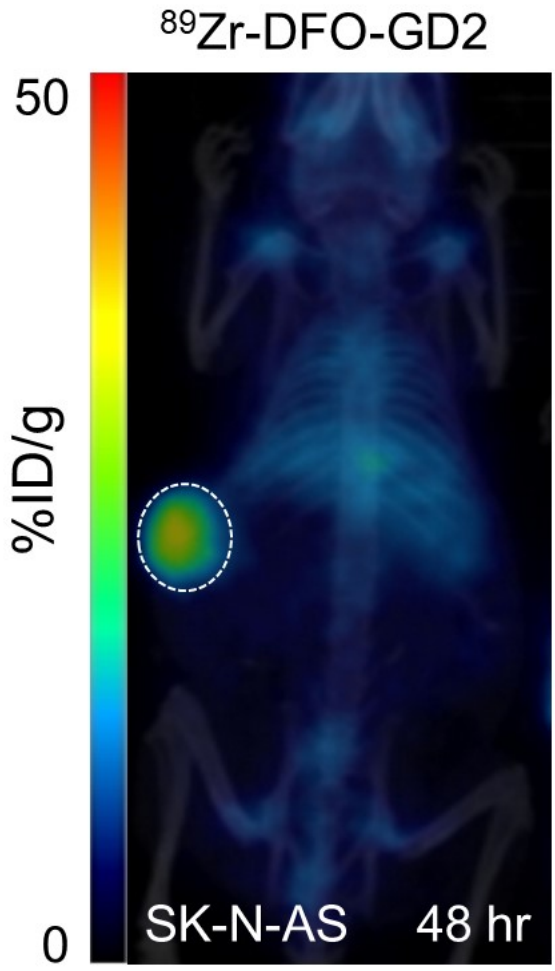
A commercial GD2-PE antibody and an in-house prepared Dinutuximab-Beta-IR700 conjugate (GD2-IR700) were used to characterise the GD2 expression by flow cytometry (FC) and confocal microscopy in Kelly, SK-N-BE(2)C and SK-N-AS NB cells. A DFO-Dinutuximab-Beta immunoconjugate was radiolabelled with zirconium-89 (^{89}Zr-DFO-GD2) and used to assess the specificity of binding on the panel of cell lines (4°C, 1 hr). For PET studies, ^{89}Zr-DFO-GD2 (10 µg, 1.8 MBq) was injected i.v. into mice bearing SK-N-AS (GD2^{high}) and SK-N-BE(2)C (GD2^{low}) tumours and images were acquired daily up to 96 hr post-injection. Biodistribution studies, autoradiography (AR), FC and immunofluorescence (IF) analyses of the resected tumours were correlated with PET imaging data.

Results

Analysis of GD2 expression by FC and confocal microscopy showed a relatively high target level in SK-N-AS compared to low expression in SK-N-BE(2)C cells. The ^{89}Zr-DFO-GD2 was prepared and isolated with a 99% radiochemical purity, and a specific activity of 0.19-0.25 MBq/µg. Cell binding of ^{89}Zr-DFO-GD2 correlated with the GD2 expression level measured via FC, and was successfully blocked with a 50-fold excess of non-labelled GD2 antibody. PET image acquisitions of SK-N-AS and SK-N-BE(2)C xenografts (24-96 hr post-injection) showed specific and receptor dependent tumour uptake of ^{89}Zr-DFO-GD2. This data was corroborated by *ex vivo* AR and IF of tumour sections. GD2-IR700 FC on fresh tumour samples correlated with ^{89}Zr-DFO-GD2 binding characteristics. The distribution of GD2 in Kelly cells was distinctly heterogeneous. Therefore our successful isolation of the more homogenous
Kelly-GD2^{high} cell population can be used for future investigations.

Summary/Conclusions

GD2-IR700 antibody can discriminate expression levels of GD2 *in vitro,* and further supports ^{89}Zr–DFO-GD2 PET to differentiate between low and high GD2 expression in tumour models of NB, as early as 48 hours post-injection. Ongoing work in mouse models and patient samples will provide insight in to the potential importance of ^{89}Zr-DFO-GD2 PET imaging to guide GD2 immunotherapy.



48 hr scan of mice bearing SK-N-AS GD2 positive tumour

Type Basic

Presentation Preference Oral presentation

Abstract nr. 192

High Throughput Proteomic Profiling of the Cell Surfaceome Identifies PTK7 as a Novel Immunotherapeutic Candidate for Neuroblastoma

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

The hallmark of targeted immunotherapy for neuroblastoma (NB) is the deployment of antibodies and host immune cells engineered to recognize tumor cell surface proteins. The introduction of the anti-GD2 antibody dinutuximab statistically improved high-risk NB patient survival. However, GD2-targeted immunotherapy is wrought with acute toxicities due to GD2 expression on normal tissues, including pain fibers and the CNS. Furthermore, recent research suggests that GD2 may be down-regulated from the NB cell surface following chemotherapy and relapse (Schumacher-Kuckelkorn, 2017). Thus, it is paramount to identify novel cell surface targets that remain upregulated throughout standard therapy and that are minimally seen on normal tissues.We therefore utilized a novel cell surface capturing (CSC) technology to define, in an unbiased fashion, the NB cell surfaceome before and after chemotherapy.

Aims

We aim to identify novel proteins that are robustly expressed on the surface of neuroblastoma cells and minimally expressed on normal tissues with the goal of nominating potential targets for antibody or cell-mediated immunotherapy.

Methods/Materials

Xenografts from 2 NB patient derived xenografts (PDXs) and one cell line xenograft (IMR5) were grown and treated with 5 days of topotecan/cyclophosphamide *in vivo*. Treated and untreated

tumors were disaggregated and glycoproteins on intact NB cells were oxidized and then selectively labeled with biocytin-hydrazide. Cells were then lysed, proteins digested, and cell surface peptides enriched using NeutrAvidin beads, followed by LC-MS analysis. We prioritized cell surface proteins that did not change under the selective pressure of chemotherapy and that were abundant on all three NB models following treatment. We then confirmed cell surface expression (ProteinAtlas.org), effects on patient survival (R2-Database), normal tissue expression (PedcBioPortal), and evaluated top hits for functional relevance in NB by genetic modification (shRNA).

Results

In two biologically replicate experiments, 292 unique cell surface proteins were identified as abundant and present across all NB models both before and following chemotherapy treatment. 34/292 proteins had a high abundance of ≥70 a.u. across all three xenograft models, including known NB surface proteins ALK and NCAM1, confirming cell location of proteins identified in this work. One protein identified was PTK7, a member of the non-canonical WNT signaling pathway. Gene expression datasets confirm that *PTK7* is highly expressed in high-risk NB tumors, with very low expression in normal tissues. High *PTK7* expression in NB is also associated with poor patient prognosis. PTK7 is widely expressed across 12 different NB cell lines and a panel of NB PDX's both at the protein (western) and cell surface (immunofluorescence) level. PTK7 promotes NB cell proliferation and colony growth *in vitro*, and *in vivo s*tudies are ongoing.

Summary/Conclusions

In summary, multiple cell surface proteins remain stable on NBs following chemotherapy treatment. PTK7 is a possible cell surface target for neuroblastoma that could be utilized for targeted therapy approaches while minimizing damage to normal tissues. Moreover, PTK7 is robustly expressed in additional pediatric and adult histotypes, suggesting the possibility that it may have broader clinical relevance. Therapeutic experiments with antibody-drug conjugate and chimeric-antigen receptor T cells against PTK7 are in progress.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 193

Intracerebroventricular Radioimmunotherapy using 131I-omburtamab for Neuroblastoma Central Nervous System/Leptomeningeal Metastases, interim results from multi-center trial 101

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Topic Other

Background/Introduction

Neuroblastoma metastasis to the central nervous system (CNS) or leptomeninges (LM) carries a dismal prognosis. In a phase 1/2 trial conducted at MSKCC, intraventricular compartmental radioimmunotherapy (cRIT) with ^{131}I-Omburtamab targeting B7-H3 was safe and improved survival.[1]

Aims

We report interim results of safety, dosimetry, pharmacokinetics and efficacy of cRIT with ^{131}I-Omburtamab on the first multi-center, international trial for patients with CNS neuroblastoma.

Methods/Materials

Y-mAbs trial 101 commenced in December 2018. Eligible patients had CNS neuroblastoma with stable systemic disease, adequate cerebrospinal fluid flow through an indwelling ventricular catheter, and adequate major organ function. A treatment cycle consisted of a 2mCi dosimetry dose and a 50mCi treatment dose, with adjustments for patients under three years or one year of age. Dosimetry by serial imaging, pharmacokinetics by CSF and blood sampling and safety were investigated during a 5-week period. Response and survival were noted by the clinical status and magnetic resonance imaging at 5 and 26 weeks with long term follow-up for overall survival up to 3 years.

Results

The interim analysis includes 17 patients (10 at MSKCC; 7 at other sites) who received at least

one cycle of ^{131}I-omburtamab before 30-Jun-2019 and have been followed for at least six months. 8 patients received 1 cycle; 9 patients received two cycles. The median follow-up time for these patients is 179 days. 15 patients remain alive at the cut-off date. The OLINDA mean absorbed doses were 3.46 mSv/MBq in the brain and 5.97 mSv/MBq in the liver. Preliminary results on dosimetry level estimates in the cerebrospinal fluid at 0.56 Gray/mCi were orders of magnitude higher than those in the blood at 0.008 Gray/mCi. The most common SAEs were related to myelosuppression. Overall, the administration of ^{131}I-Omburtamab was well tolerated.

Summary/Conclusions

Administration of cRIT with ^{131}I-Omburtamab is feasible and safe in a multi-center setting. ^{131}I-Omburtamab shows favorable dosimetry.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 194

Response Evaluation of I-131-mIBG therapy in high risk neuroblastoma

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Topic Other

Background/Introduction

I-131-mIBG-therapy is an established treatment of high-risk neuroblastoma used in initial and relapsed multimodal therapy concepts. Diagnostic I-123-mIBG-scintigraphy is a valuable tool at initial diagnosis and for response evaluation. Semiquantitative extensions scores allow estimation of the extent of mIBG-positive osteomedullary metastasis.

Aims

We were interested in the response evaluation of mIBG-therapy using semiquantitative score methods.

Methods/Materials

MIBG scans of patients treated with I-131-mIBG-therapy at our center between 2004 and 2018 were analysed. Diagnostic I-123-mIBG scans acquired before mIBG-therapy were scored and compared to I-123-mIBG scans acquired 3-8 months after mIBG-therapy for response assessment. In addition, post-therapeutic I-131-mIBG scans acquired 4h to 48hrs after I-131-mIBG injection were scored.
The SIOPEN scoring method was used for semi-quantitative estimation of osteomedullary involvement. For each pair of scans, the ratio between the score after mIBG-therapy and prior to mIBG-therapy was calculated. Response was defined as score ratio <0.8, stable disease as score ratio from 0.8-1.2 and progression as score ratio >1.2. Response was correlated to treatment episode (initial vs. relapse treatment), score prior to mIBG-therapy (<8 vs="">8), and *MYCN* status.

Results

A total of 179 scans of 50 patients undergoing 63 I-131-mIBG therapies were analysed. Median age at diagnosis was 43 months (range 2-255 months) and at mIBG-therapy 74 months (range 21-260 months). *MYCN* amplification was found in 6 out 48 evaluable patients.
Twenty-four mIBG-therapies were given during the first line treatment, 39 for relapse treatment. MIBG-therapies were applied in combination with high-dose chemotherapy either with autologous stem cell rescue (n=42) or with haplo-identlical stem cell rescue (n=3), in combination with other systemic treatment (n=10) and without additional treatments (n=8). Three patients received immunotherapy with ch14.18 between mIBG-therapy and response assessment.
The SIOPEN score of 63 pre-therapeutic I-123-mIBG scans ranged from 0 to 54 (median 1) with 27 scans revealing only uptake at the primary site (Score 0). The post-therapeutic I-131-mIBG scans identified more metastatic lesions (median 2, range 0-56, $p<0.001$). At the response assessment after mIBG-therapy, the median score score was 0 (range 0-61; $p=0.03$).
Because no metastatic osteomedullary lesion was seen in 27 pre-therapeutic I-123-mIBG scans, response could only be assessed for 36 pairs of scans revealing a median score ratio of 0.61 (range 0-3.2). A response as defined above (score ratio <0.8) was observed after 20, stable disease after 12 and progression after four out of 36 evaluable mIBG-therapies. In 10 patients, all osteomedullary lesions detected prior to mIBG-therapy (median 6.5, range 1-52) were cleared after mIBG-therapy.
Response was seen after 12 out of 17 mIBG-therapies in first-line treatment and in eight out of 19 mIBG-therapies in relapse treatment ($p=0.106$). The median score ratio of patients treated in first line treatment was 0.53 (range 0-1.9) and 1.0 (range 0-3.2) for patients treated in relapse ($p=0.22$). Similarly, neither for SIOPEN score prior to mIBG-therapy ($p=0.95$) nor for *MYCN* status ($p=0.26$) a correlation was found.

Summary/Conclusions

Semiquantitative mIBG scoring showed response to mIBG-therapy applied in combination with other systemic treatment in part of the patients. Further evaluation in larger cohorts treated with I-131-mIBG is needed.

Type Clinical

Presentation Preference Traditional poster

Abstract nr. 195

Leveraging an immunocompetent, MYCN-driven, non-germline GEM model for neuroblastoma and CyTOF mass cytometry to investigate immunosuppressive mechanisms and response to immunotherapy

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

Neuroblastoma is generally immunologically cold. Despite this observation, our understanding of how the immune system influences tumor development in this tumor has been limited by the lack of immunocompetent models in C57BL6, the work-horse mouse strain for immunological studies.

Aims

Methods/Materials

We created a non-germline GEM model in which primary trunk neural crest cells isolated from C57BL6/J embryos were modified genetically by transducing MYCN (MYCN-nGEMM), resulting in neuroblastoma after transplantation. Cell lines derived from these tumors, could be further manipulated *in vitro*, and serially transplanted in renal capsules of C57BL6 hosts. Transplanted tumors arose with 100% penetrance and short latency, making it a well-suited orthotopic model for immunotherapy studies. Both CRISPR interference CRISPR activation systems have been introduced into these lines, to enable systematic screens.

Results

RNAseq analyses revealed that the MYCN-nGEMM tumors cluster with human MYCN-amplified tumors. In comparison to the TH-MYCN model, MYCN-nGEMM tumors also
(i) expressed high levels of mesenchymal type-associated transcription factors rather than adrenergic-associated core regulatory circuitry genes, and (ii) were resistant to chemotherapy like cyclophosphamide. The MYCN-nGEMM model therefore recapitulates high-risk, treatment-resistant neuroblastoma. Analysis of the tumor microenvironment (TME) using a 40-parameter mouse-specific mass cytometry panel, revealed a highly immunosuppressive TME dominated by tumor-associated macrophages (TAMs), mostly immunosuppressive M2-like TAMs; recapitulating the TME of human high-risk neuroblastoma.
Treatment with anti-PD1 and anti-CTLA4 immune checkpoint inhibitors neither increased immune infiltration nor improved survival. In contrast, while PD-L1 is not expressed on tumor cells, treatment with anti-PD-L1 significantly delayed tumor growth by depleting PD-L1-expressing M2 TAMs. Anti-PD-L1 cooperated with a CD40 agonist to prolong survival, through decreasing immunosuppressive TAMs, increasing immuno-stimulatory TAMs and recruiting CD4+ T cells to the tumor. Despite a slight increase of memory CD8+ T cells in the tumor, these treatments however failed to induce a long-term T cells-mediated response. This confirms the need to better understand the immunosuppressive mechanisms in MYCN-driven neuroblastoma, particularly the role of macrophages. As the MYCN-nGEMM model was created on the C57BL6 background, these tumors can be transplanted into various GEM models allowing for the depletion of specific myeloid and macrophages populations, such as CD11b-DTR, CCR2-null and CD206-DTR mice. Preliminary results show a delay in tumor growth when MYCN-nGEMM cells are transplanted into CCR2-KO mice, in which recruitment of macrophages to the tumor is impaired.

Summary/Conclusions

The MYCN-nGEMM model is an easily genetically-modifiable, immune-intact model that recapitulates the highly immunosuppressive TME characteristic of MYCN-amplified high-risk neuroblastoma. Combined with mass cytometry, it constitutes a powerful tool to better understand the tumor response to various types of immunotherapy, and to identify promising therapeutic combinations.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 196

Neurologic Complications from Central Nervous System Neuroblastoma Metastases

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Topic Other

Background/Introduction

Neuroblastoma metastatic to the central nervous system (CNS NB) is associated with significant mortality (median survival <6 months, <10% survival at 36 months). Significant neurologic compromise can occur at the onset of CNS disease or during the course of treatment.

Aims

To analyze the incidence and type of neurologic complications that occur in patients with CNS NB treated at Memorial Sloan Kettering Cancer Center (MSK).

Methods/Materials

We reviewed the medical records of all patients with radiographically and/or pathologically confirmed diagnosis of CNS NB treated at MSK over a 30 year period. For this cohort, disease evaluation generally every 3 month MIBG, CT of the primary disease, bone marrow evaluation and brain CT +/- MR brain and spine. Patients were classified as having CNS NB detected while being asymptomatic and during routine extent of disease evaluations , or having symptoms that promoted further evaluation and imaging. The type of symptoms were noted.

Results

171 patients with CNS NB were evaluated and treated for CNS NB. 51 patients (30%) were asymptomatic and had disease detected by routine radiographic evaluation. The most common symptom prompting imaging was headache (N=52, 30%) +/- vomiting (N=37, 22%). New onset seizures occurred in 22 patients (13%). Altered mental status was observed in 21 patients (13%) who “felt funny” (N=1) , had slurred speech (N=1), lethargy (N=4) or became acutely obtunded (N=8) . Motor weakness occurred in 12 patients (7%), ataxia in 9 (5%), and cranial neuropathies (anisocoria, hearing deficit, facial palsy) in 13 (8%). Other uncommon symptoms included eye pain (N=1), aphasia (N=1) and opsoclonus myoclonus syndrome (N=1).

Summary/Conclusions

With the current radiographic evaluation guidelines, 30% of patients will have CNS metastases detected by routine neurologic imaging. 70% of patients are symptomatic at disease presentation with headache and vomiting being the most common sign. The most serious complication, acute obtundation, occurs in 5% of patients. Clinicians should be aware that even subtle neurologic changes can be a harbinger of CNS metastases.

Type Clinical

Presentation Preference Traditional poster

Abstract nr. 197

In vivo anti-tumour activity of the orally-bioavailable CDK2/9 inhibitor CYC065 against temozolomide-refractory neuroblastoma

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

There is a lack of characterized *in vivo* models that emulate clinical refractory and relapsed high-risk neuroblastoma, potentially affecting the predictability of the mouse hospital approach. The Th-*MYCN* mouse is the most established genetically-engineered model of neuroblastoma, which has been shown to recapitulate the major genetic and pathophysiological features of high-risk *MYCN*-amplified disease and as such, represents a relevant model to provide mechanistic insights for the development and evaluation of *MYCN*-targeted therapeutics. However, its sensitivity to frontline chemotherapy agent such as cyclophosphamide is reminiscent of the chemosensitivity of *MYCN*-amplified neuroblastomas to induction therapy rather than the chemo-resistant nature of refractory/relapsed disease. Temozolomide (TMZ) is a second-line chemotherapy agent, which shows moderate tumour control of refractory/relapsed neuroblastoma and represents, once resistance occurs, the backbone chemotherapy for the introduction of novel therapeutic strategies in European pediatric clinical trials

Aims

This study aimed to generate and characterize a TMZ-resistant model using personalised dose escalation in the Th-*MYCN* model to identify novel actionable targets against resistant/refractory disease.

Methods/Materials

Homozygous Th-*MYCN* mice with palpable tumours (n=18) were enrolled for initial screening using T_2-weighted MRI. When tumour volume reached ~ 1000 mm^3, mice were enrolled on the first cycle consisting of a 6 mg/kg daily dose of TMZ (5 days on/ 2 days off), known to only cause

partial response (RECIST). If progressive disease was detected within a cycle, the dose was increased by 50% at the start of the next cycle, until tumour growth could not be controlled anymore. On day 5 of the last cycle, mice were either *i)* sacrificed and tumours excised for molecular and pathological analysis, *ii)* treated with a high dose of TMZ (50 mg/kg p.o., n=4) or *iii)* treated with 50mg/kg CYC065 p.o. (5 days on/2 days off, n=4).

Results

We demonstrate that the Th-*MYCN* model acquired resistance to cyclic treatment with TMZ (established at 13.5mg/kg TMZ and confirmed by progressive disease during a further cycle of 50mg/kg). Resistant tumours recapitulated the known molecular and pathological hallmarks associated with TMZ resistance, including increased expression of O6-methylguanine DNA methyltransferase (*MGMT*), a DNA repair enzyme, which removes the TMZ-mediated cytotoxic O6-methylguanine DNA lesions. Interestingly, we identified deregulation of the Rb pathway as an actionable target using kinome profiling. We subsequently demonstrate that the potent CDK2/9 inhibitor CYC065 showed anti-tumour activity in TMZ-resistant Th-*MYCN* tumours, consistent with downregulation of the Rb pathways and induction of apoptosis.

Summary/Conclusions

Here, we presented a model of resistance to TMZ using the Th-*MYCN* model and demonstrated its potential as a clinically-relevant platform to evaluate novel therapeutic strategies against high-risk relapsed/refractory neuroblastoma. The anti-tumour activity of the orally-bioavailable CDK2/9 inhibitor CYC065 in this model further highlights CYC065 clinical potential in the treatment of high-risk neuroblastoma.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 198

Norepinephrine transporter-targeted macromolecular prodrug of SN-38 causes shrinkage and lasting regrowth inhibition of primary and metastatic tumors in experimental models of drug-resistant neuroblastoma

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

Uptake-1, a tissue-specific mechanism of catecholamine reuptake, can be exploited for delivery of molecular cargoes to primary and metastatic tumors. Tumor-targeted delivery of radionuclides using this process has previously been evaluated as a treatment of neuroblastoma (NB). However, due to limited tumor selectivity and non-discriminatory damaging effects of the high-dose radiation on healthy adrenergic and non-adrenergic tissues, this strategy has shown suboptimal therapeutic efficacy (≤ 30% clinical response, which is seldom curative) and serious acute and delayed toxicities, including secondary malignancies later in life.

Aims

In this study, we investigated NB pharmacotherapy targeted to *uptake-1* using an *in situ* activatable tripartite prodrug constructed with a bioeliminable polymer as a carrier. This strategy integrates a replication-dependent mode of drug action with neuroendocrine tissue selectivity, which directs the pharmacological effect to actively dividing tumor cells and minimizes toxicity to normal tissues.

Methods/Materials

We used xenograft models of refractory NB established using multidrug-resistant NB cells [BE(2)C] derived at relapse after intensive chemo-radiotherapy, paired with bioluminescent imaging to investigate tumor uptake and antitumor efficacy of the experimental prodrug. Tumor uptake analysis was carried out using an HPLC assay in orthotopic BE(2)C xenografts allowed to reach ~1 cm^3 (n=5). The effectiveness of prodrug-mediated targeted delivery against refractory

disease was evaluated in mouse models of primary (orthotopic) and metastatic, chemoresistant NB. The results were analyzed by Kruskal-Wallis one-way ANOVA and the log-rank test.

Results

Drug delivery in the form of a macromolecular prodrug, taking advantage of the *uptake-1* process, resulted in enhanced intratumoral accumulation of SN-38, a potent anticancer drug uniquely targeted to topoisomerase I. Unlike its clinically used precursor (irinotecan), SN-38 delivered as a polymer-linked prodrug was stably present in the tumor tissue at levels ≥ 100 times the drug concentration required for fully suppressing growth of chemoresistant NB tumors. Consistent with these results, SN-38 administered as the prodrug (2x/week, 4 weeks) caused rapid tumor regression, fully suppressed regrowth of chemoresistant orthotopic BE(2)C xenografts and markedly extended event-free survival (>14 weeks). At the same dosing regimen, the prodrug also caused rapid elimination of established multifocal tumor deposits in the model of refractory metastatic disease, with no detectable regrowth over >15 weeks. In contrast, irinotecan given at equivalent doses as a control had no significant effect on the disease progression in either model.

Summary/Conclusions

Tumor-guided delivery using a new prodrug design translated into rapid shrinkage and sustained regrowth inhibition of aggressive primary and disseminated tumors in the experimental settings of relapsed, multidrug-resistant NB that shows no response to conventional therapy. The results of our studies confirm feasibility and effectiveness of using macromolecular prodrugs targeted to tissue-specific mechanisms for treating localized and metastatic forms of refractory disease. Thus, targeted delivery of polymer-linked prodrug agents represents a new, promising strategy for treating high-risk NB, as well as other aggressive malignancies in children and adults.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 199

Long Term Survival for Patients with Central Nervous System Metastases Following Treatment with Intraventricular Radiolabeled Omburtamab: Results of Trial 03-133

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Topic Other

Background/Introduction

Neuroblastoma metastatic to the central nervous system (CNS NB) is associated with significant mortality (median survival <6 months, <10% survival at 36 months). Intraventricular compartmental radioimmunotherapy (cRIT) with the radio-iodinated murine IgG1 monoclonal antibody ^{131}I-Omburtamab targeting tumor cell-surface glycoprotein B7-H3 offers a therapeutic strategy.

Aims

To analyze the final results of overall survival of patients with CNS NB treated with ^{131}I Omburtamab -cRIT at a single center, Memorial Sloan Kettering Cancer Center (MSK), from 2004 - 2019.

Methods/Materials

After radiographic and/or pathologic confirmation of CNS NB , cRIT eligible patients underwent temozolomide/irinotecan-based chemotherapy, craniospinal radiation therapy, ^{131}I- Omburtamab cRIT plus systemic immunotherapy. cRIT administration involved a 2mCi tracer of intra-Ommaya ^{124}I- or ^{131}I-8H9 with nuclear imaging and CSF sampling for dosimetry followed by 1 or 2 therapeutic injections of 10-70 mCi ^{131}I-. Omburtamab. Disease evaluation included serial MR brain/spine, MIBG, CT, and bone marrow evaluation. Patients were treated on IRB-approved protocol, closed to accrual July 1, 2019. Data are presented as overall survival after detection of CNS metastasis.

Results

109 patients with CNS NB were enrolled of which 107 were treated; median follow-up 6.3 years. cRIT injections were well tolerated in the outpatient setting with myelosuppression being the most common adverse event. At analysis, the median OS is 3.7 years [95% CI: 1.9 to 7.5]. The 2-year OS rate is 57% [95% CI: 47 to 67%]. The 5-year OS rate is 41% [95% CI: 31 to 52%]. The 10-year OS rate is 37% [95% CI: 26 to 48%]. Subgroup analyses identified age at initial NB diagnosis (≤18 months), and relapse restricted to CNS as being favorable markers of long-term survival.

Summary/Conclusions

Despite advanced CNS involvement, 2 year OS is 57%, > 40% of patients treated with ^{131}I-Omburtamab remain disease free. Long term survival >10 years was observed in 37% of patients. This compares favorably to median survival of 5.5 months reported in the literature. Long term cure of CNS NB is feasible.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 200

Dual PI3K/HDAC inhibitor CUDC-907 blocks tumor growth and sensitizes MYCN amplified neuroblastoma to BCL-2 inhibition through the destabilization of MCL-1

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

Amplification of *MYCN* proto-oncogene occurs commonly in high-risk neuroblastoma and marks a particularly aggressive and high-risk form of the disease. Recent advances in therapy for neuroblastoma have been mostly through toxic escalations in conventional cytotoxic chemotherapy and radiation. Most high-risk neuroblastoma patients will relapse with highly refractory disease, therefore new therapies for neuroblastoma are focused on more targeted approaches, many of which involve MYCN regulation. Small molecule inhibitors of phosphoinositide 3-kinase (PI3K) and histone deacetylase (HDAC), which regulates MYCN expression, have shown pre-clinical efficacy in neuroblastoma and several of these are in clinical trials as monotherapy in children with neuroblastoma. However, resistance to targeted monotherapies is common in cancers. One means of bypassing resistance is to target two or more pathways simultaneously through combinations of novel drugs.

Aims

We hypothesize that inhibitors of MYCN and associated pathways each block distinct aspects of MYCN biology, and when used in combination, will both enhance efficacy and block emergent resistance. We aim to assess the efficacy and mechanism of action in the combination treatment between CUDC-907 and BH3 mimetics, venetoclax (ABT-199) and navitoclax (ABT-263), for the treatment of high-risk neuroblastomas.

Methods/Materials

We analyzed the effect of CUDC-907 on a panel of neuroblastoma cell lines; using Cell Titre-Glo assay to measure cell growth; immunoprecipitation and quantitative PCR to assess changes in protein and mRNA expression levels, respectively. Using protein arrays and cell-based assays, we

identified synergy between CUDC-907 and BCL-2 inhibitors: venetoclax and navitoclax. Other mechanistic studies include flow cytometry, caspase activation assay, and mitochondrial membrane potential assay. We assessed the efficacy of CUDC-907 in combination with venetoclax or navitoclax, *in vivo* using PDX and TH-MYCN mouse models.

Results

We show dual PI3K/HDAC inhibitor CUDC-907 can reduce neuroblastoma cell growth and is highly effective against MYC and MYCN driven neuroblastoma. CUDC-907 suppresses MYCN by both blocking histone acetylation and downregulating MYCN transcription, and destabilizes MYCN and MCL-1 proteins by regulating the phosphorylation of GSK3β. We found CUDC-907 altered several apoptotic proteins, priming neuroblastoma cells for apoptosis induced by BCL-2 inhibitors venetoclax and navitoclax. We show CUDC-907 is most potent in combination with navitoclax, significantly decreasing neuroblastoma cell growth by preventing cells from entering S-phase during mitosis and thus inducing cell cycle arrest. It also induces cytochrome c release and activation of caspase-3 leading to apoptosis. Furthermore, CUDC-907 in combination with navitoclax showed greater efficacy in our *in vivo* PDX model by significantly delaying neuroblastoma tumor growth.

Summary/Conclusions

In this study, we demonstrate that the CUDC-907 primes neuroblastoma cells for navitoclax mediated death through down-regulation of MCL-1, thus releasing the pro-apoptotic proteins, Bim and Bax. Importantly, mitochondrial priming of the cells for death by CUDC-907 requires elevated levels of MYCN or MYC, and high levels of BCL-2 or BCL-XL for synergy between CUDC-907 and navitoclax. Our research provides strong evidence demonstrating the efficacy of CUDC-907 and navitoclax as novel targeted therapy for the treatment of neuroblastoma.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 201

Overcoming Drug Resistance with Enhanced Delivery of a Novel Camptothecin to Treat High-Risk Neuroblastoma (NB)

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

Topoisomerase I inhibitors of the camptothecin family are among the most potent anticancer agents, effective against a broad range of pediatric solid tumors. However, they often show poor efficacy against high-risk NBs characterized by intrinsic or acquired drug resistance. Low drug concentrations, drug efflux by ABC transporters and glucuronidation are key mechanisms behind compromised response to camptothecins and other drugs in high-risk NB patients.

Aims

We have developed a multivalent, polymer-based prodrug of an experimental camptothecin (SN99), designed to achieve sustained intratumoral drug levels, overcome ABC-mediated drug efflux and avoid elimination by glucuronidation. We tested the hypothesis that enhanced drug design combined with prodrug-based delivery will result in significant and lasting antitumor effects of SN99 in models of high-risk NB with intrinsic and acquired chemoresistance.

Methods/Materials

We overexpressed ABCG2 in a null NB cell line (NLF) and exposed these cells to SN99 *vs*. SN38 (the active form of the clinically used camptothecin, irinotecan). We also determined tumor uptake and biodistribution of SN99 administered systemically as a polymer-based prodrug compared to irinotecan in spontaneous NBs from transgenic *TH-MYCN* mice. Tissue samples collected at predetermined times were analyzed for drug levels using HPLC. Antitumor efficacy of the prodrug was evaluated in NBs exhibiting intrinsic, ABCG2-driven multidrug resistance from homozygous *TH-MYCN* mice. We also used a model of recurrent NB by inoculating mice orthotopically with luciferase-expressing SK-N-BE(2)C cells with mutated P53. Disease progression and response to therapy were determined by monitoring the bioluminescent signal. We compared the effectiveness

of the prodrug administered by tail vein injection once a week over 4 weeks to that of irinotecan.

Results

As ABCG2 expression in NLF cells increased, SN38 was progressively less effective at inhibiting cell growth, whereas SN99 remained equally effective. SN99 delivery as a prodrug resulted in sustained intratumoral drug concentrations, significantly higher than those of irinotecan/SN38 at all time points. SN99 levels were ~50 time greater at 4 hours and 12 times greater at 168 hours compared to the highest measured SN38 level (4 hours post-delivery), with only trace amounts of SN38 remaining at 24 hours. Irinotecan modestly prolonged survival in tumor-bearing transgenic mice, but the SN99 prodrug caused complete tumor regression that persisted over 6 months after the 4-week treatment in *TH-MYCN* animals, and no tumor was detected at necropsy. Irinotecan had no effect on tumor growth in the chemoresistant orthotopic model, whereas treatment with the SN99 prodrug resulted in a rapid shrinkage and protracted suppression of the tumor regrowth for at least 14 weeks.

Summary/Conclusions

SN99 administered as a multivalent polymeric prodrug resulted in increased and protracted intratumor drug exposure compared to irinotecan. Consistent with these results, treatment with the SN99 prodrug led to long-term “cures” of primary drug-resistant tumors in the transgenic model, and to complete regression and lasting regrowth inhibition in the chemoresistant orthotopic model. We conclude that the SN99 prodrug is extremely effective in treating high-risk NBs with intrinsic or acquired drug resistance, and warrants further development for a phase 1 clinical trial.

Type Translational

Presentation Preference Traditional poster

Abstract nr. 202

The H2B deubiquitinase USP44 promotes aggressive behavior, restrains differentiation, and is associated with poor outcomes in neuroblastoma

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Recent work has implicated epigenetic regulation as having an important role in the biology of neuroblastoma. The details of which epigenetic readers and writers drive the pathology and aggression are largely unknown. Deubiquitinating enzymes (DUBs) are emerging drug targets and important gatekeepers of the ubiquitin code, including several that regulate histone mono-ubiquitination.

Aims

To identify new potential drug targets and to better understand the biology of neuroblastoma.

Methods/Materials

We analyzed existing clinically-annotated gene expression datasets using the R2 Genomics Analysis and Visualization Platform (http://r2.amc.nl) and the PREdiction of Clinical Outcomes using Genomic profiles (PRECOG; https://precog.stanford.edu/). USP44 expression was manipulated in human neuroblastoma cell lines (SH-SY5Y, IMR-32, LAN6, NGP) using RNA-interference, CRISPR gene editing, and lentiviral expression. We used an IncuCyte system to analyze cell proliferation, the transwell system to study cell migration and invasion, and image processing to analyze retinoic acid induced differentiation. We overexpressed USP44 under control of the dopamine-β-hydroxylase (dβh) promoter (Tg(*dβh:Usp44*-2A-*mCherry*)). The construct was injected into embryos from stable transgenic fish expressing *EGFP-MYCN* (Tg(dβh:*EGFP-MYCN*)) and tumor onset was monitored by detection of GFP in live fish.

Results

To identify new potential drug targets, we performed an unbiased family-wide study of the relationship between DUBs and patient survival in neuroblastoma. Using the PRECOG tool, we determined the z-scores for survival association for 95 DUBs in neuroblastoma. This revealed that

the enzyme *USP44* had the strongest association with poor survival with a z-score of 8.33 (*MYCN* z-score = 8.18). Using the R2 platform, *USP44* expression was significantly higher in tumor samples from patients with high-risk tumors compared with non-high risk. In a large clinically annotated dataset, high *USP44* was significantly associated with poor survival, metastatic disease, and unfavorable histology. The impact of *USP44* on survival was restricted to patients with *MYCN* non-amplified tumors and was also significant in non-high-risk cases. We identified cell lines with differential expression of *USP44* and found a significant acceleration of proliferation when USP44 was introduced, and slowing when it was depleted. Consistent with its association with metastatic disease, we found a significant increase in migration and invasion in cells transduced to express USP44, whereas those with depleted expression had reductions in both. As USP44 removes ubiquitin from histone H2B – a epigenetic mark associated with differentiation – we hypothesized that USP44 may restrain differentiation. Supporting this notion, we observed a significant reduction in retinoic acid induced neurite extension and growth impairment in SH-SY5Y cells expressing USP44. To determine the *in vivo* relevance of our data, we generated compound *USP44-MYCN* transgenic zebrafish and found a significant acceleration of tumor onset compared with *MYCN* alone.

Summary/Conclusions

Combining results from human datasets with our *in vitro* and *in vivo* experimental evidence, we conclude that *USP44* is a novel negative prognostic marker in *MYCN* non-amplified neuroblastoma. The expression of this epigenetic regulator promotes aggressive tumor behavior and restrains differentiation. Our work suggests that USP44 may be an attractive therapeutic target in this disease.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 203

Segmental Chromosome Aberrations and Clinical Response Impact Outcome of INSS stage 3 Patients =18 months with Unfavorable Histology and without MYCN Amplification: A Children's Oncology Group (COG) Report

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

Patients with INSS Stage 3 neuroblastoma represent a heterogeneous population with respect to disease presentation and prognosis and controversy exists regarding the most effective treatment algorithms. Patients ≥18 months of age with INSS Stage 3 tumors that are unfavorable histology (UH) and *MYCN*-non-amplified (*MYCN*-NA) represent a small cohort of patients with an outcome intermediate of those with favorable histology tumors and *MYCN* amplified tumors. The presence of Segmental Chromosome Aberrations (SCA) may predict outcome; however, their impact specifically in this cohort of patients has not been reported.

Aims

This analysis describes the event-free (EFS) and overall survival (OS) of patients with INSS Stage 3 ≥18mo patients with UH, *MYCN*-NA tumors. The impact of dinutuximab immunotherapy, clinical and biological factors including SCAs were evaluated for their impact on survival.

Methods/Materials

Eligible patients enrolled on therapeutic protocols A3973 (n=34), ANBL0532 (n=27), and biology

protocol ANBL00B1 (n=101 with 29 treated on A3973/ANBL0532) with Stage 3 disease, *MYCN* –NA, UH and age ≥18 months at diagnosis were analyzed. Patients with available high-quality tumor DNA (n=65) underwent whole exome sequencing (WES). Copy number alterations and loss of heterozygosity (LOH) for loci at chromosomes 1p, 1q, 2p, 3p, 4p, 11p, 11q and 17q were scored for gains/losses by two independent reviewers. Additional structural events including mutation analyses were carried out using established bioinformatics pipelines.

Results

The 5-year EFS/OS for children ≥18 months with stage 3, *MYCN*–NA, UH disease treated on A3973 and ANBL0532 was 73.0±8.1%/87.9±5.9% and 61.4±10.2%/ 73.0±9.2%, respectively, with no statistical differences in EFS or OS between the two cohorts (p=0.1286 and p=0.2180, respectively). In the combined cohort of patients enrolled on A3973 and ANBL0532, statistically significant differences were found (p(s) <0.0001) in patients with CR/VGPR (n=39) and PR (n=13) having better outcomes than <PR (n=5) (5-year EFS: 74.0±7.6% vs. 75.0±12.5% vs. 0%; 5-year OS: 84.4±6.2% vs. 100% vs. 20.0±17.9%). There was no difference in EFS and OS for patients who received immunotherapy vs. did not receive immunotherapy (p=0.8390 and p=0.2575, respectively). WES revealed that subjects with chromosome 11q loss/LOH had an inferior outcome in comparison to those without 11q loss/LOH (10-year EFS: 44.4+/-24.1% vs. 78.1+/-9.4%, p=0.01; 10-year OS: 62.4+/-15.9% vs. 85.9+/-7.8%, p=0.02)). Patients with 1p loss/LOH and 2p gain also showed trend towards worse event-free survival (p=0.086 and p=0.088, not statistically significant) but not in the overall-survival.

Summary/Conclusions

High-risk therapy that included single myeloablative therapy led to an 81.6±5.3%5-year OS in patients ≥18 months with UH and *MYCN*–NA Stage 3 neuroblastoma. Response to therapy is a powerful predictor of survival and the presence of chromosome 11q loss/LOH is also associated with inferior outcomes. These patients should continue to be treated on high-risk clinical trials.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 204

Low dinutuximab binding to neuroblastoma patient-derived xenografts is associated with resistance to chemoimmunotherapy and is found in patient marrow samples using multi-color flow cytometry

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

Maintenance therapy with a chimeric anti-GD2 antibody, ch14.18 (dinutuximab) combined with cytokines and isotretinoin improves outcome for high-risk neuroblastoma. A phase II clinical trial combining dinutuximab with temozolomide + irinotecan (TMZ + IRN) showed promising activity in recurrent neuroblastoma (*Lancet Oncol*, 18(7): 946-957, 2017), but a report that ~ 12% of neuroblastoma patients have low or negative GD2 expression (*Pediatr Blood Cancer* 64(1): 46-56, 2017) indicates the need to assess GD2 expression on neuroblastomas and to define expression levels necessary for activity.

Aims

Methods/Materials

Flow cytometry and directly-labeled dinutuximab quantified % positive and median fluorescence index (MFI = binding intensity) for 26 patient-derived neuroblastoma cell lines and 13 patient-derived xenografts (PDXs) established at diagnosis (n=10) or at progressive disease (PD) (n=29). Gating on neuroblastoma cells with directly-labeled non-GD2 neuroblastoma antibodies (HSAN and 459) that are negative for hematopoietic cells and gating out CD45+ cells we quantified dinutuximab binding in 59 bone marrow samples from neuroblastoma patients (41 pretherapy, 18 at PD). Event-free survival was assessed in nu/nu mice treated with temozolomide (TMZ) + irinotecan (IRN) +/- dinutuximab for neuroblastoma PDXs established from PD patients.

Results

We observed three distinct groups of cell lines based on dinutuximab binding. Cell lines with >80% of neuroblastoma cells positive for dinutuximab binding also had significantly (p=0.04)

higher density of binding (MFI). Intermediate GD2 (50-80% positive) and low GD2 (< 50% positive and MFI < 1000) was more frequent in cell lines established at PD (56% had intermediate or low GD2) compared to those established at diagnosis (14% had intermediate or low GD2). In a GD2-positive PDX (> 80% GD2-positive cells), dinutuximab + TMZ + IRN significantly ($p = 0.001$) enhanced mouse event-free survival (EFS) compared to TMZ + IRN. Compared to TMZ+ IRN alone, PDXs with 25-60% GD2-positive cells treated with dinutuximab + TMZ + IRN showed a significant but minimal increase in EFS, while a PDX with < 25% GD2-positive cells + MFI < 1000 showed no increase in EFS with TMZ + IRN + dinutuximab. Neuroblastoma cells were identified by HSAN and 459 staining in 50 of 59 patient marrow samples. Of those, 6/16 (38%) PD samples and 11/34 (32%) pre-therapy samples had neuroblastoma cells with dinutuximab MFI < 2500 and < 10% dinutuximab-positive binding.

Summary/Conclusions

Low GD2 expression can occur in neuroblastoma and may be more frequent in PD patients. Neuroblastoma PDXs in nu/nu mice provide a preclinical model to assess dinutuximab activity when combined with chemotherapy. In 34% of patient marrow samples analyzed, dinutuximab binding was comparable to levels seen in PDX's that did not benefit from dinutuximab. Quantifying GD2 expression is a potential biomarker of activity for dinutuximab that warrants evaluation in patients treated with dinutuximab combined with temozolomide + irinotecan. Funded in part by United Therapeutics Corporation.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 205

Degrading MYC by stabilizing a physiologic form of Aurora Kinase A

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

The MYC family of transcription factors/oncoprotein family members MYC and MYCN play key roles in neuroblastoma biology and oncogenesis. Increased expression of MYC proteins delineates a highly aggressive high risk subset of neuroblastoma and MYC proteins are prominent drug targets in cancer, however they are widely regarded as undruggable directly. Overexpression of MYCN and MYC is often genetic, by amplification of MYCN or enhancer hijacking of the MYC promoter, however MYC proteins are tightly regulated at the level of protein stability. Proteolysis of MYCN is blocked by interaction with the mitotic Aurora Kinase A (AURKA).

Aims

Here we aim to delineate the mechanism of action of CD532, a MYC targeted inhibitor of Aurora Kinase A, and define physiologic binding partners for MYC and MYCN in neuroblastoma.

Methods/Materials

Cell lines and tissue culture work were done under standard, previously published methods. CD532 was obtained from an in house generated supply. Proximity ligation assay and co-IP-western blots were done as previously described. Structural clustering of AURKA was done using RStudio suite and principle component analysis using FactoMineR package.

Results

We previously described CD532, an AURKA inhibitor that binds to AURKA and alters its tertiary structure to dissociate MYCN from AURKA, driving degradation. MYCN and AURKA interact in S phase, whereas MYC and AURKA interact in early G2. Nevertheless, we verify here that both

MYC and MYCN are both dissociated from AURKA by CD532, resulting in degradation of both oncoproteins. Analysis of 140 available human AURKA structures indicates that AURKA bound to CD532 closely resembles the structure of AURKA bound to its physiological interacting partner, TACC3. Through co-immunoprecipitation and proximity ligation assays, we demonstrate using proximity ligation assay and co-immunoprecipitation that CD532 destabilizes interactions between MYC or MYCN and AURKA, while in parallel stabilizing interactions between TACC3 and AURKA; and that TACC3 and MYC or MYCN interact with AURKA in a mutually exclusive manner. Our studies clarify that CD532 drives AURKA into a conformation found normally in biology. This CD532-stabilized conformation promotes interactions between TACC3 and AURKA and blocks binding of MYC or MYCN to AURKA, driving degradation of unbound MYC or MYCN in the proteasome.

Summary/Conclusions

Here we have shown that AURKA adopts a physiologically relevant structural conformation to stimulate dissociation from both MYC and MYCN in neuroblastoma and leukemia cells which is stabilized by a unique tool inhibitor, CD532, to potently promote MYC and MYCN degradation.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 206

The oncogenic transcriptional regulators CtBP-1/2 are novel potential therapeutic targets in neuroblastoma.

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Topic Gene regulatory networks and epigenetics: from basic science to therapy

Background/Introduction

Neuroblastoma (NB) is a relatively rare disease, representing 6-10% of all childhood tumors, but a significant ~15% of childhood cancer deaths. NB is a solid tumor that arises from the developing sympathetic precursors of the neural crest. CtBP-1 and -2 constitute an evolutionarily conserved family of redox-sensing transcriptional regulators with pleiotropic roles during development and adult lifespan. A very unique feature of CtBPs is the presence of a catalytic dehydrogenase domain, requiring NAD(P)H as a cofactor. CtBP-2 has been shown to modulate neural crest development by repressing melanocyte lineage differentiation and promoting commitment towards sympathoadrenal fates. More recently, accumulating evidence support a role for CtBPs as oncogenic factors that are over-expressed in many cancer types, and whose expression levels significantly correlate to worse prognosis and more aggressive phenotypes. CtBPs can simultaneously repress the transcription of several tumor-suppressive genes (e.g. p53) while inducing pro-oncogenic functions (e.g. c-Myc upregulation), such as cell proliferation, EMT and migration. In the NB cell line SH-SY5Y, RNAi-mediated CtBP2 downregulation resulted in decreased expression of c-Myc, MMP9 and MMP2 proteins, with concomitant reductions of *in vitro* cell growth, proliferation, migration and invasion. Thus, development of highly selective and specific inhibitors targeting CtBPs transcriptional functions could lead to improved therapeutic strategies in NB and other cancers. In this context, inhibition of CtBPs' catalytic domain has proven beneficial in murine *in vivo* models of colon cancer, pancreatic ductal adenocarcinoma and intestinal adenomatous polyposis. The most promising inhibitory molecules targeting this domain are derivatives of the substrate competitor 2-hydroxy-imino phenylpyruvate (HIPP), but their current potency and specificity require further improvement.

Aims

Several evidences suggest a role for CtBPs in cancer, and more specifically in NB, but many details of the molecular mechanisms remain to be elucidated. Thus, we sought to assess the effect of CtBPs downregulation and/or pharmacological inhibition on different NB cellular processes. Moreover, we have established a collaboration with the Department of Biochemistry and Molecular Pharmacology (UMass Medical School) whereby novel CtBP inhibitor (HIPP) derivatives are being designed, synthesized and tested on NB cells.

Methods/Materials

We use inducible shRNA-expression systems and pharmacological inhibitors together with high throughput imaging cytometry to assess the effect of CtBP downregulation/inhibition on several NB cell biology. Luciferase reporter assays and RT-qPCR are being used to assess CtBP transcriptional output in response to the different treatments to test. Bioinformatic analysis of available NB patient databases (R2 Platform). RNA-seq analysis will be performed to further characterize the transcriptional landscape regulated by CtBPs in NB cells.

Results

We observe significant decreases in NB cell proliferation and viability upon shRNA downregulation of either CtBP-1 or CtBP-2. As shown for other solid tumors, we demonstrate that CtBPs expression negatively correlate with NB patient survival probability in multiple annotated clinical cohorts. We have recently started screening a novel set of HIPP chemical derivatives by means of biophysical studies (isothermal titration calorimetry, multi-angle light scattering and protein crystallography) as well as cell-based approaches to optimize performance as inhibitors of CtBPs function. CtBPs-dependent transcriptional activity and oncogenic functions in response to most promising compounds will be assessed in a panel of NB cell lines.

Summary/Conclusions

Our preliminary analysis confirm a role for CtBPs as regulators of NB cell homeostasis and warrant for further characterization. Current and future efforts will contribute to clarify the mechanisms by which these transcriptional modulators mediate NB initiation, progression and/or metastasis, as well as to uncover improved inhibitory molecules with enhanced therapeutic value.

Type Basic

Presentation Preference Rapid Fire Poster Presentation

Abstract nr. 207

DYRK family kinases are critical modulators of Neuroblastoma cell homeostasis and tumorigenicity.

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Topic Gene regulatory networks and epigenetics: from basic science to therapy

Background/Introduction

Neuroblastoma (NB) is the most common solid malignancy in early childhood and high-risk disease accounts for a disproportionately high mortality when compared to other childhood cancer types. The *MYCN* oncogene is amplified and/or overexpressed in 25% of patients with this childhood cancer, making it the most powerful prognostic marker to date. MYCN drives the initiation and progression of neuroblastoma through a transcriptional program regulated by multiple protein–protein and protein–DNA interactions in malignant cells. Our group recently described a novel MYC(N) interaction with the tumor suppressor p53, leading to alterations in its transcriptional behavior. In search for potential modulators of this interaction, we focused on the '**D**ual specificity t**y**rosine phosphorylation-**r**egulated **k**inase' (DYRK) family, with particular attention to members DYRK1A and DYRK2. Interestingly, DYRK kinases have been shown to phosphorylate and modulate both p53 and MYC, constituting potential molecular regulators of the MYC(N)-p53 interaction. DYRK1A has been shown to play a critical role for neural development from flies to human. Altered expression has been linked to Down Syndrome and Autism Spectrum disorders as well as some types of cancers. An increasing body of literature suggests several roles for DYRK2 in the regulation of cancer cell homeostasis and tumor progression. In particular, treatment of NB cell lines with the DYRK family inhibitor Harmine, resulted in caspase activation and subsequent cell death in a MYCN amplification-sensitive fashion.

Aims

We sought to characterize the function of the DYRK family of kinases (DYRK1A and DYRK2 members in particular) as modulators of different aspects of NB cell biology and tumorigenicity.

Methods/Materials

We used lentiviral-mediated shRNA expression systems to constitutively or inducibly

downregulate DYRKs expression in NB cell lines *in vitro* and in mouse xenograft tumor models. Pharmacological inhibition or shRNA knockdown were used to evaluate effects on cell viability, proliferation and apoptosis in vitro. Bioinformatic analysis of NB cell/patient databases was used for expression-survival correlation plots.

Results

Here, we demonstrate that expression and function of either DYRK1A or DYRK2 is essential for proper NB cell survival and proliferation *in vitro*. Importantly, DYRK2 expression shows a significant negative correlation to survival probability of NB patients. This is in agreement with the fact that NB cell lines show the highest *DYRK2* mRNA expression among all other cancer cell lines analyzed (Cancer Cell Line Encyclopedia, The Broad Institute).Ongoing experiments will evaluate the impact of conditional DYRK1A/2 knockdown in orthotopic xenograft models. Current efforts also aim at elucidating the transcriptional mechanisms regulated by these kinases in NB cells by means of RNA-seq analysis.

Summary/Conclusions

Altogether, our findings demonstrate that DYRK kinases indeed are critical modulators of NB cell pathophysiology. We anticipate that additional preclinical studies will support the potential translation of these findings into clinical therapeutic tools.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 208

Regulation of MYCN/MAX interaction and transcriptional function by the PAK2 stress kinase.

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Topic Gene regulatory networks and epigenetics: from basic science to therapy

Background/Introduction

The MYC oncoprotein family (MYCN, c-MYC, and MYCL) are short-lived transcription factors (TF) that are collectively overexpressed or deregulated in more than half of all human cancers, thus constituting potential therapeutic targets of crucial relevance. In neuroblastoma (NB), MYCN drives tumor initiation and progression through a transcriptional program regulated by multiple protein–protein and protein–DNA interactions. MYC family TFs require hetero-dimerization with its transcriptional partner MAX to exert their functions. c-MYC/MAX dimerization is regulated in part via phosphorylation of 3 residues within the C-terminal basic Helix-Loop-Helix domain (bHLH) of c-MYC (S358, S373 and T400) by the stress-induced ‘p21-activated kinase 2’ (PAK2). Such post-translational modifications disrupt c-MYC/MAX interaction (S373 and T400) and DNA binding (S358), resulting in inhibition of c-MYC-dependent transcription, proliferation and transformation. Furthermore, PAK2-mediated disruption of c-MYC/MAX interaction was shown to trigger a switch on Retinoic Acid Receptor a (RARa) from repression to induction of differentiation on leukemic cells, constituting a novel and unexpected MAX-independent c-MYC tumor-suppressor function. We observed that two of such c-MYC residues (S373 and T400, which mediate interaction with MAX) are conserved in both MYCN and MAX bHLH domains, suggesting that they may also be targets of PAK2 kinase activity and thus mediate the functional behavior of the MYCN/MAX transcriptional complex.

Aims

We aimed to test whether PAK2 has the ability to interact with and phosphorylate MYCN and/or MAX on these and/or other residues, and ultimately modulate their function.

Methods/Materials

Bioinformatic analysis of sequence alignments and NB cell/patient databases (Cancer Cell Line Encyclopedia, The Broad Institute and R2 Platform), co-immunoprecipitation assays, in vitro

kinase assays from endogenously and exogenously expressed proteins, site-directed mutagenesis and pharmacological inhibition of kinase activity.

Results

We have confirmed PAK2/MYCN/MAX interaction by co-immunoprecipitation. Furthermore, we show that a constitutively active PAK2 mutant can specifically phosphorylate MYCN in *in vitro* kinase assays. Moreover, we have generated MYCN point-mutant versions of the two PAK2 target residues to phosphorylation-mimetic Aspartic Acid residues (S400D, T427D or both) and confirmed their role in the modulation of MYCN/MAX interaction and function. Finally, we propose a potential role for the related PAK family member PAK3, given its remarkably high expression level in NB cell lines (CCLE) and its very significant correlation to NB patient survival probability (R2 platform).

Summary/Conclusions

Present and future efforts will contribute to fully elucidate the role of PAK kinases on MYCN and MAX phosphorylation and function in *in vivo* NB mouse models. Our preliminary results highlight the PAK family kinases as novel regulators of oncogenic MYC(N)/MAX transcription and open new roads to the design of improved translational strategies to fight NB.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 209

Targeting thymidylate biosynthesis pathway in MYCN-amplified neuroblastoma cells

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

MYCN gene amplification is consistently associated with poor prognosis in patients with neuroblastoma. Because transcriptional factors (e.g., N-Myc and c-Myc) are thought to be “un-druggable” targets, alternative approaches are required to develop new treatments. Recently, synthetic lethal (SL) approaches are emerging as a promising strategy for cancer therapy. For instance, poly ADP-ribose polymerase inhibitor “Olaparib” has been approved for treating *BRCA*-deficient ovarian cancer, which can induce cancer cell-specific cell death.

Aims

Based on the SL approach, we sought to identify new SL genes in *MYCN*-amplified neuroblastoma cells, in which elevated levels of DNA damage and dysregulated cell division are observed.

Methods/Materials

We performed a genome-wide shRNA library screening by using a commercial library that consists of over 80,000 shRNA constructs targeting about 16,000 human genes. Briefly, IMR-32 cells (*MYCN*-amplified) and SH-SY5Y cells (*MYCN* single-copy) were transduced with lentiviruses carrying the shRNA sequences. The relative abundance of shRNA constructs in each cell line was then quantified by next-generation sequencing. shRNA sequences with a false discovery rate <0.1 from an exact test analysis in edgeR software were selected.

Results

In addition to already known synthetic lethal genes, about 130 genes were identified as new candidates. Based on experimental validations using siRNA or chemical inhibitors, we identified some mitotic kinases and enzymes involved in *de novo* thymidylate (dTMP) biosynthesis pathways (e.g., dUTPase and deoxythymidine kinase) as new SL target molecules in *MYCN*-amplified neuroblastoma cells. Compounds targeting the dTMP biosynthesis pathways, such as fluoropyrimidines and antifolates, are considered to be one of the major class of anticancer drug, however, their usefulness for neuroblastoma patients have not been intensively studied. Of note, it was reported that methotrexate, an antifolate drug, can specifically suppress the cell growth of

MYCN-amplified neuroblastoma cells (Lau DT *et al*., Oncotarget, 2015), and thus we investigated the efficacies of other antifolates, raltitrexed (RTX) and pemetrexed, on neuroblastoma cell lines. Our cell growth-inhibitory assay revealed that RTX showed superior inhibitory activity against *MYCN*-amplified cell lines. Because this can be rescued by adding thymidine to the culture medium, a salvage pathway to produce dTTP was active in neuroblastoma cells and the shortage of dTTP was a sole cause of the observed suppression. Although RTX treatments induced high levels of single-stranded DNA induced DNA damage response in IMR-32 cells, this was not clearly observed in SH-SY5Y cells. Interestingly, the expression level of replication protein A2 (encoded by *RPA2* at 1p35.3), a DNA binding protein to protect the exposed single-strand DNA at damaged site, was low in IMR-32 cells. It is widely known that the loss at chromosome 1p is often correlated with *MYCN* amplification, and therefore RTX might be more effective for the treatment of such patients.

Summary/Conclusions

Pre-existing antifolate drugs targeting dTMP biosynthesis pathways would be useful for the treatment of high-risk neuroblastoma.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 210

Identification of tumor infiltrating T cells in neuroblastoma by single cell sequencing

Author - Prof. Dong, Rui, Children's Hospital of Fudan University, Shanghai, China (Presenting Author)

Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

Neuroblastoma (NB) is the most common extracranial solid tumor in children, with highly variable clinical features and outcomes. Anti-GD2 immunotherapy has been proven effective in high-risk neuroblastoma, which highlights the significance of immunotherapy in the treatment of NB. Systematic identification of tumor-infiltrating lymphocytes especially T cells in NB is the footstone of the development and application of immunotherapy.

Aims

To identify tumor infiltrating T cells in neuroblastoma at single cell level, and to promote the understanding of the functional state and dynamics of T cells in NB, which might pave the way for new immunotherapy for NB.

Methods/Materials

We performed single cell sequencing on 11 NB patients and isolated all together 12,909 T and NK cells, which went through qualification control and unsuperviesed clustering. The inferred developmental trajectory was performed by Monolce2. Expression scores were calculated by mean expression of specific markers of different cell types. RNA-seq mRNA expression data and clinical parameters were retrieved from cBioportal. CIBERSORT was used to infer T cell compositions in RNA-seq data from TCGA.To correct the effect of T cell levels within each sample, the expression of selected genes in tumor were divided by that of mean of *CD3* genes (*CD3D, CD3E* and *CD3G*).

Results

Ten different large subgroups were identified by distinct expression patterns between clusters, with three subsets CD4+ T cells, six subsets of CD8+ T cells and one subset of NK cells. In general, much more naïve T cells than effector T cells were observed. In terms of effector T cells, a group of CD4+ NKT cells with high expression of *KLRB1* and *GZMK* was identified, while there are three groups of CD8+ cytotoxic T cells, with CD8-APOD group more naïve than CD8-NKG7 cells. We also found that a group of CD4+ exhausted cells, highly expressed *TIGIT.* There was almost no expression of *PD1* and *CTLA4*, which may be why anti-PD1/PDL1 or anti-CTLA4

therapy received no satisfactory results in NB. Cell trajectory was delineated, and moreover with the surge of cytotoxic score in pseudotime, there was a rather slower increase of exhausted score. Several genes such as *APOD, CCL4, CST7* might be positively correlated with the expression of inhibitory markers. We used CIBERSORT to infer the relative ratio of T cells in Rna-seq, finding that compared with poorly differntiatied NB, there was more NKT cells and CD8+ cytotoxic T cells in ganglioblastoma. Futhermore, patients with higher expression of NKT signature gene-*KLRB1* in tumor had a significantly higher survial.

Summary/Conclusions

Single-cell RNA-seq reveals distinctive functional composition of T cells in NB. There was almost no expression of *PD1* and *CTLA4*.The majority of T cells are in the un-activated naïve state. Therefore, promoting these cells to more cytotoxic phenotype might be a potentially effective strategy for cancer immunotherapy. A group of CD4+ NKT cells was identified and its signiture gene-*KLRB1* was correlated with lower risk stratification and better prognosis.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 211

Temporal clustering of neuroblastoma in children and young adults from Ontario, Canada

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Topic Other

Background/Introduction

The aetiology of neuroblastic tumours is likely to involve both environmental and genetic factors. A number of possible environmental risk factors have been suggested, including infection. If an irregular temporal pattern in incidence of cases of a disease is found, this might suggest that a transient agent, such as infection, is implicated. Previous work has found that such an agent might have been involved in the aetiology of neuroblastic tumours in children and young adults living in Northern England, where evidence of temporal clustering of cases between months and between fortnights was found.

Aims

The aim of this analysis was to see if there is evidence of similar temporal clustering of neuroblastic tumours in children and young adults living in Ontario, Canada.

Methods/Materials

We examined data from the population-based Pediatric Oncology Group of Ontario Networked Information System (POGONIS) registry. All cases of neuroblastic tumours diagnosed in children and adults aged 0-19 years between 1985 and 2016 were extracted from the registry, which covers a population of 3.1 million young people resident in Ontario, Canada at the time of diagnosis. A modified version of the Potthoff-Whittinghill method was used to test for temporal

clustering. Estimates of extra-Poisson variation and standard errors were obtained.

Results

876 cases of neuroblastoma (481 [55%] males) were diagnosed during the study period. No evidence of temporal clustering was found between fortnights within months, between months within quarters or between quarters within years in the study period. However, significant extra-Poisson variation was found between quarters within the 32 year study period (extra-Poisson variation=0.41 SE =0.13; p=0.002) and between years within the study period (extra-Poisson variation =1.05 SE =0.25; p=0.005). When the analysis was repeated for males and females separately extra-Poisson variation was found between quarters within the study period (0.31 SE =0.13; p=0.012) and between years within the study period (0.79 SE =0.25; p=0.005) in females, but no extra-Poisson variation was apparent in males (extra-Poisson variation between quarters -0.11 SE =0.13; p=0.81 and between years -0.05 SE=0.25; p=0.54).

Summary/Conclusions

The findings are consistent with the possibility that a transient agent, such as an infection, that is characterised by ‘peaks and troughs’ in its occurrence, might be implicated in the aetiology of neuroblastic tumours. It is notable that in this population temporal clustering arose over longer time periods than was reported in the previous work undertaken in Northern England, A putative explanation for this is the large geography of Ontario in comparison to Northern England, which might impact on the time it takes for infectious agents to spread. Further work is required to explore why temporal clustering was apparent in females but not males and to examine potential differences between children diagnosed in infancy and those diagnosed later in childhood.

Type Basic

Presentation Preference Traditional poster

Abstract nr. 212

ABYSMAL OUTCOME OF HIGH RISK NEUROBLASTOMA : EXPERIENCE FROM A CENTRE IN INDIA

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Topic Other

Background/Introduction

The outcome of children with high risk neuroblastoma has improved dramatically in the developed countries with introduction of immunotherapy. Such treatment is not available and is prohibitively expensive to patients from LMIC. This study was done to look at the overall and disease free survival of children who were treated in a centre in India, where even myeloablative chemotherapy is too expensive for majority of our patients.

Aims

The aims and objectives of this analysis was to assess response to rapid COJEC chemotherapy in children treated for high risk neuroblastoma (HR-NBL) and the final outcome after surgery, radiotherapy, MAT and differentiation therapy.

Methods/Materials

Children >18 months who were diagnosed to have HR-NBL and completed rapid COJEC chemotherapy from 2008- 2019 were included in the analysis. Standard criteria was used to assess response as complete or good partial response.

Results

40 children, 26 boys and 14 girls, aged 19 months to 13 years were included. Primary site of tumour was adrenal gland in 22, thoracic 6, retroperitoneal 4, paravertebral 4 and unknown in 4. Metastatic site of involvement were bone marrow -36 and bone-28, lymphnodes-12, sphenoethmoid sinuses-2 and liver in one. Myc-N was tested in 28 patients; amplified- 5, negative 19, test failed in 2 and report not available in 2.
Response assessment after COJEC complete/good partial response in 32 children; 30/36 bone marrow negative, 20/28 bone metastasis showed partial response. 25 children underwent excision of tumour; debulking was done in 3 of them. 8 had inoperable tumour, 2 opted against further treatment, 2 had no detectable residual disease and another 2 progressive disease, hence were not operated. 30 children had radiation to primary site and 3 had MIBG therapy. 9 children had

MAT and 30 received cis-retinoic acid.
Mean follow up period of 19 months (median of 24.7m), 10 children are alive, 29 died and there was no information about one child who was well at end of treatment. Among the 10 alive, only 4 are disease free. Mean time to relapse post rapid COJEC was 12.48 months (3-34 months).

Summary/Conclusions

With rapid COJEC 80% of patients achieved complete /good partial response. However, the overall survival of this group was only 25% and disease free survival was only 10%. Immunotherapy and tumour vaccine if made available will improve survival of children with high risk neuroblastoma in India.

Type Clinical

Presentation Preference Traditional poster

Advances in Neuroblastoma Research (ANR) Meeting
ANR 2020
Amsterdam
11-14
May
www.anr2020.org

Abstract nr. 213

Exosomics of Neuroblastoma: the role of exosomal proteins in risk stratification and induction-chemotherapy response

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

High-risk neuroblastoma (HR-NB) patients receive aggressive treatment that includes intensive chemotherapy, radiotherapy, surgery, autologous blood stem cell transplantation and immunotherapy with anti-GD_2 antibody. However, biological parameters enabling a dynamic early prediction of inadequate response, which would justify upfront different therapy, are lacking. The need of novel diagnostic molecular tools in oncology has led to increasing interest in liquid biopsies as a source of biomarkers, as they provide a minimally invasive method. Body fluids are a source of exosomes, nanosized extracellular vesicles that can drive tumor growth and chemoresistance. We previously identified exosomal microRNAs (exo-miRNA) indicative of HR-NB patient sensitivity/resistance to chemotherapy. As exosomes from cancer patients carry proteins that reflect the surface and cytoplasm content of parental cancer cells, including immunosuppressive molecules known to be associated with cancer progression and/or response to therapy, we extended our study to the exosomal proteins (exo-proteins) to assess whether they can predict induction-chemotherapy response and patient eligibility to immunotherapy.

Aims

The purpose of the study is to identify exo-proteins that (i) are associated with the tumor

phenotype, in order to improve risk stratification, (ii) can be indicative of induction chemotherapy response and, therefore, be integrated with the exo-miRNA profile, and (iii) can serve as biomarkers of patient eligibility to immunotherapy.

Methods/Materials

We isolated exosomes from plasma specimens of HR-NB patients before and after induction chemotherapy (n=48), stage 1 (n=8), stage 2 (n=7) and stage 4S (n=8) NB patients, and 24 controls. Exo-protein expression was evaluated by liquid chromatography-mass spectrometry (LC-MS).

Results

We identified exo-proteins associated with tumorigenesis that were specifically expressed in NB plasma specimens at diagnosis and not in control samples. Moreover, we identified exo-proteins differentially expressed in plasma from stage 1 and 2 patients compared to HR patients: such proteins could contribute to the development of aggressive metastatic NB tumors. The principal component analysis (PCA) showed that the group of stage 4S patients was clearly different from both localized and HR-NB cases. We also identified exo-proteins differentially expressed after induction chemotherapy in HR-NB patients, suggesting that exo-protein modulation could be indicative of different response to treatment. By integrating these data with our previous results obtained on exo-miRNA, we found a specific cellular pathway associated with p53/Ras as predictive model to stratify patients according to treatment response. This analysis was paralleled by the phenotyping of relevant exosomal surface molecules using a panel of fluorochrome-conjugated antibodies against 37 selected surface markers to define immunotherapeutic targets. We identified exosomes positive for GD2 and B7-H3, two critical NB markers.

Summary/Conclusions

Our studies demonstrate that exo-proteins can be employed to improve patient stratification and can give deeper insights into the mechanisms mediating the development of aggressive metastatic NB tumors. The results show that exo-proteins, together with exo-miRNA, can be markers of HR-NB patients response to induction chemotherapy and pave the way for the application of such molecules to define targeted therapeutic approaches in NB. To this purpose, we are currently extending the study of the exosome surface proteins to identify new immune-checkpoint molecules for patient eligibility to immunotherapy.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 214

Identification of TIMP3 as a regulator for metastasis in neuroblastoma

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Neuroblastoma (NB) is the most common extra-cranial solid tumor in children. Despite improvements in available treatment options, the prognosis for high-risk neuroblastoma patients remains unfavorable, with metastatic spread being one of the reasons behind it. New therapeutics for patients with advanced NB are urgently needed. MYCN is an oncogene whose amplification is associated with high-risk NB. Additionally, EGFR and ErbB2 were both shown to be highly expressed in NB cells, and have been implicated in metastasis of various tumors. Therefore, targeting MYCN or EGFR and ErbB2 may have the therapeutic potential for the treatment of NB metastasis.

Aims

In this study, we tested the ability of JQ1, a Bromodomain and Extra-Terminal (BET) inhibitor, and CL-387,785, an EGFR/ErbB2 dual inhibitor, to block metastatic properties of NB cells. In addition, the underlying mechanism was verified.

Methods/Materials

The tumor metastasis-related cell behaviors were analyzed by wound healing, transwell assays and colony formation assays. A QPCR-based tumor metastasis array was performed to identify the differentially expressed genes (DEGs) in the NB cells treated with JQ1 and CL-387,785. The clinical significance of selected DEG was further analyzed by the web-based R2 platform and a Taiwanese cohort of neuroblastoma patients.

Results

Both JQ1 and CL-387,785 significantly reduce migration and invasion of NB cells. Consistently, both compounds can inhibit anchorage-independent growth of NB cells. We further identified tissue inhibitor of metalloproteinases 3 (TIMP3) as one of the significantly upregulated genes in NB cells treated with JQ1 or CL-387,785, indicating that TIMP3 may involve in the mechanism of

NB cell metastasis. Lastly, both clinical datasets demonstrate that high expression of TIMP3 positively correlated with favorable prognosis of patients and the differentiation histology of the NB tumors.

Summary/Conclusions

Our findings provide the proof-of-principle evidence that JQ1 and CL-387,785 could be further developed to become the target therapies for high-risk neuroblastoma and also verify the prognostic merit of TIMP3 in neuroblastoma.

Type Translational

Presentation Preference Traditional poster

Abstract nr. 215

The role of PCP4 and its potential gene regulation network in neuroblastoma

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Topic Gene regulatory networks and epigenetics: from basic science to therapy

Background/Introduction

Neuroblastoma (NB), the most common malignant solid tumor in children's abdomen, accounts for 8%-10% of childhood malignant tumors. But the molecular mechanism of NB tumorigenesis is still unclear. Our research shows that the *PCP4* and its gene network may play an important role in NB. PCP4 (Purkinje cell protein 4), also named Calmodulin regulator protein, stands on the top of Calmodulin (CaM) signaling cascade, implying it may have a complex downstream regulation network in NB.

Aims

To investigate the functions of potential *PCP4* gene network in NB, and to promote the understanding of the molecular mechanism of the tumorigenesis in NB.

Methods/Materials

Immunofluorescence and Western blot were used to detect the expression of *PCP4* in the NB pathological sections and tissues. And the *PCP4* overexpression or knockdown and other genes overexpression or knockdown in NB cell lines, SK-N-BE(2)C and the SK-N-SH, were achieved by lentiviral transduction or plasmid transfection. Functions of cell lines were analyzed by the abilities of proliferation, migration and other abilities. Next, we performed a differential proteomics analysis to figure out the potential gene network of *PCP4*, and then verify the expression by the qPCR, Western Blot and immunohistochemical staining.

Results

According to the immunofluorescence results, *PCP4* expression was down-regulated in NB tumor cells. And compared with the control, *PCP4*-overexpressed NB cell lines showed a poor proliferative and migration abilities, while *PCP4* knockdown cell lines obtained a more invasive phenotype. We performed the differential proteomics analysis to investigate the downstream gene network of *PCP4*. Then we got 289 different expressed genes as potential targets. After the target gene expression detection and bioinformatics analysis, we may construct a *PCP4* gene network in

NB.

Summary/Conclusions

We found that *PCP4* plays a tumor suppressive role in NB with its potential gene network. This would help us further understand of the mechanism of NB.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 216

Single cell transcriptome analysis decodes development trajectory of neural crest cells

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Topic Neuroblastoma as developmental disease

Background/Introduction

Neuroblastoma is an embryonal tumor in which the normal developing process of adrenal medulla and paravertebral sympathetic ganglia is affected. The cell origin and initiation mechanism of neuroblastoma has been long-standing mysteries. Therefore, investigation of differentiation and migration of neural crest cells no doubt will promote futher understanding of initiation of neuroblastoma. Single cell transcriptome analysis is a powerful tool in unconvering the differentiation process of neural crest cells.

Aims

To delineate development trajectory of human neural crest cells at single cell level and to infer putative cell origin of NB

Methods/Materials

We performed single cell sequencing on two human embryos at gestational age (GA) of about 4-5 weeks and two fetal adrenal glands at GA of 10 weeks and 16 weeks respectively. Development trajectory was delineated using Monocle. Cell types was identified by well-defined markers. CIBERSORT was used to infer NB expression pattern of RNA-seq data by utilizing diffentially expressed genes of neural crest cell derivatives as reference.

Results

After qualification control and unsupervised clustering, there are all together 36,839 cells from embryos divided into 10 cell subgroups and 25,615 cells from fetal adrenal glands with 13 cell types. Cell trajectory reveals the cell fate transition from Schwann cell progenitors to chromaffin cells, through the stage of chromaffin progenitors. Along with this process, there is a synergetic expression of *PHOX2B* and proliferation genes such as *MK167*. By analyzing pseudotime-dependent transcription factor (TF), several previously unknown TFs associated with chromaffin progenitors, such as *SOX11* and *TCF4*, were identified. Putative cell origin of neuroblastoma was inferred using RNA-seq data of NB, which shows that inspite of primary site, either sympathetic

ganglia or adrenal medulla, the expression pattern of tumor cells is more similar to more mature chromaffin cell type.

Summary/Conclusions

Schwann cell progenitors, belonging to the late migrating neural crest cells, differentiate into chromaffin cells, through the stage of chromaffin progenitors. Several TFs are dynamically expressed during the process. Inspite of primary site of the tumor, the expression pattern of NB cells resembles more mature chromaffin cell type, implying a putative cell origin.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 217

Randomized Phase II Trial of MIBG vs. MIBG/Vincristine/Irinotecan vs. MIBG/Vorinostat for Patients with Relapsed/Refractory Neuroblastoma: A Report from the New Approaches to Neuroblastoma Therapy (NANT) Consortium

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

The radiopharmaceutical ^{131}I-metaiodobenzylguanidine (MIBG) remains one of the most active single agents for patients with neuroblastoma. Phase 1 trials have evaluated MIBG combined with potential radiation sensitizers (irinotecan as a DNA-damaging agent; vorinostat as an agent to increase norepinephrine transporter and decrease DNA damage repair gene expression). It is not clear if addition of these agents improves upon the single agent activity of MIBG.

Aims

The primary aim was to identify the MIBG treatment regimen associated with highest response rate among these three regimens: MIBG monotherapy (Arm A); MIBG/Vincristine/Irinotecan (Arm B); MIBG/Vorinostat (Arm C). The secondary aim was to compare toxicity across regimens.

Methods/Materials

We conducted a multicenter, open label, randomized phase II clinical trial through the NANT consortium. Patients 1-30 years old with relapsed, refractory, or persistent high-risk neuroblastoma were eligible if they had at least one MIBG-avid site of disease and adequate autologous stem cells (ASCs). Patients were allowed to have received prior MIBG monotherapy if at least 6 months prior and response other than progression.

All patients received MIBG 18 mCi/kg on Day 1 and ASC on day 15. Patient on Arm A received only MIBG; patients on Arm B additionally received vincristine (2 mg/m^2) IV on Day 0 and irinotecan (50 mg/m^2) IV daily on Days 0-4; patients on Arm C additionally received vorinostat (180 mg/m^2) orally once daily on days -1 to 12. Disease was evaluated on Days 43-50.

The primary endpoint was response after one course according to NANT response criteria. The trial was designed as a pick-the-winner study with a maximum of 105 eligible and evaluable patients to ensure an 80% chance that the arm with highest response rate is selected, if that response rate is at least 15% higher than the other arms.

Results

114 patients enrolled. Three patients were ineligible, 6 eligible patients never received MIBG, and 3 patients had not reached end of first course at time of this report, leaving 102 eligible and evaluable patients (35 Arm A; 34 Arm B; and 33 Arm C). The median age was 6.6 (range: 1.8 - 28.1) years. 49 patients had marrow involvement. 9 patients had received prior MIBG monotherapy, 62 prior irinotecan, and 7 prior vorinostat. After one course, the response rates (Partial Response or better) on Arms A, B, and C were 17% (95% CI 7-34%), 15% (6-32%), and 30% (16%-49%). An additional 3, 4, and 7 patients met NANT Minor Response criteria (partial response in one disease domain and stable disease in other disease domains) on Arms A, B, and C, respectively.

On Arms A, B, and C, rates of any grade 3+ non-hematologic toxicity were 20%, 50% and 33%; rates of grade 3+ diarrhea were 0%, 12%, 0%; and rates of grade 3+ febrile neutropenia were 3%, 9%, and 0%.

Summary/Conclusions

The combination of vorinostat/MIBG had the highest response rate, with manageable toxicity. Vincristine and irinotecan do not improve the response rate to MIBG and are associated with increased toxicity. These data provide response rates for MIBG monotherapy in a contemporary patient population assessed with current response criteria.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 218

Variations of DNA mismatch repair genes in hypermutated neuroblastoma

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Next generation sequencing (NGS) approaches, aiming to describe the overall genomic landscape, highlight a generally low mutational burden in neuroblastoma (NB), with a mean of 15 non-synonymous single nucleotide variants (SNVs) per coding genome per sample, or <1 variant per megabase (Mb) of coding genome, with a paucity of recurrently altered genes. Interestingly, previous NGS studies have indicated that a small group of NB patients display a high tumor mutational burden (TMB), which has been associated with a poor outcome. We hypothesize that these patients might constitute a distinct clinical subgroup, characterized by alterations in common biological pathways.

Aims

Methods/Materials

Cases with an intermediate to high TMB, defined as >5 somatic variants/Mb, were selected from different sequencing programs (MICCHADO NCT03496402; MAPPYACTS NCT02613962, or in-house sequencing programs). Molecular analysis consisted of standard Illumina© 100PE 100x whole exome sequencing (WES) and/or 100x whole genome sequencing (WGS) of tumor tissue and paired germline material. Following filtering on germline, somatic SNVs/mutations were called using GATK-UnifiedGenotyper, GATK-HaplotypeCaller, Samtools and Mutect. Copy-number profiles were generated using Varscan and DNAcopy.

Results

Among 150 NB cases (55 analyzed at diagnosis, 95 at relapse) 10 cases with an intermediate to high TMB > 5 somatic variants/Mb were identified (4 at diagnosis, 6 at relapse). Median age was 45 months at diagnosis (range 14-155), all but one had INSS stage 4/INRG stage M disease. Three had primary refractory disease, 7 relapsed after a median of 20 months (range 8-55) from diagnosis. Three out of 10 patients had an amplification of *MYCN*.

MLH1 was found to be altered in 4 cases: 2 patients had a pathogenic germline variant, whereas in 2 cases known/predicted pathogenic monoallelic somatic variants of *MLH1* were observed; two of them displayed microsatellite instability. One tumor had a somatic variation of *MSH6*. Interestingly, another tumor had a homologous deletion of *NBN*, a gene of the double-strand repair complex involved in Nijmegen breakage syndrome. Other variants of unknown significance were found in DNA repair genes such as *RERE, BRCA1* and *BRAT1*.

Among the other observed variants, genes previously described to be recurrently altered in NB were identified, including mutations of *ALK* in 2 cases, and mutations of RAS-MAPK pathway genes in 3 cases. Furthermore, variants of genes involved in chromatin remodeling were observed in 4 cases, including variants in *ARID1A* (n=2), *SMARCA4* and *IDH1* (n=1 each). In addition, somatic pathogenic variations of *TP53* were observed in 3/7 cases studied at relapse.

Analysis of paired diagnosis-relapse samples is currently ongoing (n=4) with an aim to compare the overall TMB and mutational signatures between primary tumor and relapse, and to search for potentially therapy-induced mutational profiles at relapse.

Summary/Conclusions

Hypermutated neuroblastomas are a small subset of tumors that need to be explored more thoroughly. Further studies are necessary to determine if hypermutation which might be associated with expression of new epitopes, is associated with response to checkpoint inhibitors and to determine if patients with hypermutated NB might benefit from an alternative treatment approach.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 219

Application of a sustained release carrier containing the Okinawan bioresource having anticancer activity

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

Background
Midkine is a growth factor expressed in various tumors including neuroblastoma, and its expression positively correlates with poor prognosis of the patients. We previously investigated antitumor activities of natural products obtained in Okinawa, a subtropical region in Japan, using midkine as an indicator. We obtained a candidate material which suppressed midkine secretion by human neuroblastoma cells. Further we found the hydrogel prepared from carboxymethyl cellulose (CMC) (Carbohydrate Polymers 106 (2014) 84-93) could be useful as a drug delivery system (DDS) carrier matrix for these bioresources, which gradually releases materials over a long period of time, and therefore could be a potential a DDS.

Aims

Aim
The purpose of this study is to evaluate and apply a DDS for controlled release of anticancer bioresource products. This substance already has been confirmed to show sustained release of lysozyme and BSA. In the current study, we investigated the efficacy of the newly-developed DDS on neuroblastoma.

Methods/Materials

We prepared the powder-like CMC based hydrogel as a DDS matrix. To assess the DDS, we used a neuroblastoma cell line;SK-N-SH. We assessed the effects of the CMC based hydrogel

conjugated with Jabuticaba(*Myricaria cauliflora*) leaf ethanol extract and with carboplatin on neuroblastoma cell growth.

Results

The CMC based hydrogel conjugated with Jabuticaba significantly suppressed neuroblastoma cell growth, and its effect is comparable with the CMC based hydrogel conjugated with carboplatin. Our DDS using electrospinning for carboplatin efficiently suppressed in situ tumor growth without any serious side effects of the anticancer drug.

Summary/Conclusions

At ANR2018, we have shown that our DDS using electrospinning for carboplatin efficiently suppressed in situ tumor growth without any serious side effects of the anticancer drug. But, electrospinning type DDS had to be manufactured at high heat. Therefore, it was not possible to use a substance that is weak to heat. On the other hand, the new DDS is not affected by heat. This DDS may be useful for cancer treatment including neuroblastoma.

Key words
Jabuticaba(*Myricaria cauliflora*), drug delivery system (DDS), CMC based hydrogel

Type Basic

Presentation Preference Traditional poster

Abstract nr. 220

Multi-plex Imaging to dissect Bone Marrow Disseminated Disease in Neuroblastoma at the Single Cell Level

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

Disseminated tumor cells (DTCs) in the bone marrow (BM) are detected in the majority of stage M (metastatic) neuroblastoma patients who have a high risk of relapse and low survival rates. Currently, automatic immunofluorescence (IF) imaging is state-of-the-art to evaluate treatment response and identify DTCs in the BM, however this method allows visualization of only up to three biomarkers on single cells, hampering a comprehensive spatially and time-resolved characterization of DTC heterogeneity and the local immune response in the BM microenvironment.

Aims

Thus, the aim was to identify as well as validate additional relevant biomarkers, visualize these by multi-plex imaging and thereby characterize DTCs and the metastatic BM microenvironment using machine-learning based methods.

Methods/Materials

Based on data mining of RNA-sequencing data-sets of stage M NB (tumors, DTCs, BM-derived

non-tumor cells) and proteomics data (NB tumors, cell lines, fibroblasts), and guided by public databases, relevant biomarkers were selected. Sample preparation and IF-staining protocol were optimized to validate specificity of identified biomarkers and allow the assessment of protein expression profiles by multi-epitope ligand cartography (MELC). Single-cell morphological and fluorescence features were computed and validated on processed and segmented MELC images to be further used for exploration of distinct cell populations by dimensionality reduction based methods.

Results

Herein, three novel potential DTC biomarkers are proposed, namely DCLK1, FAIM2 and TAG1, which give new insight into NB heterogeneity. By addition and validation of further biomarkers to characterize the cell composition in the BM microenvironment and potential therapeutic targets, three individual 23 biomarker IF-panels were established. Based on machine learning methods, the qualification of computed features as single-cell descriptors was proven and the latter were employed for visualization of distinct cell populations, e.g. lymphocytes, myeloid cell types, stem/progenitor cells, mesenchymal and tumor cell phenotypes, by stochastic neighbor embedding approaches.

Summary/Conclusions

Our results demonstrate proof-of-principle for the assessment of cell composition in the metastatic BM microenvironment and illustrate tumor plasticity on a single cell level. This approach complements sequencing based approaches to study tumor heterogeneity and will help to refine diagnostics for stage M NB patients.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 221

ScRNA-seq of MYCN and LMO1 driven zebrafish neuroblastoma reveals intra- and inter-tumoral heterogeneity

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

The cellular composition of primary and metastatic neuroblastoma (NB) tumours and their microenvironment has an essential impact on drug response and survival rate for young patients. Transgenic animal models such as zebrafish expressing human MYCN, which have been shown to resemble human NB tumours genetically and histologically, can help to understand the origin and consequence of inter- and intratumoral heterogeneity.

Aims

With single-cell RNA sequencing analysis of primary tumours from different transgenic zebrafish NB models, we are untangling the transcriptomic landscape of NB tumours and their microenvironment with the aim to understand inter- and intratumoral heterogeneity and the potentially resulting therapy resistance and disease relapse.

Methods/Materials

We sequenced 7k cells on average of four primary tumours from transgenic zebrafish expressing either MYCN alone or together with LMO1 under the control of the dopamine ß-hydroxylase (dbh) promoter and compared these to scRNA-seq data from two control fish expressing only a fluorescent reporter. The gene-expression pattern for different cell types of the prepared tumour-mass was locally resolved via fluorescent in situ hybridization (FISH) and antibody staining.

Results

We found an average of 20 cell subpopulations in each dissected tumour, including diverse cell types of the microenvironment. The tumours displayed heterogeneous degrees of immune infiltration as well as association of various fibroblast subtypes that were not present in control tissue. The gene expression profiles of neuroblastoma cells recapitulate their common sympathoadrenal origin, but also reveal transcriptionally distinct subgroups. As previously described by the group of Rogier Versteeg (Van Groningen et al., *Nat. Gen.*, 2017), we find subpopulations of neuroblastoma cells that express mesenchymal marker genes. The MYCN/LMO1 double transgenic fish develop tumours at two separate sites that we prepared and sequenced separately. ScRNA-seq data of both tumours from the same individual provides information on the differences in neuroblastoma transcriptional profiles arising from different cells of origin.

Summary/Conclusions

Our data provides an in-depth look into intra- and inter-tumour heterogeneity of transgenic neuroblastoma models on a single-cell level. The NB zebrafish with diverse patterns of immune infiltration and tumour cell heterogeneity make an excellent model system for studying the effects of chemotherapeutic treatment and knock-out of distinct cell populations. Moreover, we confirm that neuroblastoma cells show varying degrees of mesenchymal characteristics.

Type Basic

Presentation Preference Traditional poster

Abstract nr. 222

Rapid in vivo validation of HDAC inhibitor-based treatments in pediatric nervous system tumors

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

Background: High-risk neuroblastoma patients respond poorly to multimodal chemotherapy, thus the development of novel drug combinations and improved preclinical drug testing models are necessary to effectively target the disease. Histone deacetylase (HDAC) activity has an important role in numerous cellular processes, and aberrant expression of HDACs has been associated with a variety of malignancies, including neuroblastoma. We have previously reported selective inhibition of HDACs as a promising approach to sensitize aggressive neuroblastomas to chemotherapy.

Aims

Aim: Establishment of zebrafish neuroblastoma, glioblastoma and medulloblastoma xenograft models, and *in vivo* evaluation of combination treatments involving standard chemotherapy and

selected HDAC inhibitors (HDACis).

Methods/Materials

Methods: A suspension of fluorescently labeled tumor cells is injected two days post fertilization (dpf) into the yolk sac (tumor volume studies), perivitelline space (cell dissemination studies) or brain of zebrafish larvae. At 3dpf compounds of interest are added to the maintenance water and tumor volume or disseminated cells are monitored by confocal microscopy 48h after treatment start (5dpf). Tumor cells were also co-stained with, tetramethylrhodamine ethyl ester (TMRE) and DiD to detect the amount of viable cells as well as the total amount of injected cells. Moreover, cell proliferation was assessed by quantifying the expression of Ki67 and phospho-histone H3, immunomarkers specific for cells undergoing mitosis, on sections of the xenografted tumors.

Results

Results: On average, 40-60% of tumor cells remain mitotically active following injection, and we are able to monitor cancer progression, including disseminating of single tumor cells, with single-cell resolution in an intact environment. HDACis proved on-target activity via substrate acetylation and induced the shrinkage of the xenografted tumors. HDACis in combination with doxorubicin substantially affected tumor growth as well as tumor cell dissemination to the fish tails.

Summary/Conclusions

Summary: Our findings indicate that engraftment of pediatric tumor cells into zebrafish larvae can facilitate fast results on drug responsiveness, before moving on to more costly and time-consuming mammalian studies.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 223

Mitochondrial unfolded protein response (UPRmt) and spatial localization of the UPRmt proteins in neuroblastoma treated with 2-DG, poly(I:C) or cisplatin

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Topic Other

Background/Introduction

Glycolysis and other metabolic event are deeply involved in tumor growth including neuroblastoma (NB), which is also highly dependent on the proper function of mitochondria. Mitochondrial unfolded protein response (UPR^{mt}) is known to maintain proteostasis of the mitochondria through chaperones and proteases.

Aims

We conducted the study to unveil if UPR^{mt} and spatial organization of UPR^{mt} proteins in response to 2DG, poly(I:C) and cisplatin is involved in NB cell life and death.

Methods/Materials

Two NB cell lines without (SK-N-AS) and with (SK-N-DZ) *MYCN* amplification were treated with 2DG, poly(I:C) and cisplatin in different doses, combinations for different time points. Mitochondrial chaperones (HSP10, HSP60) and proteases (CLPP, AGF3L2 and LONP1) as well as their regulators (CHOP, ATF4, ATF5) were studied *in vitro* and the spatial localization of HSP60 was studied in animal xenografts.

Results

Treating SK-N-AS and SK-N-DZ cells with increasing dose of 2DG from 2 to 20 mM, resulted in up-regulation of CHOP, ATF4, ATF5 and AGF3L2, while at the same time significant and progressive decrease of CLPP, but no change in HSP10. In SK-N-DZ, LONP1 is also up-regulated. On the contrary, treatment of SK-N-AS poly(I:C) from 5-20 mg/ml, showing dose-

dependent increase of HSP10, while there is no significant change in CLPP. Treating SK-N-AS xenograft with 2DG, cisplatin and poly(I:C) resulting in significant expression of HSP60 in NB cells. The same is true for SK-N-DZ xenografts treated with cisplatin, and the distribution of HSP60 is mainly peri-vascular.

Summary/Conclusions

The results suggested that there is cell-type specific response of UPR^{mt} proteins to 2DG, CDDP and poly(I:C).The distribution of UPR^{mt} proteins in animal xenografts deserves future study to unveil their clinical significance.

Type Basic

Presentation Preference Traditional poster

Abstract nr. 224

Longitudinal ctDNA measurements in a multiply relapsed neuroblastoma patient detects disease progression earlier than image guided diagnostics

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

Neuroblastoma is the most common extracranial solid tumor occurring in the pediatric age group. There are increasing reports on the use of liquid biopsies for the mutation profiling, treatment monitoring and relapse detection in neuroblastoma

Aims

The aim of this study was to examine the accuracy and clinical usefulness of serial ctDNA (circulating tumor DNA) measurements for relapse surveillance and treatment response in a multiple relapsed neuroblastoma patient

Methods/Materials

A 5 year old girl initially treated for Stage Ms neuroblastoma at 4 months of age and recently treated for a second relapse of metastatic disease, without *MYCN* amplification or segmental chromosomal aberrations, was discovered to harbor a somatic activating *ALK* mutation (c.3824G>A, p.R1275Q) in her relapsed tumor sample. Due to some residual NBL cells in her bone marrow (complete remission by imaging) after end of relapse treatment, she was started on monotherapy with a first generation ALK-inhibitor, Crizotinib (280 mg/m2 x 2), and entered into our liquid biopsy project for surveillance of treatment response and early relapse detection. EDTA-blood was collected approximately every second month, and plasma was purified within two hours by centrifugation at 820 G, 10 min, followed by 10 000 G, 10 min and storage at -80°C. Cell free DNA was collected using the QIAamp Circulating Nucleic Acid kit (Qiagen), and ctDNA was evaluated in triplicate by digital droplet PCR (ddPCR) using the PrimePCR ddPCR Mutation Assay *ALK* p.R1275Q (dHsaMDV2516768, Bio-Rad) on a QX200 AutoDG Droplet Generator and QX200 Droplet Reader (Bio-Rad). PCR program was as follows: 95°C 10 min, 94°C 30 sec and 56°C 1 min for 40 cycles, 98°C 10 min, 4°C ∞, ramp rate was adjusted according to Bio-Rad's recommendations. Results were analyzed using Quantasoft version 1.7.4 (Bio-Rad), and fractional abundance (mutant DNA/total DNA) was reported. Samples having more than three positive

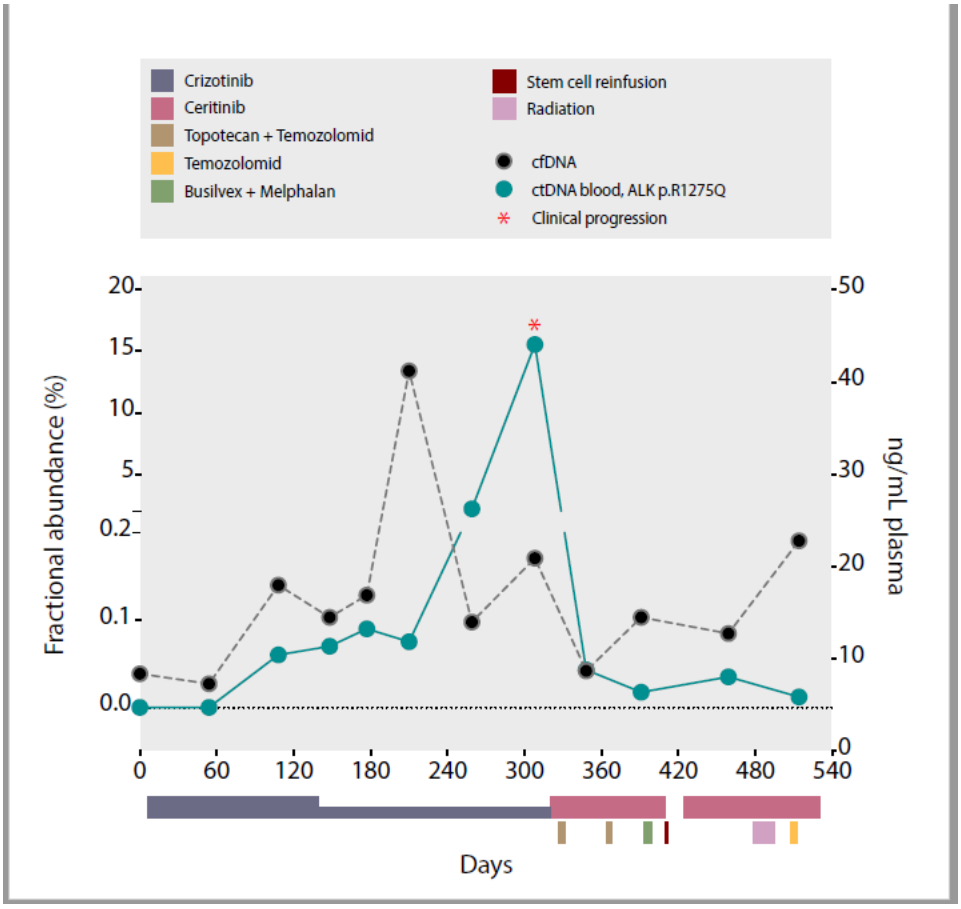
droplets were defined as ctDNA-positive

Results

After 3.5 months of Crizotinib monotherapy, the ctDNA assay showed a slight increase in ctDNA (without clinical evidence of a new relapse) that stayed stable for about 3.5 months before showing a marked increase 1.5 months prior to a clinically evident relapse. The new relapse was treated aggressively with re-induction chemotherapy, followed by high dose chemotherapy with autologous stem cell support and change to a second generation ALK inhibitor. ctDNA was negative (0/3211 detected droplets) after the first course of re-induction chemotherapy and change of ALK inhibitor, and stays detectable but stable and low since, which is in accordance with imaging and clinical conclusion of continued complete remission

Summary/Conclusions

Monitoring of serial ctDNA over a period of 17 months showed clear signs of a new relapse before it was clinically evident (Figure 1), allowing for treatment adjustment and avoiding the need for a re-biopsy of the third tumor relapse. The patient is now in radiological, clinical and biochemical remission after a fourth treatment regimen including maintenance treatment with a second generation ALK inhibitor and low dose metronomic oral chemotherapy, a total of 6 years after the primary presentation



Timeline of ctDNA, cfDNA and tumor treatment

Type Translational

Presentation Preference Traditional poster

Abstract nr. 225

Epgenetic Differentiation Therapy: A preclinical study and methylation analysis in phase I study

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

The CpG island methylator phenotype (CIMP) in neuroblastoma (NBL) is strongly associated with poor prognosis, giving larger HR than *MYCN* amplification [Abe, Cancer Res, 65:828, 2005; Abe, Cancer Lett, 247:253, 2007; Banelli, Lab Invest, 92:458, 2011; Kiss, BMC Med Genet, 13:83, 2012]. Therapeutically, treatment with a DNA demethylating agent, decitabine (DAC), and 13-*cis*-retinoic acid had synergistic effect on differentiation of NB-39nu cells [Abe, Oncology, 74:50, 2008].

Aims

We aimed to demonstrate the potential of a combination therapy of DAC and a synthetic differentiation agent, tamibarotene (TBT) *in vitro* and *in vivo*. We also aimed to monitor the effect of DNA demethylation in peripheraly leucocyte in phase 1 study.

Methods/Materials

Gene expression was analyzed by qRT-PCR, and DNA methylation was analyzed by quantitative MSP in cell lines. Demethylation in peripheral leucocytes was analyzed by pyrosequencing of LINE1 sequence.

Results

DAC suppressed the growth of 12 NBL cell lines by inducing demethylation and expression of genes related to cell death and neurological processes. TBT induced neural extension and upregulation of differentiation markers, such as *HOXD4,NGFR,* and *NTRK1*, in five NBL cell lines. Pretreatment of the five cell lines with DAC enhanced the upregulation levels of differentiation markers, showing that DAC enhanced TBT-induced differentiation. The tumor suppression effect of DAC and TBT was finally confirmed *in vivo* using a mouse xenograft model of NB-1 cell line. Based upon these preclinical fidings, an investigator-initiated phase I trial of TBT as a single agent was completed in 2017, determining a recommended dose of TBT for pediatric patients. Then, a phase I trial of a combination of DAC and TBT was started in 2018, detail of which will be reported elsewhere. Successful demethylation of LINE1 sequence in peripheral leukocytes was observed in three of five patients at level 0, and in three to four of four patients at level -1 of DAC.

Summary/Conclusions

A combination therapy of DAC and TBT was effective *in vitro*, and that DAC and TBT could suppress growth of NBL cells as a single agent *in vivo*. In our phase I study involving DAC and TBT, demethylation in peripheral leukocytes was confirmed.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 226

Study of clonal evolution and resistance mechanisms of high-risk neuroblastoma under targeted therapies

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

An improvement of survival of high-risk neuroblastoma (HR-NB) requires the development of new overall treatment strategies. Potentially promising strategies are based on the matching of somatic genetic alterations as molecular targets with targeted therapies. However, following the introduction of targeted therapies, in many instances, after initial response, treatment resistance and disease progression can occur. The potential mechanisms of resistance after initial sensibility to targeted therapies remain to be fully elucidated. The most plausible hypotheses favor 3 underlying mechanisms: (1) tumor heterogeneity with the presence of different subclones characterized by distinct molecular profiles might lead to a differential sensitivity with response of one versus emergence of another clone; (2) the emergence of new mutational events leading to treatment resistance in a major clone, or (3) signaling pathway alterations (up/dowregulation). Indeed, different large scale next-generation sequencing (NGS) approaches have demonstrated the presence of tumor heterogeneity and the existence of distinct clones in HR-NB at diagnosis and at relapse.

Aims

The aim of our work is to study the mechanisms of resistance and escape to treatment of HR-NB based on the study of clonal evolution by extensive genetic profiling in HR-NB murine models under targeted therapy, alone or combined with standard chemotherapy.

Methods/Materials

We used HR-NB patient-derived xenografts (PDX) (n=5) harboring “targetable” mutations identified by whole-exome sequencing (WES) and RNAseq, treated by targeted therapies alone or combined with standard chemotherapy. To study clonal evolution, we performed sequential mice plasma sampling during the treatment and at sacrifice for circulating-free DNA (cfDNA) analysis by WES. For bioinformatics analysis, we developed a specific pipeline enabling to focus on human (tumor) content.

Results

To date results are available for 1 PDX model harboring *CDK4* amplification and an *ATRX* mutation, treated by the CDK4 inhibitor ribociclib with and without standard chemotherapy. The analysis is ongoing for 4 other PDX models carrying, respectively, an *ALK* amplification, an *ALK* F1174L mutation (both treated by ALK inhibitor lorlatinib), *HRAS* mutation (treated by MEK inhibitor trametinib) and *ARID1A* mutation (treated by EZH2 inhibitor EPZ6438), each +/- chemotherapy combinations.

We were able to extract cfDNA from mouse plasma and to perform WES on the ctDNA. Median plasma volume was 62.5 µL [25-170]; the median amount of cfDNA was 792.88 ng/mL [87.84-7542.86], with a median proportion of ctDNA in cfDNA of 25% [1.57-93.64]. The comparative copy-number and single nucleotide variation (SNV) analyses confirmed the presence of the same tumor specific genetic abnormalities in the ctDNA of the mouse carrying the PDX, as compared to the primary tumor and the PDX itself. Most of the mutations were shared between the control and treated groups (including *ATRX* c.5242G>A, *CDK2* c.643G>C and *ETV1 c.708C>G*), and were mostly clonal, with a median variant allele fraction (VAF) of 0.43 [0.01-1]. The analysis of the clonal evolution confirmed the presence of resistant mutations (which persisted in both treated and control groups), the emergence of new/induced mutations (which appeared only in treated tumors, such as *GRIN2B* c.1584delC and *MAP3K7* c.631G>C) and sensitive mutations (which disappeared in treated tumors, such as NR2C2 c.1265C>G). While resistant mutations were mostly clonal (median VAF 0.23 [0.01-1], induced and sensitive mutations appeared to be mostly subclonal (median VAF 0.04 [0.01-0.18] and 0.05 [0.01-0.13] respectively.

Summary/Conclusions

This approach will contribute to the definition of optimal treatment combinations in a particular genetic context based on the study of clonal evolution under targeted therapy. This knowledge will be important in particular when considering future approaches for HR-NB treatment and when discussing possibilities of introducing targeted therapies in upfront (during the induction or the maintenance phase), in parallel or sequentially with standard chemotherapy in the management of HR-NB.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 227

Immune correlative markers in refractory or relapsed neuroblastoma patients treated with irinotecan/temozolamide/dinutuximab immunotherapy

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

In the Children's Oncology Group (COG) ANBL1221 trial (NCT01767194) the partial or complete response rate following treatment with irinotecan/temozolomide/dinutuximab (I/T/DIN) was 41% in patients previously treated for high-risk neuroblastoma and enrolled at the time of first relapse or first declaration of refractory disease. In light of this impressive response rate, identification of predictive biomarkers is critical.

Aims

This study sought to identify biomarkers associated with patient response. These included plasma dinutuximab levels, human anti-chimeric antibody (HACA) status, levels of immune cell markers and regulators, and KIR-KIR-ligand genotype combinations.

Methods/Materials

Plasma, mononuclear cells, and nucleic acids were isolated from blood samples of patients

enrolled on ANBL1221. Forty-five patients who received chemo-immunotherapy provided consent for biology studies. NK marker levels were determined by flow cytometry. CD276, HACA and dinutuximab plasma levels were evaluated using ELISA. NKp30 isoform status and KIR/KIR-ligand status were assessed using RNA and DNA analyses, respectively. Preliminary statistical analyses are reported here and will be finalized prior to the ANR meeting.

Results

The median dinutuximab trough level (measured prior to start of cycle 2) in the 20 patients with objective responses (CR or PR) was 852 ng/ml (range = 132-12,595). This was higher than the median level in non-responders (21 patients with stable or progressive disease) which was 511 ng/ml (range 101 – 1553; $p<0.001$, Mann Whitney-Wilcoxon test). A HACA response was detected in only 2 patients. Both HACA+ patients were among the 13 that had received dinutuximab prior to entry; none of the dinutuximab-naïve patients developed HACA. Although the number of patients was small, HACA+ patients had very low plasma dinutuximab levels following detection of HACA, consistent with the hypothesis that the HACA was neutralizing detectible dinutuximab *in vivo*. Variation in the levels of NK cell marker levels (KIR, NKp44, CD161 and CD226) within individual patients during therapy was limited (ANOVA $p>0.05$). Based on preliminary analyses, the number of NK cells expressing KIR, CD161 and CD226 (by flow cytometry) appear to be higher in responders than non-responders. In contrast, there were no statistically significant differences between responders and non-responders based on: total NK cell (CD3-/CD56+) number, the NKp44+ cell populations, NKp30 isoform or the genotypes for KIR/KIR-ligand. Pretreatment plasma levels of CD276 did not appear to correlate with clinical responses, although preliminary analyses suggest that high plasma levels of CD276 may be associated with poor progression-free survival.

Summary/Conclusions

In this study, higher dinutuximab trough levels appear to correlate with complete/partial response. HACA positivity was strikingly rare, potentially due to the concurrent chemotherapy administered with the dinutuximab. KIR/KIR-ligand status did not correlate with outcome in this setting. NK cell surface marker expression of KIR, CD161 and CD226 may be associated with response, though further evaluation is needed. Additional study of these biomarkers in a larger group of patients treated with chemo-immunotherapy may aid in identification of patients most likely to respond to this therapy.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 228

Telomerase inhibition acts synergistically with chemotherapy in preclinical high-risk neuroblastoma models

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

Telomerase is activated in the majority of high-risk neuroblastomas. We have recently demonstrated that telomerase-interacting compounds, such as 6-thio-2'-deoxyguanosine (6-thio-dG), significantly impair growth of neuroblastoma cell lines harboring *TERT* rearrangements, *MYCN* amplification, or high *TERT* expression in the absence of these alterations both *in vitro* and *in vivo*.

Aims

We here aimed to examine whether telomerase-interacting compounds in combination with established chemotherapy or *ALK* inhibitors act synergistically on neuroblastoma growth *in vitro* and *in vivo*.

Methods/Materials

Growth-inhibitory effects of varying concentrations of 6-thio-dG in combination with etoposide, doxorubicin or ceritinib on neuroblastoma cell lines with *TERT* rearrangements (CLB-GA, GI-ME-N), high *TERT* expression (SH-SY5Y) or *MYCN* amplification (SK-N-Be(2)c, IMR-32, Kelly, LS, TR-14) were determined after 96 hours of substance incubation using CellTiter-Glo®. Drug synergy was calculated using the Chou-Talalay method. Tumor growth inhibition of subcutaneous xenografts was assessed upon treatment with 6-thio-dG, imetelstat, etoposide, or combinations of these compounds. Telomerase activity in imetelstat-treated and control tumors was measured using a TRAP assay.

Results

*S*trong synergies were observed between 6-thio-dG and etoposide or doxorubicin in most telomerase-positive neuroblastoma cell lines *in vitro*. By contrast, we did not observe a synergistic effect of 6-thio-dG in combination with ceritinib in *ALK*-mutated or *ALK*-wildtype cell lines *in vitro*. Treatment of mouse xenografts with combinations of 6-thio-dG and etoposide or imetelstat and etoposide significantly attenuated growth of cell lines bearing a *TERT* rearrangement (CLB-GA; $p<0.001$ and $p=0.001$ respectively) or high *TERT* expression (SH-SY5Y; $p=0.001$ and 0.011 respectively) in comparison to xenografts treated with single compounds. Telomerase activity in xenograft tumors treated by imetelstat was decreased by roughly 50%, indicating intermediate on-target specificity in this setting.

Summary/Conclusions

Telomerase inhibition by imetelstat or 6-thio-dG act synergistically with genotoxic drugs in a fraction of telomerase-positive neuroblastoma and may thus represent a promising treatment option for high-risk patients. Our data also suggest that the development of telomerase-interacting compounds with higher on-target specificity may further improve the efficacy of such treatment strategies.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 229

High-throughput drug screening of high-risk neuroblastoma PDX-derived tumor cells

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

Pediatric precision oncology platforms in Europe have analyzed over 1,000 pediatric cancers by next generation sequencing. While potential targets can be identified in 50% of cases, the remaining patients lack actionable alterations. This occurs particularly in high-risk neuroblastoma (HR-NB), indicating significant, currently unmet needs in precision medicine. Patient-derived tumor xenograft (PDX) mouse models have been established within these platforms. Translating *ex vivo* drug profiling results in combination with genetic data into clinical application has been successful in small pilot series, providing proof-of-concept for further systematic evaluation of the functional precision medicine approach.

Aims

The aim of this project is to validate the feasibility of high-throughput drug screening on HR-NB PDX-derived tumor cells (PDTCs). Our ultimate goal is to improve current therapeutic approaches in HR-NB by establishing next generation drug sensitivity and resistance profiling in PDTCs.

Methods/Materials

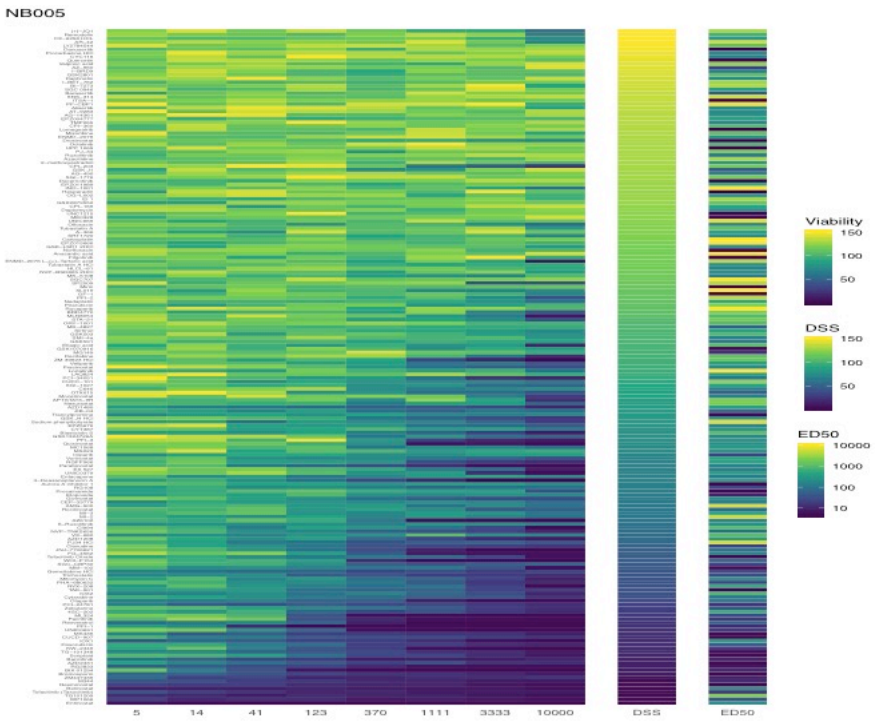
We established a biobank of 15 HR-NB PDXs with known genetic profiles and at least 1 actionable mutation (range 1-4). PDTCs were obtained by mechanical/enzymatic dissociation and plated in 384-well plates. The proportion of tumor content in PDTCs was determined by RT-qPCR. Drugs were added 24 hours after plating (D1) at 8 different concentrations each. We screened four HR-NB models using commercially available arrayed drug library representing 182 epigenetic compounds and evaluated their effect on cell survival. We also compared the effect of those drugs to common chemotherapy used in NB and other selected targeted agents. Drugs were administered in monotherapy, unless chemotherapy (monotherapy/combination). Cell viability was measured by Cell-titerGlo® at plating (D0), before drug addition (D1) and at D4. Drug efficacy was assessed by quantitative drug sensitivity scoring and half-maximal effective concentration.

Results

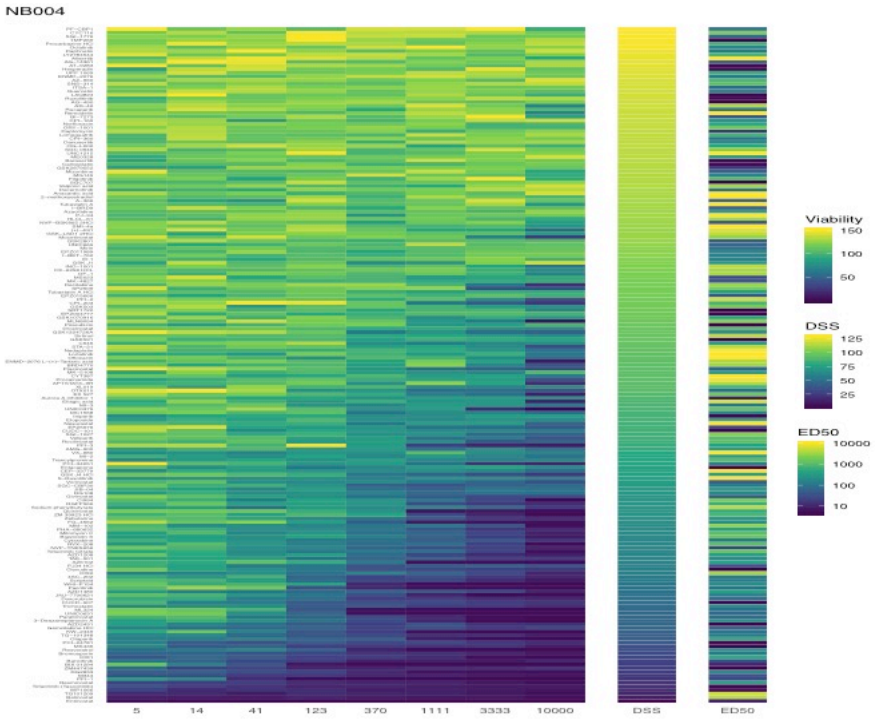
A median number of 143.10^6 [42.10^6-376.10^6] cells/tumor was obtained, with a median viability of 83,5% [82-88]. Models HSJD-NB004 and HSJD-NB005, *MYCN* amplified-NB PDX established at diagnosis and relapse from the same patient, harbored *TP53* (c.517G>T), *ARID1B* (c.3968G>A) and *NF1* (c.7669C>T) mutations, with HSJD-NB005 harboring additional *ROS1* (c.1114A>G) mutation. MAP099 model harbored *MYCN*/*ALK* amplifications, with homologous deletion of *CDKN2A/B*. Model MAP422 showed high mutational load (33 mutations/Mb).
Our drug sensitivity results, available for 2 models, showed differential effects on the proliferation of HSJD-NB004 and HSJD-NB005 models. Surprisingly, both models were resistant to etoposide and carboplatin in monotherapy, and were also relatively resistant to the combined treatment. In contrast, both models responded at relatively high concentrations of doxorubicin. The effect of doxorubicin combined to etoposide was very similar to the effect induced by doxorubicin-carboplatin, in a dose-dependent manner. High sensitivity (top 10 efficacy drugs) was found to HDAC, JAK/STAT and BET inhibitors. Interestingly, lorlatinib showed no effect in HSJD-NB-005. Additional studies are needed to explain the different sensitivity of both models. Taken together, our results suggest that inhibition of HDAC, JAK/STAT pathway or BET proteins may provide an effective strategy to treat a subset of HR-NB.

Summary/Conclusions

We propose that drug response profiling of PDTCs by high-throughput drug screening will provide additional key information for precision pediatric oncology. We think this approach will push forward the development of innovative clinical trials involving personalized drug sensitivity profiles as biomarkers for trial inclusion.



Heat-map of cell viability based on DSS at 8 different concentrations of 200 compounds for PDX model HSJD-NB-004



Heat-map of cell viability based on DSS at 8 different concentrations of 200 compounds for PDX model HSJD-NB-005

Type Translational

Presentation Preference Oral presentation

Abstract nr. 230

Impact of anti-GD2 antibody at induction on ability to achieve gross total resection of high-risk neuroblastoma

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

High-risk neuroblastoma requires multi-modality therapy including intensive chemotherapy, surgery, high-dose chemotherapy with stem cell rescue, radiation and biologic agents. The addition of anti-disialoganglioside mAb (anti-GD2 mAb) immunotherapy has improved response rates. In previous trials, anti-GD2 antibody therapy was used at the end of therapy to treat minimal residual disease. Two current trials administer the anti-GD2 antibody upfront, during induction, with encouraging results.

Aims

The aim of this study was to evaluate the impact of anti-GD2 antibody given during induction on the completeness of surgical resection. Our hypothesis was that an increased percentage of patients would have a gross total resection (>90%) with this induction regimen compared to historical controls. Our control cohort consisted of patients with high risk neuroblastoma treated on regimens that did not include anti-GD-2 antibody administered during induction, operated on by the same surgeon at our institution.

Methods/Materials

Following IRB approval, we retrospectively reviewed charts of patients treated on our, institutional phase II trial (NB2012) using a humanized anti-GD2 mAb (hu14.18K322A) in combination with granulocyte–macrophage colony-stimulating factor (GM-CSF) and low-dose IL2 during induction together with induction chemotherapy agents cyclophosphamide, topotecan, cisplatin, etoposide, doxorubicin, and vincristine. We compared these patients to those treated on our previous institutional high-risk neuroblastoma protocols where induction therapy did not include anti-GD-2 mAb immunotherapy. The number of image-defined risk factors (IDRF) at diagnosis, the number

of pre-operative cycles of chemotherapy, and the time to resumption of chemotherapy post-operatively were determined. Tumor volume on immediate pre-operative computed tomography (CT) and end of induction CT (after surgical resection of primary tumor) was measured by a single pediatric radiologist and the percent decrease of tumor volume was calculated. Patients who did not have surgery or who had upfront surgery prior to chemotherapy were excluded.

Results

There were 36 patients eligible to be reviewed on historic trials from 1999-2009 and 47 on NB2012, after excluding 21 and 18 patients, respectively. The number of patients with 3 or more IDRFs at diagnosis was significantly higher in NB2012 patients (84.8%) compared to those on prior trials (56.7%, p=0.009). In the older trials, the mean percent reduction of tumor volume after surgery compared to pre-operative imaging was 88.0% (±21.2%) with 66.7% achieving a >90% resection. For patients on NB2012, the mean percent reduction was 90.7% (±17.6%) with 70.2% achieving a >90% resection. The percent reduction (p=0.52) and ability to achieve >90% resection (p=0.813) were not significantly different. There was no difference between the two groups in the number of cycles of chemotherapy administered pre-operatively (mean 3.7±1.6 historic vs 3.2±1.2 NB2012, p=0.10). Patients in both groups resumed chemotherapy within similar timeframes post-operatively (12.3 days historic versus 11.9 days on NB2012, p=0.742).

Summary/Conclusions

Despite a patient population/referral pattern with significantly more IDRFs at diagnosis, the use of anti-GD2 antibody during induction allowed for similar gross total resection rates without requiring an increased number of pre-operative treatment cycles or resulting in delays in resumption of chemotherapy post-operatively.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 231

A phase I/II proof-of-concept study using novel hybrid imaging technique [124I]mIBG PET/CT and PET/MRI in improving imaging of neuroblastoma.

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Topic Other

Background/Introduction

Functional imaging plays a critical role in prognostic stratification, response assessment and treatment decisions makings for patients with high-risk neuroblastoma. Meta-iodobenzylguanidine(*m*IBG) is a well-established imaging and molecular radiotherapy agent in neuroblastoma, for which 95% of cases are *m*IBG-avid at diagnosis. Planar scintigraphy using *m*IBG labelled with Iodine-123([^{123}I]-*m*IBG) is the current imaging gold standard for staging and response assessment, but the technique has relatively poor resolution and is not easily

standardised/quantified despite the use of semi-quantitative scores. Recently, newer hybrid functional/anatomical imaging technologies have become available whose potential in neuroblastoma imaging requires evaluation.

[124I]*m*IBG as a novel PET tracer is hypothesised to improve diagnostic accuracy, prognostic stratification and treatment decisions and potentially minimise the risk of overtreatment in neuroblastoma patients, and additionally will simplify their staging/ restaging work-up by decreasing the number of investigations and the need for sedation/general anaesthesia in children.

Aims

The primary objective of this study is to compare the diagnostic performance of [^{124}I]-*m*IBG PET/CT, with conventional [^{123}I]mIBG imaging, in detecting an equal or greater number of neuroblastoma lesions.
Secondary and tertiary objectives include the tolerability and safety of administration of [^{124}I]*m*IBG, and comparison of the number of lesions detected as positive by [^{124}I]*m*IBG hybrid PET/MRI with [^{124}I]*m*IBG PET/CT and conventional imaging with [^{123}I]*m*IBG planar scintigraphy, SPECT/CT, routine CT and MRI.

Methods/Materials

A multi-centre, non-therapeutic Phase I/II clinical trial (NCT02043899) directly comparing [^{124}I]-*m*IBG PET/CT and conventional [^{123}I]*m*IBG imaging in patients with newly diagnosed, relapsed or refractory metastatic neuroblastoma was conducted over a period of 5 years. [^{124}I]-*m*IBG PET/CT scans were acquired 24 hours post injection. Results were assessed by four independent reviewers. Parameters include lesion number, site, Standardized Uptake Value-SUV(mean, max and peak), Metabolic Tumour Volume(MTV). PET/MR data includes maximum axial diameter(MAD) and mean apparent diffusion coefficient(ADC). Inter-observer agreement in scoring the number of positive lesions, confidence scores, SIOPEN and CURIE scores between observers using kappa statistics and percentage agreement were measured.

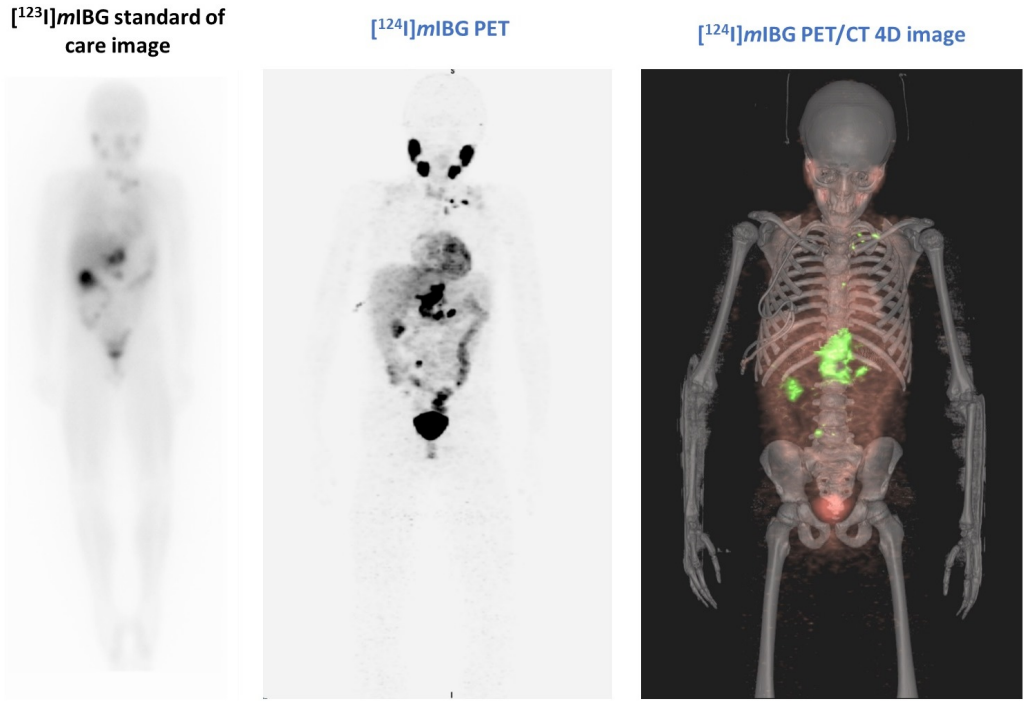
Results

Nine evaluable patients (4 to 41yrs) with metastatic neuroblastoma were scanned with [^{123}I]*m*IBG planar scintigraphy and SPECT/CT, and [^{124}I]*m*IBG PET/CT. Five patients also underwent hybrid PET/MRI scans after the PET/CT scan allowing the direct comparison of the two imaging modalities. The mean injected activity was 283 MBq (92.3 to 450 MBq) for [123I]-*m*IBG and 35 MBq (17.8 to 52 MBq) for [^{124}I]-*m*IBG. No adverse effects related to the imaging agents were observed. Qualitative and semi-quantitative imaging analysis results will be available in May 2020.

Summary/Conclusions

[^{124}I]*m*IBG PET/CT and PET/MRI are feasible and safe imaging modalities for children with high risk neuroblastoma that could potentially improve diagnostic accuracy relative to currently employed conventional imaging techniques. They could improve the accuracy of response assessment to existing and novel therapies, and reduce both the number of imaging investigations and sedation/ general anaesthetics required in young children. Logistically, hybrid imaging offers a

“one-stop shop” assessing morphological and functional information simultaneously and can also provide additional information on tumour cellularity. Both [^{124}I]*m*IBG PET/CT and PET/MRI are promising techniques worthy of further evaluation in neuroblastoma, in particular in the context of [^{131}I]-*m*IBG radioisotope therapy.



[124I]mIBG vs [123I]mIBG images

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 232

Survival Impact of Anti-GD2 Antibody Response – A Phase II Ganglioside Vaccine Trial in Relapsed Neuroblastoma

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

High-risk neuroblastoma (HR-NB) relapse carries a dismal prognosis. Recent experience, however, suggests that cure is possible when remission is achieved with salvage therapy that includes anti-GD2 monoclonal antibody (mAb) (*OncoImmunol 2015*). The favorable survival outcome and safety profile after vaccination with gangliosides GD2 and GD3 in a Phase I trial (n=15, *Clinical Cancer Res 2014)*, and the importance of seroconversion (mounting anti-vaccine antibody response) need to be confirmed.

Aims

To analyze the kinetics of seroconversion and its prognostic impact on survival in a Phase II trial (Clinicaltrials.gov NCT00911560) amongst HR-NB patients with prior disease progressions.

Methods/Materials

A cohort of 102 HR-NB patients who achieved complete remission after salvage therapy were enrolled in this trial. They received 7 subcutaneous vaccine injections (week 1-2-3-8-20-32-52) plus oral beta-glucan (starting week 6 at 40 mg/kg/day, 14 days on/14 days off). Each vaccine injection consisted of 30 µg each of GD2 and GD3, lactonized and conjugated to the immunogenic carrier protein keyhole limpet hemocyanin and mixed with the saponin OPT-821 adjuvant (150 $\mu g/m^2$). Serum anti-vaccine antibody titers were quantified by ELISA. Kaplan-Meier analyses and multivariable prognostic models were used for survival estimates.

Results

Among 102 patients, 63% had one, 21% had 2, and 16% had 3-6 prior disease progressions. 83/100 patients had failed prior anti-GD2 mAb (m3F8, dinutuximab, or naxitamab) therapy before vaccine: one mAb (n=62), two mAb (n=15), or all three (n=6). Common toxicities were self-limited injection-related local reaction and fever. No pain, neuropathy, or grade 3/4 toxicities occurred during or post treatment. Progression-free survival (PFS) was 44%±5% and overall survival (OS) was 88%±4% at 2 years, and 36%±7% and 70%±8% at 5 years, respectively. Serum anti-GD2 (IgG1 and IgM) and anti-GD3 (IgG1) titers had marked increases following the initiation of beta-glucan at week 6. In univariate analyses, favorable prognostic factors included: one versus ≥2 prior disease progressions (PFS p=0.005, OS p=0.04); none versus any prior anti-GD2 mAb failures (PFS p=0.003, OS p=0.01); and the induction of ≥150 ng/ml anti-GD2-IgG1 titers by week 8 (PFS p=0.02, OS p=0.06). Factors not prognostic included: time to first NB progression, *MYCN* amplification status, anti-GD2-IgM, anti-GD3-IgG1 or anti-KLH-IgG1 titers, and treatment with anti-GD2 mAb right before vaccine. In multivariate analyses, week 8 anti-GD2-IgG1 titer ≥150 ng/ml yielded a hazard ratio (HR) of 0.41 [0.20, 0.83], p=0.01 for PFS, and HR=0.15 [0.02, 1.12], p=0.06 for OS. The second independent prognostic variable was the number of prior disease progressions (≥2 vs 1), yielding HR of 2.12 [1.26, 3.58], p=0.005 for PFS, and HR =2.77 (1.03, 7.47), p=0.04 for OS.

Summary/Conclusions

The absence of toxicities despite seroconversion contrasted sharply with the side effects of conventional anti-GD2 mAb therapy. Even with prior disease progressions and prior failures from anti-GD2 mAb therapy, anti-GD2 (though not anti-GD3) seroconversion was associated with notable long-term survival among HR-NB patients previously thought to be unsalvageable. A randomized trial to assess the role of beta-glucan in seroconversion is actively accruing patients.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 233

Risk factor evaluation in high-risk neuroblastoma patients receiving dinutuximab beta (DB) based immunotherapy: A report from the HR-NBL-1/SIOPEN Trial

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

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Prognostic factors at diagnosis were previously incorporated into a multi-variable pseudo-value regression model in HR-NBL1/SIOPEN patients treated without DB to develope a simple score including age, serum serum lactate dehydrogenase (LDH) and metastatic site index (MSI). Points were allocated in proportion to the log-cumulative hazard ratio (cHR): cHR for LDH >1250U/L was

1.33 (score of 1), cHR for age >5 years and metastatic site involvement (MSI) >1 were 1.79 and 1.86 (scores of 2).

Aims

...We tested if this score (Morgenstern, PBC 2018) would provide meaningful information in DB treated HR-NBL1/SIOPEN patients (validation cohort).

Methods/Materials

....High-risk patients (stage 4 ≥1yr.; stage 4 <1yr. with MYCN amplification (MNA); stage 2, 3, 0-21y with MNA) received intensive induction (rapid COJEC or N5-MSKC and TVD for insufficient response), surgery, high dose therapy with BUMEL or CEM and local radiotherapy (21Gy). Patients ≤9 months intervall till HDT/SCT with ≥ partial response prior and no progression thereafter received up to 5 cycles of 100mg/m^2 DB: 2006 to 2013 as short-term (STI: d 8-12) or 2014 to 2018 as long-term infusion (LTI: d8-17) ± 6 (STI) or 3 (LTI) x 10^6 IU/m^2 subcutaneous interleukin 2 (scIL2 over 2 x 5 days) followed by 160mg/m^2 oral isotretinoin (d19-32).

Results

....1018 patients [512 males; 749 Stage 4(89%), 13 Stage 4s(1%), 86 loc. MNA(10%); median age at diagnosis 2.8 yrs. (1 day – 20 yrs.)] received DB in 18 countries participating. Their 2-yrs. event-free (EFS)/overall survival (OS) was 0.65±0.02/0.78±0.01 and the 5-yrs. EFS/OS of 0.56±0.02/0.63±0.02. 891/1018 patients were evaluable for risk factor and score analysis. STI was used in 541(61%) patients, LTI in 350(39%). DB without scIL2 was given to 550(62%) patients and with scIL2 to 341(38%). There was no difference in EFS/OS neither regarding infusion schedule (5-yrs. EFS of 0.58±0.02 for STI and 0.64±0.03 for LTI) nor for the addition of scIL2 any schedule (5-yrs. pEFS of 0.57±0.02 without and 0.61±0.03 with scIL2). Patients in complete remission prior DB treatment had better outcomes than those with residual disease (in CR: 5-yrs. EFS/OS of 0.60±0.03/ 0.68±0.03; for non-CR: 5-yrs. EFS/OS of 0.54±0.03/0.57±0.03). Younger patients faired better than older ones (5-yrs. EFS: <1.5-yrs: 0.74±0.06, 1.5-yrs. to 5-yrs: 0.58±0.02 and >5-yrs. 0.48±0.04) as did localised ones or those with only 1 MSI (5-yrs. pEFS: 0.77±0.05, 0.78±0.05, 0.54±0.02 >1 MSI). EFS multivariate analysis included age, MSI, LDH ≥1250U/L, HDT, response prior DB, DB schedule and use of scIL2: only age >1.5yrs.(p=0.002) and MSI >1 (p= 0.002) had independent prognostic prediction. If restricted to stage 4 only, LDH ≥1250U/L was an additional independent factor (p=0.049). Using the score (0 to 5) relevant risk groups can be identified: 5-yrs. EFS/OS for scores 0&1 are 0.80±0.06&0.77±0.04, score 2/3 are 0.58±0.03&0.57±0.04 and for scores 4/5 are 0.50±0.06&0.43±0.08. Similar outcome proportions apply for OS as well as for EFS/OS for stage 4 only.

Summary/Conclusions

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The score adds value in DB treatment groups as it differentiates prognostic subgroups facilitating decision making for future DB add on treatments.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 234

Early molecular characterization and monitoring of neuroblastoma by circulating tumor DNA analysis

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Molecular aspects of neuroblastoma establish a wide clinical variability. Given the oncogenic role of *MYCN*, *ALK* or genes involving the hemizygous deletion in chromosome 11q (del11q), characterization and monitoring of these molecular biomarkers are crucial for neuroblastoma patients. Recent data indicate that implementing molecular characterization of tumors by circulating tumor DNA (ctDNA) analysis, will improve disease assessment. Certainly, plasma ctDNA liquid biopsy (LB) testing using high sensitivity molecular assays would allow early detection of tumor growth through a non-invasive peripheral blood extraction. In fact, the invasiveness of surgical biopsies complicates disease monitoring and cannot allow molecular characterization for extremely difficult access or poor quality of surgical samples.

Aims

This work aims to perform a methodological strategy for neuroblastoma molecular characterization and patient monitoring using plasma LB.

Methods/Materials

The study included 26 pediatric patients with neuroblastoma that were diagnosed or referred to the Pediatric Oncology Unit of Hospital La Fe (58% at diagnosis and 42% from relapse), from 2017 to 2019.

Peripheral blood samples were collected into EDTA tubes and separated into plasma and blood cells within 24 h (1,900 g 10 min 4 ºC; 16,000 g 10 min 4 ºC). Circulating cell-free DNA (ccfDNA) from plasma and germinal DNA from blood cells were isolated with *QIAmp Circulating Nucleic Acid* and *QIAmp DNA mini* kits (Qiagen), respectively. Tumor DNA (tDNA) from matched tumor samples was extracted with *QIAamp DNA Investigator kit* (Qiagen). The DNA size distribution and

quantification were performed in a Bioanalyzer TapeStation (Agilent Technologies) or Qubit (Invitrogen).
DNA samples were sequenced in a Illumina NGS platform (NextSeq500), using a NGS custom panel and *SeqCap EZ HyperCap kit* (Roche). The panel allowed coding (with flanking intronic) regions and copy number variation (CNV) analysis in 408 genes related to pediatric cancer. *ALK* oncogenic mutations, *MYCN* or *ALK* amplifications and del11q were monitored by ddPCR in ccfDNA, using a QX200™ Droplet Digital™ PCR platform and specific assays (Bio-rad).

Results

In all cases, somatic genetic variants identified in tDNA were also detected in matched ctDNA samples by NGS panel testing. Also, CNV in matched ctDNA were detected when there was more than 20% ctDNA in ccfDNA. In two patients, both somatic variants and CNV were detected in ctDNA but unnoticed in matched tDNA (highlighting the oncogenic *ALK*:p.R1275Q variant in one patient and *MYCN* amplification in the other).
Monitoring was performed in 45% (9/20) patients with *MYCN* amplification, 15% (3/20) with *ALK* amplification, 40% (8/20) with del11q and 30% (6/20) with oncogenic somatic *ALK* mutations (83% ALK:p.R1275Q and 17% p.F1245C). A total of 62 serial ccfDNA samples were analyzed by ddPCR during patient follow-up. The results were plotted in follow-up graphs, showing clonal evolution of tumors carrying the specific oncogenic alteration. The results agreed with the disease status and predicted recurrences in all cases.

Summary/Conclusions

The methodological strategy performed to analyze plasma LB by our custom NGS panel and specific ddPCR assays proved to be a potencial non-invasive alternative to characterize neuroblastoma tumors and monitor the disease during follow-up, showing new oncogenic biomarkers not seen in tumor biopsies.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 235

Adjusted administration of a T-cell utilizing bispecific anti CD3/GD2 construct and CH14.18mAb in relation to immune recovery after alloSCT in patients with relapsed neuroblastoma

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

Whereas immunotherapy in high-risk/relapsed neuroblastoma with standard antibody formats targeting the tumor associated antigen GD2 is currently used after autologous/allogeneic stem cell transplantation (SCT), T-cells have rarely been addressed as effectors. Bispecific antibodies can bring T-cells into close proximity to tumor cells and activate them through the CD3 region, which ultimately leads to target cell elimination. They lack the Fc-domain and cannot trigger ADCC or CDC as NK-cells. An example for bispecific antibodies is NG-CU (developed at the Department of Immunology, University of Tuebingen), which recognizes CD3 and GD2 as target antigens. The tumor antigen specific antibody domain, derived from a fab fragment of GD2 14.18 antibody and the T-cell recruiting antibody domain, derived from single chain variable fragment (scFv fragment) of UCHT-1 (CD3) are linked by modified CH2 domain.

Aims

We investigated whether the bispecific antibody NG-CU might be an alternative to the therapeutic monoclonal antibody CH14.18, which mediates CDC and ADCC through NK-cells.

Methods/Materials

Different antibody concentrations and effector to target ratios (E:T) were evaluated using the xCELLigence RTCA system, PBMCs (healthy donors and patients after allogeneic/autologous SCT) and the neuroblastoma cell lines LS and LAN-1. CDC was evaluated using autologous serum.

Results

NG-CU showed enhanced cytotoxicity compared to CH14.18. Median specific lysis (n=3) after 12/24/48 hrs (effectors: healthy PBMCs, E:T=5:1) with LS cells was: 55/73/77% (CH14.18, 1 µg/ml) vs. 70/100/100% (NGCU, 100ng/ml). P values with Mann-Whitney test: p=0,0244; p<0,0001; p<0,0001. With LAN-1 cells: 56/69/88% (CH14.18, 1 µg/ml) vs. 9/46/99% (NGCU, 100 ng/ml). P values with Mann-Whitney test: p<0,0001; p=0,1139; p=0,0464.
Taking CDC into account by adding autologous serum, cytotoxicity of CH14.18 was drastically increased. ADCC and CDC eradicated targets with 64% after 4 hrs and 93% after 48 hrs (E:T=5:1). However, using a lower E:T ratio (1:1) lysis of CH14.18 remained static, but not for NG-CU. Median lysis (n=3) after 12/24/48 hrs: 71/74/77% (CH14.18) vs. 25/42/67% (NG-CU). After >48 hrs targets were completely eradicated by NG-CU, while targets treated with CH14.18 still proliferated.
Using patient PBMCs, significant lysis with both constructs was detected dependent on percentages and total numbers of T- and NK-cells.
In the first wave of immune regeneration after SCT, NK-cells represented the majority of effectors. Here, CH14.18 produced higher lysis, whereas later on, associated with increasing T-cell counts, NG-CU was more effective. With both antibody constructs complete eradication of neuroblastoma cells (LS+LAN-1) was detectable.

Summary/Conclusions

Using healthy PBMCs, NG-CU showed enhanced cytotoxicity compared to CH14.18 even in lower concentrations and E:T ratios. Dependent on the differential recovery of effector cells posttransplant either NK-cell based or T-cell based antibody constructs might result in optimal anti-tumor activity. An adjusted administration of both antibody constructs in relation to the state of immune recovery after SCT could be an alternative to classical neuroblastoma approaches.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 236

Single cell DNA sequencing in neuroblastoma: zooming in on intratumor heterogeneity

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

Intratumor genetic heterogeneity has been widely reported in neuroblastoma (NB) and can contribute to treatment failure following clonal selection during disease evolution. Single cell (SC) DNA sequencing techniques now allow a precise description of genetic features at a single cell level, enabling to characterize tumor cell populations in detail.

Aims

We sought to identify genetic alterations at a SC DNA level, aiming to reconstruct NB phylogeny.

Methods/Materials

Samples from 3 NB PDX models (IC-pPDX-17, HSJD004 and NB-422) and from one primary tumor studied relapse (NB1483) were included in this study, and the bulk of these 4 samples was whole-exome sequenced (depth of coverage 100x).
Two different SC DNA sequencing methods were then applied: a manual approach and the commercialized 10X Genomics® method.
In the manual method, the tumor sample was dissociated enzymatically and suspended for SC isolation. Tumor cells were stained with GD2 and CD81 antibodies and then flow sorted in 96-well plates. DNA from single cell was extracted and whole-genome amplified; 50ng of amplified DNA was then used for library construction.
For the 10X Genomics® method, PDX samples were enzymatically digested and 1000 single cells were isolated using the commercially available CNV kit. Libraries were whole genome sequenced, and raw sequencing data was processed through barcode-aware bioinformatic analysis using the Cell Ranger DNA (10X Genomics®) pipeline. Copy number alterations (CNA) and variants were called using FREEC and Mutect2 respectively at single cell resolution, and by creating a pseudo-bulk of defined cell clusters. Clusters were inferred from CNA information by hierarchical clustering.

Results

From IC-pPDX-17, NB-422 and HSJD004, a total of 662, 332 and 131 single cells were scDNA sequenced, respectively. Altogether, 1151 single cells could be analyzed, (excluding ~5% of noisy cells), with a mean depth of coverage of 0.33x to 1.6x. Ploidy was distributed around 2 for a majority of cells in HSJD004 and NB-422, and around 4 in IC-pPDX-17. CNAs, including amplifications of *MYCN* and *CDK4* in HSJD004 and IC-pPDX-17 respectively, were observed in all single cells, confirming the tumor origin of the analyzed cells (Figure-A). Comparing CNA profiles between SCs and the bulk of the same PDX, single cell-specific CNAs could be detected (Figure-A). Distinct clusters were defined in each sample mainly based on aneuploidies (3 clusters in IC-pPDX-17 and NB-422 and 2 in HSJD004).
For the NB1483 sample, 21 SC were analyzed (mean depth of coverage of 0.4x). A focal deletion on chr9 p observed in the bulk was clearly confirmed in all 21 SC.
Importantly, in all 4 cases, all somatic single–nucleotide variations (SNVs/Indels) detected in the bulk were also seen in the SC samples, with a total of additional 42 SNVs detected only at a SC level, further highlighting intratumor heterogeneity. Detailed analysis of inferred clusters is ongoing.

Summary/Conclusions

ScDNA sequencing approaches constitute a powerful method to study intratumor genetic heterogeneity in NB. With the potential to identify CNAs as well as SNVs at a SC level, these are promising tools to identify different clones in a given sample and to reconstruct tumor phylogeny.

Figure A

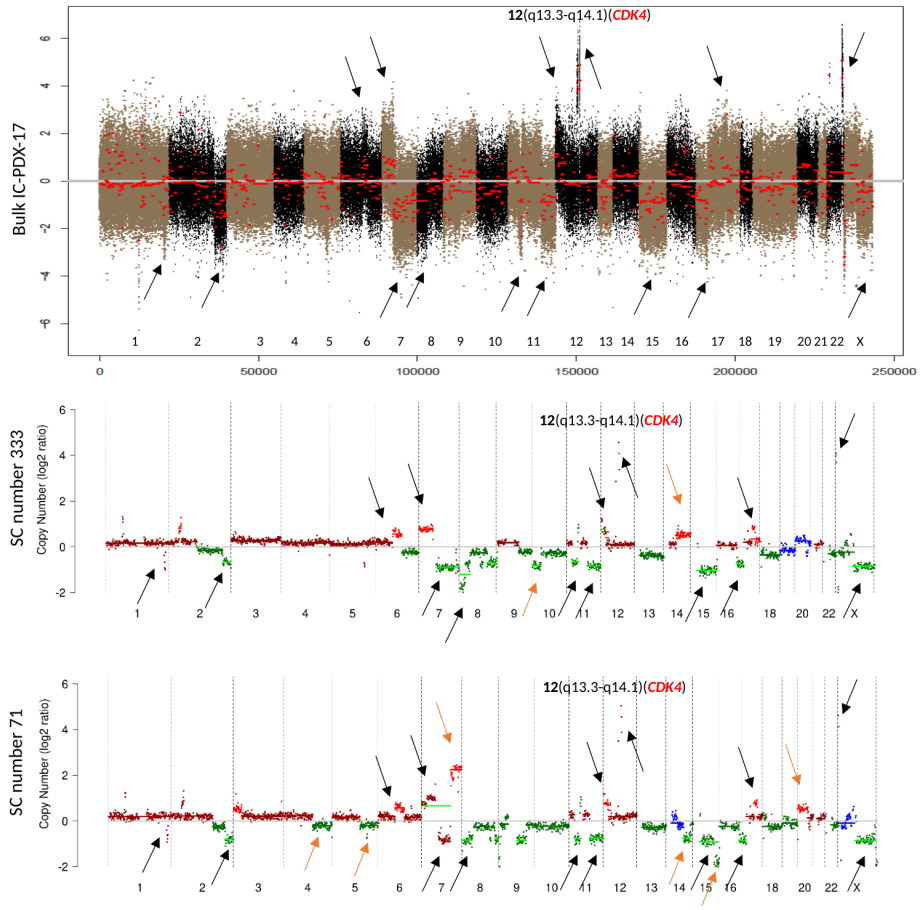

Figure A. Comparison between CN profiles of 2 single cells (333 and 71) and the bulk of IC-PDX-17 sample. Black arrows show segmental CNAs that are in commun between bulk and the 2 single cells. Orange arrows show additional distinct CNAs that we noticed in the 2 single cells but that can not be distinguished in the bulk

Figure A. Comparison between CN profiles of 2 single cells (333 and 71) and the bulk of IC-PDX-17 sample. Black arrows show segmental CNAs that are in

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Type Translational

Presentation Preference Oral presentation

Abstract nr. 237

Addition of Lorlatinib to induction chemotherapy significantly prolongs survival in ALK-mutant murine neuroblastoma models

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

14% of high-risk neuroblastoma tumours carry activating mutations in *ALK* at diagnosis. This genotype carries an increased risk of relapse after first-line treatment, and evidence from paired tumour biopsies shows enrichment of *ALK*-mutant clones at relapse.
ALK mutation provides a survival advantage to neuroblastoma cells via both pro-proliferative signalling, and inhibition of apoptosis. Selection for *ALK*-mutant sub-clones at relapse may therefore be caused by their relative resistance to cell death during cytotoxic treatment.
Targeted ALK inhibitors have been trialled in relapsed paediatric cancers and the first-generation inhibitor Crizotinib has now entered a Phase III study for newly-diagnosed *ALK*-mutant neuroblastoma. However, our laboratory has previously demonstrated increased efficacy of the third-generation inhibitor Lorlatinib compared to Crizotinib as a single agent *in vivo.*

Aims

To evaluate the preclinical efficacy of Lorlatinib in combination with cytotoxic chemotherapy in *in vivo* models of *ALK*-mutant neuroblastoma, and to test the hypothesis that Lorlatinib is more effective than Crizotinib when given in combination with multi-agent induction chemotherapy.

Methods/Materials

Mice from the Th-*ALK*^{F1174L}/Th-*MYCN* murine neuroblastoma model were enrolled with established intra-abdominal tumours. Each received a single intraperitoneal dose of chemotherapy (Vincristine, Doxorubicin and Cyclophosphamide, ‘VAC’) on day 1. Oral Lorlatinib (10mg/kg/day)

or vehicle was commenced on day 2 and continued until mice required euthanasia. MRI was performed weekly to measure tumour volume.
Syngenic mice were injected subcutaneously with untreated cells derived from the Th-*ALK*^{F1174L}/Th-*MYCN* model to provide a predictable, robust i*n-vivo* model for multi-arm, multi-agent preclinical studies. When tumours were >8mm mean diameter, they received a single dose of VAC or vehicle, followed by continuous oral dosing of either Lorlatinib (10mg/kg/day), Crizotinib (100mg/kg/day) or vehicle from day 2 until day 56 (or until euthanasia was required).
Frozen tumour tissue was collected at necropsy for RNA-Seq analysis and compared to publically-available data from human neuroblastoma.

Results

Addition of Lorlatinib to chemotherapy significantly prolonged median survival in the Th-*ALK*^{F1174L}/Th-*MYCN* transgenic mouse from 13 days to 33.5 days (p=0.0005). MRI showed prolonged reduction in tumour burden in animals receiving the combination.
In subcutaneous Th-*ALK*^{F1174L}/Th-*MYCN* allografts, this effect was confirmed (median survival 22.4 weeks vs 5.9 weeks, p=0.0067). 3 of 4 animals receiving VAC and Lorlatinib combined had durable complete responses, with no tumour evident at necropsy. For animals treated with Crizotinib in addition to VAC, no survival benefit was observed (median survival 5.8 weeks vs 5.9 weeks).
RNA-Seq of untreated Th-*ALK*^{F1174L}/Th-*MYCN* tumours showed increased expression of anti-apoptotic *BCL2* family members compared to control tumours from Th-*MYCN* single-transgenic murine neuroblastoma. We identified the same anti-apoptotic pattern in human neuroblastomas expressing high levels of *ALK* mRNA, suggesting this is a clinically-relevant effect of ALK activation. We are currently analysing these anti-apoptotic markers in treated murine tumours to confirm their relationship to treatment response.

Summary/Conclusions

The addition of Lorlatinib, but not Crizotinib, to VAC chemotherapy significantly prolongs survival in *ALK*-mutant murine neuroblastoma. This strongly supports development of clinical trials combining Lorlatinib with standard-of-care induction chemotherapy in children with high-risk neuroblastoma that is *ALK*-mutant at diagnosis.

Type Translational

Presentation Preference Rapid Fire Poster Presentation

Abstract nr. 238

Tumor suppressive functions of an identified trunk neural crest specific gene

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Topic Neuroblastoma as developmental disease

Background/Introduction

Neuroblastoma (NB) is a childhood cancer with origin in the developing sympathoadrenal lineage in the early neural crest.

Aims

To investigate the role of and potential tumor suppressive functions in NB of a gene recently identified as trunk neural crest (NC) cell specific.

Methods/Materials

In silico of gene expression in NBs from publicly available RNA sequencing data. We overexpressed the identified NC specific gene in NB cell lines and performed RNA sequencing, analyzed tumor growth *in vivo*, and cell movement, clonogenic capacity, differentiation and proliferation *in vitro*. *In vivo* experiments modulating gene expression in chick embryo NC cells were also performed.

Results

The trunk NC specific gene is expressed in a time- and spatially restricted pattern during early human (embryonic week 5 and 6) and avian (E1.5 and E3) NC development. Tissue specific knockdown of the gene during normal avian development resulted in delayed embryonic growth. Gene overexpression in NB cells with no endogenous expression resulted in less tumor burden by decelerated tumor growth and prolonged survival *in vivo* as well as decreased proliferation *in vitro* . Through RNA sequencing we found a defined set of eight genes involved in cell invasion, cell adhesion, angiogenesis, and differentiation. Our identified trunk NC specific gene further discriminates between the mesenchymal- and adrenergic phenotype in NB being virtually exclusively expressed in mesenchymal, neural crest like NB cells and, in addition, in Schwann cell precursor cells.

Summary/Conclusions

We present evidence of tumor suppressive functions of this trunk NC specific gene. Gene knockout in chick embryonic NC cells with endogenous expression as well as overexpression in NB cells lacking the endogenous protein suggest important functions during normal and tumor development. The genetic profile depicts a sympathoadrenal origin and our data can aid in defining a subtype of NB driven by loss of trunk NC specific genes.

Type Basic

Presentation Preference Traditional poster

Abstract nr. 239

TIAL1 is a MYCN-regulated alternative splicing protein that modifies NF1 to increase RAS-MAPK signaling in neuroblastoma

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

Activation of RAS-MAPK signaling occurs commonly in relapsed neuroblastoma. Recent studies suggest that increased RAS-MAPK signaling confers aggression in primary and relapsed tumors. NF1 is a GTPase activating protein (GAP) that negatively regulates RAS-MAPK signaling, and is mutated in relapsed neuroblastoma. Inclusion of exon 23a in NF1 mRNA generates an $NF1^{23a}$, a hypomorphic GAP that inefficiently blocks RAS-MAPK signaling. Inclusion of NF1 exon 23a is regulated in-part by the splicing protein TIAL1. Amplification of *MYCN* drives approximately half of high-risk neuroblastoma, and is further known to transcriptionally regulate the alternative splicing landscape. We hypothesize that TIAL1 is a central effector of primary *MYCN*-amplified neuroblastoma, driving RAS-MAPK through negatively regulating NF1.

Aims

The aims of this study were to 1) evaluate TIAL1 as a transcriptional target of MYCN, 2) determine whether TIAL1 regulates RAS-MAPK signaling through alternative splicing of NF1, and 3) analyze functional relevance of the MYCN-TIAL1-NF1 axis.

Methods/Materials

We developed a system implementing lentiviral doxycycline-inducible dCas9 with a secondary lentiviral sgRNA. This CRISPRi system allows us to reversibly regulate TIAL1 knockdown in human patient cell lines and patient derived xenograft lines. We have also introduced these systems into neural crest cells of the sympathoadrenal lineage derived from human iPSCs. We cloned lentiviral constructs to overexpress TIAL1 and MYCN. We analyzed ChIP-seq and RNA-seq results from existing neuroblastoma patient tumor sets to assess MYCN binding and MYCN, TIAL1, and $NF1^{23a}$ vs $NF1^{WT}$ expression. We performed growth and signaling studies. We

injected our modified cells orthotopically into the renal capsules of NSG mice, as an *in vivo* mouse model.

Results

We found that MYCN binds multiple sites in the TIAL1 promoter in a variety of patient derived xenograft tumor lines and cell lines. High expression of TIAL1 correlated inversely with survival. Relapsed patient tumors showed higher levels of incorporation of higher NF1 exon 23a compared to primary tumors. We found that lentiviral misxpression of MYCN increased expression of TIAL1, and also increased ratios of $NF1^{23a}/NF1^{WT}$. Knockdown of TIAL1 by CRISPRi decreased relative incorporation of NF1 exon 23a. Further, knockdown of TIAL1 led to decreased levels of RAS-GTP. Conversely, we also found that TIAL1 overexpression led to increased abundance of activated Erk. Knockdown of TIAL1 decreased proliferation more effectively in MYCN-high cell lines compared MYCN-low cell lines. Our preliminary results *in vivo* suggest that TIAL1 knockdown increases tumor latency.

Summary/Conclusions

Our study explored if alternative splicing of NF1 could potentially modulate a MYCN-driven axis in aggressive neuroblastoma tumors. We have identified TIAL1 as an alternative splicing protein which is regulated by MYCN and which affects NF1 exon 23a inclusion to regulate overall NF1 activity and downstream RAS/MAPK signaling. Thus, TIAL1 could be a potentially novel targetable component acting in high-risk and relapsed neuroblastoma. We are continuing to delve into mechanisms of TIAL1 in neuroblastoma aggression and therapy resistance.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 240

Integrated molecular analysis from SIOPEN HR-NBL1 trial identifies an association between multi-exon deletions in ATRX and a poor response to induction chemotherapy

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

With intensification of therapy, patients with *MYCN* amplified neuroblastoma now have similar survival to patients with non-*MYCN* amplified high-risk disease. However, age at diagnosis remains an important prognostic factor and increasing age at diagnosis is strongly associated with worse survival.

Older age at diagnosis is associated with specific biological features including 11q loss of heterozygosity (LoH), *ATRX* alterations and alternative lengthening of telomeres (ALT). 11q LoH, *ATRX* alterations, and ALT have all individually been associated with adverse outcomes although there is significant overlap amongst these genetic features and the relative influence of each factor

on clinical phenotype is unclear.

Aims

To determine *ATRX*, 11q and ALT status in patients with non-*MYCN* amplified neuroblastoma, treated on the SIOPEN HR-NBL1 clinical trial and link molecular findings to the clinical phenotype.

Methods/Materials

Eligibility criteria were *MYCN* non-amplified neuroblastoma, aged >18 months, and treated on the SIOPEN HR-NBL1 trial. ALT status was evaluated by c-circle assay. Tumour DNA was sequenced using a 91-gene custom paediatric solid tumour hybrid capture next generation sequencing (NGS) panel. The NGS panel detects single nucleotide variants small insertions/deletions, copy number and structural variants. 11q status was determined by existing array-CGH/SNP-array data from SIOPEN biology laboratories, NGS panel, or where sufficient DNA available, by low coverage whole genome sequencing.

Results

36/64 tumours fulfilling the eligibility criteria were ALT positive by c-circle assay. However, c-circle stability experiments showed that c-circles rapidly degrade if DNA is left at ambient temperature for 1 week, highlighting the probability of false negatives in these archival samples. In cases confirmed as ALT positive, the most commonly altered gene was *ATRX* (13), followed by *SMARCA4* (4) and *ALK* (3).

The most common *ATRX* variant detected was a multi-exon deletion (13), followed by missense alterations (5). One tumour had a frame-shift *ATRX* alteration and another a splice site variant. There was no significant difference in EFS according to *ATRX* status, although median time to 1st event was significantly shorter in the *ATRX* deleted group (189 vs 465 days, p=0.0192). Consistent with this, patients with *ATRX* multi-exon deletions had much lower overall response rates to induction chemotherapy (46% in patients with *ATRX* deletions, 83% in patients with other *ATRX* mutations, 85% in *ATRX* wild-type group). Overall response rate to induction chemotherapy in c-circle positive patients in the absence of an *ATRX* deletion was 84%.

Information on both 11q and *ATRX* status was available in 58 patients. 80% of patients with *ATRX* alterations had concurrent 11q loss. Patients with 11q loss who were *ATRX* wild-type (n=22) had an induction response rate of 90%.

Summary/Conclusions

Preliminary SIOPEN HR-NBL1 trial data identifies a strong association between *ATRX* deletions and a poor induction chemotherapy response, however patients with 11q LoH and/or ALT have an excellent response to induction therapy in the absence of a concurrent *ATRX* deletion.

This data will be integrated into a larger on-going SIOPEN biology study. Over 400 patients enrolled on the SIOPEN-HR-NBL-1 trial have now had tumour DNA sequenced by NGS panel, which will be linked with telomere status and clinical outcome data.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 241

High-level MYCN expression in neuroblastoma impairs L1CAM-directed CAR T cell therapy in vitro

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

Current treatment protocols have only limited success in patients with neuroblastomas harboring amplifications of the central oncogenic driver, *MYCN*. CAR T cell therapies have recently entered neuroblastoma clinical trials after their success against hematological malignancies. Initial responses have however been disappointing.

Aims

We investigated the influence of *MYCN* amplification on tumor cell response to neuroblastoma-specific CAR T cell therapy with the aim to illuminate mechanisms inhibiting CAR T cell success against neuroblastoma.

Methods/Materials

As a starting point, we used the SK-N-AS neuroblastoma cell line with the normal diploid *MYCN* complement ($MYCN^{non\text{-}amp}$) and overexpressed MYCN ($MYCN^{amp}$) in these cells using a tetracycline-inducible system. Neuroblastoma cells with the different *MYCN* backgrounds were co-cultured with second-generation L1CAM-targeting CAR T cells harboring either the CD28 or 4-1BB costimulatory domain. L1CAM-CAR T cell function was assessed in assays for activation/inhibitory

receptor expression, cytokine release and tumor cell cytotoxicity. Combination of CAR T cells with the indirect MYCN inhibitor, MLN8237, was assessed via combinatorial cytotoxic effects and synergism was calculated using R.

Results

Co-culture with $MYCN^{amp}$ SK-N-AS cells reduced activation of L1CAM-CAR T cells (decreased CD25 and CD137 expression) compared to parental $MYCN^{non\text{-}amp}$ cell co-culture. Inhibitory Tim3 receptor expression was increased by almost 2-fold on L1CAM-CAR T cells and they released up to 2-fold less IFNG and IL2 in co-culture with $MYCN^{amp}$ SK-N-AS cells compared with parental $MYCN^{non\text{-}amp}$ cells. The L1CAM-CAR T cells co-cultured with $MYCN^{amp}$ SK-N-AS cells also demonstrated less killing potential against the $MYCN^{amp}$ SK-N-AS cells in comparison to the parental cell line. All effects were observed for L1CAM-CAR T cells, regardless of which costimulatory domain was used in the CAR construct, but CD28 costimulation did produce higher efficacy in all functionality assays. Interestingly, co-culture with L1CAM-CAR T cells downregulated the L1CAM target antigen on the $MYCN^{amp}$ tumor cells. First hints from patient data emphasizes that lower L1CAM expression is associated with *MYCN* amplified high-risk tumors. Indeed, stable L1CAM expression on tumor cells rescued the negative effects exerted on CAR T cells. A combinational therapy approach using an aurora a kinase inhibitor, indirectly inhibiting MYCN function, and L1CAM-specific CAR T cells restored L1CAM expression on $MYCN^{amp}$ neuroblastoma cells in our *in vitro* model. Antigen loss on neuroblastoma cells was not recovered after combination treatment, but L1CAM-CAR T cell-directed $MYCN^{amp}$ SK-N-AS cell killing was increased by up to 40%. Nevertheless, a synergistic effect was detected exclusively for MYCN overexpressing cells when the indirect *MYCN* inhibitor and L1CAM-targeted CAR T cell therapy were combined.

Summary/Conclusions

MYCN-driven L1CAM downregulation on neuroblastoma cells may be one cause for impaired efficacy of L1CAM-specific CAR T cell therapy in patients with neuroblastoma. Whether target antigen downregulation on the tumor cells is the only escape mechanism exerted within *MYCN*-amplified tumors remains elusive. We provide preclinical evidence that combining L1CAM-specific CAR T cells with an aurora a kinase inhibitor might be an option to increase CAR T cell efficacy for patients with *MYCN*-amplified neuroblastomas.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 242

Optimizing sustained release local therapy: single versus dual chemotherapy for the treatment of neuroblastoma

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

Neuroblastoma is the most common pediatric extracranial solid malignancy with limited effective treatment. We have shown that single drugs delivered locally in a sustained release manner through a silk-based biomaterial are effective in decreasing orthotopic neuroblastoma xenograft growth. We further optimized this approach and hypothesized that increasing doses of local chemotherapy or delivering two chemotherapeutic agents simultaneously could inhibit additional tumor growth.

Aims

To investigate the effectiveness of local sustained released therapy using two chemotherapeutic agents.

Methods/Materials

MYCN-amplified (KELLY) and non-MYCN-amplified (SKNAS) neuroblastoma cells were treated with combinations of cisplatin, vincristine, doxorubicin, and etoposide to determine cytotoxicity and synergy. Drug-loaded silk platforms were created, and the amounts of drug released from the material over time was recorded. Murine orthotopic neuroblastoma xenografts were generated and tumors were implanted with silk platforms loaded with single or dual chemotherapies. Ultrasound monitored tumor growth. Histologic evaluation of tumors was performed.

Results

In vitro, vincristine/cisplatin combination was synergistic, significantly decreasing cell viability relative to other combinations. After loading onto silk platform, both drugs could be released effectively. Locally implanted vincristine/cisplatin silk platform induced increased tumor growth suppression compared to either agent alone in KELLY tumors ($p<0.05$). The dose-dependent effect seen in KELLY tumors treated with combination therapy diminished at higher doses in SKNAS tumors, with little benefit with doses >50/500µg for vincristine/cisplatin, respectively. H&E staining of tumor sections demonstrated tumor cell necrosis adjacent to drug-loaded silk platforms.

Summary/Conclusions

Local delivery of sustained release chemotherapy can suppress tumor growth especially at high doses or with two synergistic drugs. Customized dual therapy is a promising approach for future clinical testing.

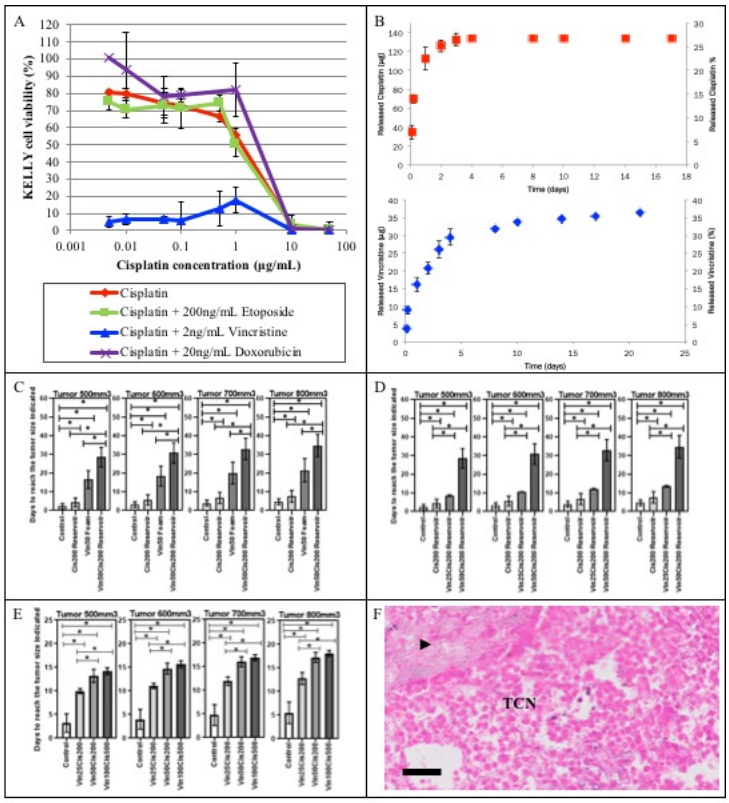

Figure 1. (A) Cytotoxicity profile of KELLY cells treated with cisplatin and doxorubicin, etoposide or vincristine. (B) Amount (µg) and percentage of drug released from silk platform loaded with a combination of 100 µg vincristine and 500 µg cisplatin. (C) Growth kinetics, (displayed as the number of days for tumor to reach a given size) for KELLY tumors treated with single vs dual agent silk reservoir; (D) Substantial Kelly tumor growth inhibition caused by increasing doses of dual therapy. (E) Growth kinetics of SKNAS tumor treated with increasing doses of vincristine and cisplatin demonstrate limited therapeutic benefit at the highest dose range. (F) KELLY tumors treated with local combination therapy were sectioned and stained with H&E. The histology showed tumor cell necrosis adjacent to the drug-loaded silk platform. Asterisk (*) indicates p-value < 0.05; scale bar = 100 µm; arrow (►) indicates drug loaded silk platform; TCN, tumor cell necrosis.

Type Translational

Presentation Preference Traditional poster

Abstract nr. 243

Analyzing the impact and mechanism of CAR T cell impairment by soluble neuroblastoma antigen

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

Despite high remission rates for hematological malignancies following CAR T cell therapy, targeting solid tumors remains challenging. The hostile and immunosuppressive tumor microenvironment contributes to reduced CAR T efficacy against solid tumors, but the nature of the target antigen still plays a crucial role. L1CAM (formerly CD171), an adhesion molecule playing a fundamental role during brain development, is overexpressed by different tumor entities including neuroblastoma. The cancer-specific glycosylation of the CE7 epitope makes it a suitable target antigen, and CAR T cells targeting it are currently in a phase I clinical trial for high-risk neuroblastoma at the Seattle Children’s Hospital (USA). Constitutive ADAM metalloprotease-directed cleavage of the L1CAM extracellular domain releases the L1CAM ectodomain containing the CE7 epitope into blood and ascites, which we propose could impair CAR T cell performance either by neutralizing CAR T cells before they reach the tumor site or exhausting CAR T cells more quickly via direct or excessive CE7 CAR domain binding.

Aims

The extent and underlying mechanism of how soluble L1CAM impacts L1CAM-directed CAR T cell therapy will be analyzed to improve this therapeutic strategy for patients with neuroblastoma.

Methods/Materials

The extent of constitutive L1CAM shedding during *in vitro* culture of SK-N-BE(2) ($L1CAM^{high}$) and SK-N-AS ($L1CAM^{low}$) neuroblastoma cell lines was assessed using western blotting. L1CAM- or CD19-directed CAR T cells were cultured with increasing amounts of soluble recombinant L1CAM or followed by co-culture with CD19-expressing SK-N-BE(2) cells. Expression of the

CD137 activation marker and effector cytokine production were subsequently flow cytometrically analyzed.

Results

Soluble L1CAM is constitutively shed *in vitro*, and shedding remains stable over time, leading to soluble L1CAM accumulation. Exposing L1CAM-targeting CAR T cells to soluble recombinant L1CAM concentrations as low as 333 ng/ml upregulated CD137 on the T cell surface, indicating that soluble L1CAM caused L1CAM-targeting CAR T cell activation. T cell activation was higher with increasing concentrations of L1CAM. Interestingly, L1CAM-targeting CAR T cells exposed to high levels of soluble L1CAM then challenged with SK-N-BE(2) cells in the presence of soluble L1CAM were incapable of producing IFNG, suggesting that if levels of soluble L1CAM reach a critical amount, it has a neutralizing effect on L1CAM-targeting CAR T cells. Soluble L1CAM had no affect on CD19-directed CAR T cells at any concentration used, indicating a CAR-specific effect.

Summary/Conclusions

Here we show that L1CAM is constitutively shed by neuroblastoma cells, and that soluble L1CAM specifically impairs L1CAM-directed CAR T cells by neutralization and early activation distant from the tumor site. Knowing how soluble L1CAM affects L1CAM-directed CAR T cell therapy will enable us to implement strategies to avoid CAR T cell impairment and improve CAR T cell therapy for patients with neuroblastoma.

Type Translational

Presentation Preference Traditional poster

Abstract nr. 244

Metachronous bilateral adrenal neuroblastoma or a coincidence?

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

Bilateral adrenal neuroblastoma is very rare and occurs in infants. Its presentation suggests synchronous multifocal primaries rather than metachronous metastases. The therapeutic strategy is similar to that used against unilateral tumors.

Aims

We present a case of a 5.5 y old boy with Metachronous bilateral adrenal neuroblastoma.

Methods/Materials

SNParray Array analysis was performed on genomic DNA extracted from peripheral blood and bilateral adrenal tumors using Affymetrix CytoScan HD array.

Results

The patient was first diagnosed at the age of 3.5 years with a localized right adrenal MYCN not - amplified neuroblastoma. Two years after a complete resection of the right adrenal mass, an expanding mass was observed in the left adrenal. Metastatic work up was negative. The mass was completely resected. The pathology of the first adrenal tumor demonstrated a poorly differentiated neuroblastoma with diffuse necrosis, some calcifications, high cellularity, high mitotic activity and low MKI. The pathology of the second tumor demonstrated differentiating neuroblastoma, low mitotic activity, low MKI, and less necrosis.
Cytoscan HD (affymetrix) SNP array was done on both tumors and blood. In both tumors and blood samples we found several LOH regions (6p21.1-q15; 9q 22.-31.2), 11p15.4 deletion, 14q32.33 gain and more changes. We also found the following changes in both tumors: 12q24.12

LOH (PTPN11) and 19p13.2-13.3 deletion. However there were changes that were found only in the tumor in 2017: 2p(11.2-24.3) gain (NBAS, DOX1, PAM49A, SMC6); whole chromosome 7 gain; 17q(11.2-25.3) gain and the following changes only in the tumor in 2019: 7q(31.33-36.2) gain; 3q gain (GAP43). WES is ongoing on both tumors and blood.

Summary/Conclusions

According to these changes we assume that the patient has a background of predisposing syndrome and develop two different neuroblastoma tumors at two different time points. WES is ongoing and the results will be presented.

Type Clinical

Presentation Preference Traditional poster

Abstract nr. 245

Clinical and genomic differences according to the metastatic burden in stage 4 high-risk neuroblastoma

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Stage 4 high-risk neuroblastoma is a remarkably heterogeneous disease with varying outcomes. Previous analyses of several cohorts have reported that metastatic burden and/or patterns have prognostic significance in stage 4 neuroblastoma patients. However, we need further detailed information on clinical behavior and genetic aberrations in tumors with different metastatic spread to explain different treatment outcomes and to inform additional risk stratification.

Aims

The aim of this study was to identify subgroups of stage IV high-risk neuroblastoma based on pattern and burden of metastatic spread and compare their clinical and genomic differences.

Methods/Materials

Patients with stage 4 mIBG-avid neuroblastoma (age≥18 months) were categorized by Curie score (8< vs. ≥8) at diagnosis and their clinical and genomic differences were compared (n=117). The optimum cutoff value of Curie score was determined by Fine and Gray competing-risks analysis with respect to progression free survival. We reviewed their medical records and, also performed whole transcriptome analysis with RNA-Seq to explore gene expression differences.

Results

Patients with a higher Curie score (≥ 8) at diagnosis had a significantly worse progression-free survival (PFS) than those with a lower score (< 8) (3-y PFS: 59.9%±6.6% vs. 84.4±6.6%, respectively; P = 0.004). Patient with a higher Curie score at diagnosis were more likely to have a

smaller primary tumor size (244±304cm^3 vs. 475±461cm^3, *P* = 0.003), a non-MNA tumors (*P* = 0.032) and elevated serum ferritin and urine VMA levels. (*P* <0.001, *P* = 0.001, respectively). And patients with a higher Curie score also had a higher score of metastatic site index (*P* <0.001) which is also representing metastatic burden. Meanwhile, better reduction in MIBG score (%) after 3 cycles of chemotherapy was observed in patients with a higher Curie score (64.7%±4.3 vs. 36.7%±5.3, *P* = 0.015). However, there was no difference in primary tumor volume reduction after 3 cycles of chemotherapy. In genomic study, we identified 13 differentially expressed genes in patients with a higher Curie score as compared to the patients with a lower Curie score (p-value < 0.01, |FC| > 2). Among those genes, *MAGEA1*, *MAGEA2*, *MAGEA6*, *MAGEA12, CSGA1* and *C1QTNF9* were expressed differentially with the highest significance between two groups.

Summary/Conclusions

We found differences in clinical behavior and gene expression according to the extent of metastatic neuroblastoma which carries prognostic significance. We believe that our findings contribute to the understanding of stage 4 high-risk neuroblastoma and provide ideas for further studies.

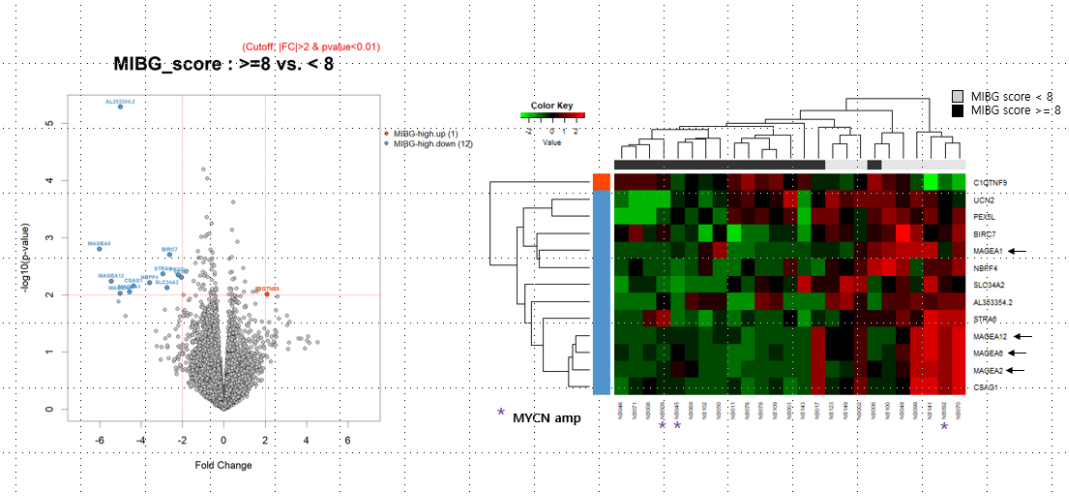

Figure 1. Whole transcriptome analysis

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 246

P-glycoprotein overexpression is a resistance mechanism for induction chemotherapy in high-risk neuroblastoma

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Topic Other

Background/Introduction

High-risk neuroblastoma (HR-NB) patients undergo intensive therapeutic intervention which is heavily reliant on combination chemotherapy. Despite this, these patients have a survival rate of only ~50%, largely due to intrinsic or acquired drug resistance. P-glycoprotein (P-gp; *ABCB1*) is a multidrug transporter known to efflux multiple conventional agents used in HR-NB induction therapy including doxorubicin, vincristine and etoposide and has been reported as a resistance mechanism for the ALK inhibitor crizotinib. We observed surprisingly high P-gp expression in HR-NB cell lines and patient-derived xenograft (PDX) models, so investigated whether P-gp expression was relevant for response to therapy in this disease.

Aims

To determine: i) what drives high P-gp expression in HR-NB and if P-gp levels influence the effectiveness of chemotherapy; ii) whether P-gp inhibition can increase the therapeutic window for standard-of-care chemotherapies and targeted agents.

Methods/Materials

To determine the prevalence of high *ABCB1*/P-gp expression we performed RNA-sequencing, immunohistochemistry and western blot on panels of neuroblastoma tumour samples, PDX models and cell lines. We also interrogated *ABCB1* expression in larger cohorts using the R2 database (https://hgserver1.amc.nl/cgi-bin/r2/main.cgi) and are investigating *ABCB1* regulation using publicly available ChIP-Seq and RNA-Seq data. To assess the influence of P-gp in resistance to standard of care and targeted therapies, cytotoxicity assays were performed on high P-gp expressing HR-NB cell lines with or without the P-gp inhibitor tariquidar. Following *in vitro* assessment with western blot, proliferation and cytotoxicity assays, P-gp knockdown (shRNA), *ABCB1*-null (CRISPR/Cas9) or control neuroblastoma cells were engrafted into NOD-SCID Gamma (NSG) mice then treated with vincristine (P-gp substrate) or cisplatin (non-substrate). To determine feasibility of combining tariquidar with vincristine *in vivo*, we examined the influence of tariquidar on vincristine pharmacokinetics using volumetric absorptive microsampling and liquid chromatography-tandem mass spectrometry.

Results

RNA-sequencing, immunohistochemistry, western blot and R2 database interrogation showed high *ABCB1*/P-gp expression is frequently observed in HR-NB and is more common than in most other cancers ($p<0.0001$). High relative expression at diagnosis is associated with poorer patient outcome (NCI TARGET, n=217, $p=0.04$), consistent with its multidrug transporter function. *In vitro* , tariquidar and P-gp knockdown both sensitize high P-gp expressing neuroblastoma cells to vincristine, doxorubicin and etoposide, but not the ALK inhibitors crizotinib, ceritinib and alectinib. P-gp knockdown sensitized neuroblastoma xenografts to vincristine, extending survival, suggesting P-gp levels are sufficient to confer resistance to standard-of-care therapies. An *ABCB1* -null neuroblastoma cell line was sensitized to P-gp substrates *in vitro*, however *ABCB1*-null xenografts were not sensitized to vincristine, suggesting that additional resistance mechanisms can rapidly develop. In the presence of tariquidar, plasma vincristine exposure was approximately doubled in mice. We are currently assessing whether tariquidar can provide an increased therapeutic window in xenograft models.

Summary/Conclusions

Elevated P-gp expression is common in HR-NB and can be sufficient to confer resistance to standard-of-care chemotherapies in model systems. Our findings suggest that tumour P-gp levels might be used to guide treatment options for individual patients to avoid ineffective treatments. The potential of P-gp inhibitors as adjuncts to conventional chemotherapy for HR-NB should be further investigated.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 247

Insights into the biology of neuroblastoma exosomes

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

The interaction between the tumor and its microenvironment plays a key role in regulating neuroblastoma progression. In particular, neuroblastoma cells may create a supportive milieu contributing to tumor growth and metastasis through the secretion of tumor-derived factors, such as exosomes. Exosomes are the smallest extracellular vesicles of endocytic origin, released by both normal and cancer cells. Tumor-derived exosomes can affect the biological activities of recipient cells via transfer of their content, encouraging neoplastic transformation and survival. Moreover, the molecular composition and surface profile of exosomes is highly heterogeneous and dependent on the parental cell type, tumor stage and other parameters.

Aims

We set out to identify exosomal signatures specifically associated with genetic neuroblastoma subtypes.

Methods/Materials

Six human neuroblastoma cell lines reflecting the three major genetic subgroups of high-risk

neuroblastoma were selected, including IMR5-75, BE(2)-C, LAN-6, SK-N-FI, GI-M-EN, CLB-GA. Exosomes were isolated from medium conditioned by the cell lines by OptiPrep density gradient ultracentrifugation (DGUC) followed by size-exclusion chromatography. Exosomes were characterized using transmission electron microscopy, nanoparticle tracking analysis and western blotting. The proteomes of exosome contents and surface profiles were investigated using liquid-chromatography tandem mass spectrometry and a novel multiplex bead-based flow cytometric assay, respectively.

Results

The workflow to isolate exosomes from conditioned medium was first established and optimized. A combination of DGUC and size-exclusion chromatography produced well-purified exosome samples. Neuroblastoma-derived exosomes exhibited the typical cup-shaped morphology in transmission electron micrographs, and showed an enrichment of exosome-specific markers (CD63, CD81, PDCD6IP, FLOT1). Absence of the endoplasmatic reticular protein, calreticulin, demonstrated that our exosome preparation contained no contamination from apoptotic bodies or organelle derived-vesicles. Nanoparticle tracking analysis showed that vesicle size ranged from 30 to 150 nm, consistent with that of exosomes. Proteomic analysis and surface profile of IMR5-75 - derived exosomes revealed the presence of well-known exosome-associated proteins, as reported by the *ExoCarta* and *Vesiclepedia* databases. Proteins associated with the biological processes of cell adhesion, proliferation or the inflammatory response in the target cells were detected in exosomes. The proteomic contents of exosomes overlapped with the cell lines from which they were derived, and experiments are currently underway to compare the proteomic cargo among the six matched exosome/cell line pairs and define exosome heterogeneity from one neuroblastoma cell line and among cell lines and genetic high-risk subtypes.

Summary/Conclusions

An in-depth view on exosomes cargo and heterogeneity is crucial for a better understanding of the role of these vesicles in neuroblastoma pathogenesis. This may also produce new potential biomarkers and molecular targets for high-risk subtypes.

Type Basic

Presentation Preference Rapid Fire Poster Presentation

Abstract nr. 248

Cellular and gene signatures of tumor-infiltrating dendritic cells and natural killer cells predict favorable clinical outcome of neuroblastoma

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Tumor-infiltrating T cells (TILs) play an essential role in improving clinical outcomes in neuroblastoma (NB). Low-risk NBs were characterized by a higher number of proliferating T cells and a more structured T-cell organization, which was gradually lost in highly aggressive tumors. However, the underlying mechanisms governing the establishment of the T cell-inflamed phenotype in NB remain poorly understood.

Aims

Herein, we sought to evaluate other immune cell populations involved in T cell-mediated antitumor immune responses to human NB.

Methods/Materials

In situ IHC staining for dendritic cells (DC) and natural killer (NK) cells was performed on 104 NB specimens, previously characterized for the density of TILs and the expression of immune checkpoint molecules, and correlated to each other and with the clinical outcome. Multiplex immunofluorescence analysis was performed to evaluate the spatial distribution of intratumoral $CD8^+$ T cells, DCs and NK cells in T cell-enriched NB samples. The expression of 770 immune-related genes was assessed on fresh frozen NB samples with the Nanostring PanCancer Immune Profiling assay. Subcellular localization of target genes was determined by RNAscope technology. Transcriptomic profile was validated in two human primary NB datasets from SEQC-GEO.

Results

We showed that DC and NK cells are positively correlated with T-cell infiltration in human NB, both at transcriptional and protein levels, and associated with a favorable clinical outcome of NB patients. Multiplex imaging displays DCs stably conjugated with T cells and NK cells in the tumor microenvironment of low risk NBs. This connection is further strengthened by the identification of two gene signatures related to DC and NK cells able of predicting survival of NB patients. Remarkably, these gene signatures were strongly correlated with the expression of PD-1 and PD-L1.

Summary/Conclusions

In summary, our findings unveil a key prognostic role of DC and NK cells and indicate their related gene signatures as promising clinical biomarkers to predict prognosis and immunotherapy efficacy in NB patients.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 249

Anti-tumor immunity in neuroblastomas associated with opsoclonus myoclonus ataxia syndrome (OMAS) is mediated by B lymphocytes

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

OMAS is a paraneoplastic disorder that affects about 2% of children with neuroblastoma. It is associated with excellent tumor-related outcomes, but devastating neurological sequelae impacting cerebellar and brain stem functions.

Aims

To discover the molecular basis of OMAS in order to understand adaptive immunologic recognition of neuroblastoma as a window into novel therapeutic strategies for both diseases.

Methods/Materials

We retrospectively procured all primary tumor samples (N=38) available from the COG ANBL00P3 clinical trial testing the efficacy of IVIg in neuroblastoma patients with OMAS (de

Alarcon, 2018) and 13 low- and 13 high-risk tumor samples from neuroblastoma patients without OMAS as a comparator. We first performed immunohistochemical analyses with anti-CD3, anti-CD20, and anti-Ki67 antibodies. Next, all samples were subjected to bulk RNA sequencing (Illumina RNA Access platform), as well as T and B cell receptor repertoire analyses using the Adaptive Immunoseq platform. Finally, we carried out an association study to identify HLA alleles enriched in neuroblastoma patients with OMAS compared to neuroblastoma patients without OMAS symptoms.

Results

OMAS-associated tumors were enriched for germinal center-like structures with maturing B lymphocytes, as well as neuronal localization of both B cells and T cells. RNAseq showed B and T cell infiltration and activity and interferon gamma signaling as the dominant distinguishing features of OMAS tumors. We observe striking diversity of both T and B cell repertoires from OMAS-associated tumors, with no detectable clonal enrichment of any T or B cell receptors. We confirm and extend prior observations of particular MHC Class II alleles being significantly associated with OMAS (DOB*01:01; DRB1*13:02; DRB1*01:01), implicating B cells and CD4+ helper T cells in OMAS neuropathology and tumor immunity.

Summary/Conclusions

The absence of dominant T or B cell clones in OMAS tumors suggests that no single antigen is responsible for driving an anti-tumor immune response. Instead, the essential nature of B cells in OMAS neuroimmunity, and the observations of enriched MHC Class II allele expression, and increased B cell infiltration and activation in OMAS tumors, support a central role for B cells in OMAS anti-tumor immunity. We propose a model in which B cells acting as antigen presenting cells promote improved and sustained T cell activation via antigen presentation by their MHC, acting as potentiators of already broad T cell specificity that accompanies OMAS autoimmunity.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 250

Successful treatment of central nervous system relapses in metastatic high-risk neuroblastoma after KIR-ligand-mismatched allogeneic cord blood transplantation without anti-GD2 antibody therapy

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

Although intensive care has improved the survival of high-risk neuroblastoma (NB) patients, recurrent NB has a poor prognosis. In particular, central nervous system (CNS) relapse of NB is difficult to rescue. In previous reports, 14 cases of CNS relapse of NB were fatal, with a median survival duration of 3.6 months from relapse (range: 10 days to 25 months) (Kramer et al. Cancer. 2001; Astigarraga et al. Med Pediatr Oncol. 1996). We previously reported that KIR-ligand-incompatible allogeneic umbilical cord blood transplantation (CBT) reduces bone marrow relapse and improves survival in children with high-risk metastatic NB because NB is susceptible to KIR-mismatched NK cells (Nishio et al. Advances in Neuroblastoma Research. 2018).

Aims

We report the successful treatment of three children with CNS relapse of NB after KIR-ligand-mismatched allogeneic CBT without anti-GD2 antibody treatment.

Methods/Materials

Case 1: A 2-year-old boy with stage 4 NB received multiple courses of combined chemotherapy, BU/L-PAM followed by auto-peripheral blood stem cell transplantation (PBSCT), Flu/L-PAM/TBI (2 Gy) followed by KIR-ligand-mismatched CBT, and focal radiotherapy (19.8 Gy). Ten months after CBT, isolated CNS relapse was diagnosed. He underwent metastatectomy, proton beam craniospinal irradiation (CSI; 19.8 Gy), and focal radiotherapy (10.8 Gy). He has been living without disease progression for 4 years after CNS relapse.

Case 2: A 3-year-old boy with stage 4 NB received multiple courses of combined chemotherapy, BU/L-PAM followed by auto-PBSCT, L-PAM/TBI (12 Gy) followed by KIR-ligand-mismatched CBT, and focal radiotherapy (18 Gy). Eleven months after CBT, CNS relapse was diagnosed. He underwent metastatectomy, proton beam CSI (18 Gy), and focal radiotherapy (12.6 Gy), and a second KIR-ligand-mismatched CBT with Flu/L-PAM/TBI (2 Gy) was performed because of positive bone marrow minimal residual disease (BM-MRD). He also received intrathecal radioimmunotherapy involving 131I-omburtamab. He has been living without disease progression for 22 months after CNS relapse.

Case 3: A 3-year-old girl with stage 4 NB received multiple courses of combined chemotherapy, IFO/L-PAM followed by auto-PBSCT, and Flu/L-PAM/VP-16 followed by KIR-ligand-mismatched CBT. Seven months after CBT, isolated CNS relapse was diagnosed. She underwent metastatectomy, CSI (21 Gy), and focal radiotherapy (10 Gy). She also received three cycles of TMZ/CPT11 and intrathecal 131I-omburtamab. She has been living without disease progression for 10 months after CNS relapse.

Results

We reported three children who developed CNS relapse of NB at 10, 11, and 7 months after KIR-ligand-mismatched CBT. Tumor resection and CSI were performed in all cases. One patient underwent a second KIR-ligand-mismatched CBT because of positive BM-MRD, and two patients received intrathecal omburtamab. Without anti-GD2 antibody treatment, bone marrow relapse was controlled with KIR-ligand-mismatched CBT. The three children have been living without disease progression for 4 years, 22 months, and 10 months after CNS relapse.

Summary/Conclusions

Our findings suggest that KIR-ligand-mismatched CBT could control bone marrow relapse without anti-GD2 antibody treatment, and CNS relapse could be rescued by local control therapy involving metastatectomy, CSI, and intrathecal omburtamab.

Type Clinical

Presentation Preference Traditional poster

Abstract nr. 251

Multiplexed quantification of four DNA targets in a single reaction using droplet digital PCR

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Major challenges of tumor-derived cell-free DNA (cfDNA) analysis purified from blood samples are small sample volumes and low cfDNA concentrations. Droplet digital PCR (ddPCR) is a suitable technology to analyze low levels of cfDNA.

Aims

We intended to establish two quadruplexed ddPCR assays that (i) reliably quantify *MYCN* and *ALK* copy numbers in a single reaction together with two reference genes and (ii) accurately estimate ALK^{F1174L} and $ALKR^{1275Q}$ mutant allele fractions using cfDNA or genomic DNA (gDNA) as input.

Methods/Materials

We optimized separation of positive and negative droplets to detect two targets in each ddPCR fluorescence channel by adjusting probe and primer concentrations for each target molecule. Accuracy and sensitivity was assessed in both assays using a panel of 10 neuroblastoma cell lines and blood plasma samples paired with corresponding primary tumor tissue from 9 neuroblastoma patients.

Results

Assay conditions were first established for a triplexed reaction, detecting 2 reference genes (*NAGK* and *AFF3*) in channel 2 and *MYCN* or *ALK* in channel 1. Droplet clusters were accurately separated for *AFF3* and *NAGK* in channel 2 as well as *ALK* or *MYCN* in channel 1. Primer/probe concentrations for simultaneously detecting *ALK* and *MYCN* in channel 1 were next established. Following optimization, QuantaSoft Analysis Pro software accurately distinguished 16 different droplet clusters that included negative, single-, double-, triple- and quadruple-positive droplets. The quadruplexed reaction maintained the same linearity as duplexed and triplexed reactions within the range of 0.5 to 100 ng DNA of input DNA. *MYCN* and *ALK* copy number status was re-analyzed in 8 neuroblastoma cell lines to demonstrate that quadruplexed ddPCR clearly distinguished between amplification, gain and diploid status. We next applied quadruplexed ddPCR-based *MYCN* and *ALK* copy number assessment to blood plasma samples paired with corresponding primary neuroblastoma tissue from 3 patients. Simultaneous *MYCN* and *ALK* copy number quantification was feasible using cfDNA purified from plasma and tumor-derived genomic DNA as input. We also established a quadruplexed ddPCR protocol detecting ALK^{F1174L} together with the respective $ALK^{wildtype}$ sequence in channel 1 and ALK^{R1275Q} and with its corresponding $ALK^{wildtype}$ sequence in channel 2. Detection of both *ALK* hotspot mutations maintained linearity within the range of 0.5 to 130 ng of input DNA. Sensitivity thresholds were set for the quadruplexed assay, and resembled those for the respective duplexed assays. Data from 6 neuroblastoma cell lines and matched blood plasma samples paired with corresponding primary neuroblastoma tissue from 6 patients confirmed that quadruplexed ddPCR assays accurately distinguished among ALK^{F1174L}, ALK^{R1275Q} and $ALK^{wildtype}$ status and correctly estimated allelic fractions of both mutations using up to 20 ng of input DNA.

Summary/Conclusions

We present two robust quadruplexed ddPCR protocols to assess *MYCN* and *ALK* copy numbers as well as ALK^{F1174L} and ALK^{R1275Q} mutant allele fractions in cfDNA or gDNA that are applicable in the routine clinical setting.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 252

Level of droplet digital PCR-detected seven neuroblastoma-associated mRNAs in peripheral blood stem cells predicts tumor relapse/regrowth of high-risk neuroblastoma patients

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

More than 50% of highrisk neuroblastoma (NB) patients experience tumor relapse due to the activation of minimal residual disease (MRD).MRD is defined conceptually as residual tumor cells that persistently reside in cancer patients after local and systemic cancer therapies. In high-risk NB patients, MRD can be assessed in peripheral blood stem cell (PBSC) in addition to BM and PB because almost all of patients are subjected to autologous PBSC transplantation. Although the detection of several sets of NB-associated mRNAs (NB-mRNAs) by quantitative real-time PCR (qPCR) is reported to have a prognostic power in NB patients, the impact of NB-mRNAs detection PBSC is not clear. Conflicting results have been reported in the previous literatures. Whereas high level of B4GALNT1 or TH mRNA in PBSC samples did not affect survival, high level of CHGA, DCX, DDC, PHOX2B, or TH mRNA in PBSC samples was associated with worse outcome. In the present study, level of 7NB-mRNAs (CRMP1, DBH, DDC, GAP43, ISL1, PHOX2B, and TH mRNAs) were determined by droplet digital PCR (ddPCR) that is an adaption of qPCR and potentially provides more simple and reproducible detection of low-level of mRNAs.

Aims

To determine the impact of ddPCR MRD detection by 7NB-mRNAs in PBSC on tumor relapse/regrowth of high-risk NB patients.

Methods/Materials

PBSC samples were collected from 20 high-risk NB patients treated based on the high-risk NB protocols (JN-H-07, JN-H-11, JN-H-15) of the Japanese Children's Cancer Group (JCCG) Neuroblastoma Committee (JNBSG) at Kobe Children Hospital and Kobe University Hospital between June 2011 and January 2018.Expression of CRMP1, DBH, DDC, GAP43, ISL1, PHOX2B, and TH mRNAs were determined by ddPCR using a QX200 ddPCR system (Bio-Rad Laboratories, Hercules, CA). HPRT1 was used as an endogenous reference for ddPCR. Level of 7NB-mRNAs (combined signature) was defined as the weighted sum of 7 relative copy numbers.

Results

Among 20 high-risk NB patients included in the present study, 8 patients had tumor relapse/regrowth and 12 patients remained relapse/regrowth-free for more than 2 years. In 20 PBSC samples from 20 patients, 8 relapse/regrowth samples expressed significantly higher level of 7NB-mRNAs than 12 non-relapse/non-regrowth samples. Receiver operating characteristic (ROC) analysis was then performed for level of 7NB-mRNAs in 8 relapse/regrowth and 12 non-relapse/non-regrowth PBSC samples. Area under curve (AUC) was estimated to be 0.7812>0.7with the significant accuracy.

Summary/Conclusions

The present study suggests that level of ddPCR-detected 7NB-mRNAs in PBSC is significantly associated with tumor relapse/regrowth of high-risk NB patients.

Type Translational

Presentation Preference Traditional poster

Abstract nr. 253

Submyeloablative Doses of [131I]MIBG Therapy for Relapsed/Refractory Neuroblastoma with Segmental Chromosomal Alterations in Taiwan

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

Catecholamine synthesis and metabolism are hallmarks of neuroblastoma (NB). Iodine-131-labeled meta-iodobenzylguanidine ([^{131}I]MIBG) targets the Norepinephrine Transporter, accumulates in NB cells, and serves as a retrieval therapy in NB.

Aims

To evaluate the feasibility and clinical benefit of [^{131}I]MIBG therapy at submyeloablative doses (<12 mCi/kg) for patients with relapsed/refractory, *MYCN*-non-amplified NB with segmental chromosomal alterations (SCAs) in Taiwan.

Methods/Materials

In 2015, an [^{131}I]MIBG Treatment Facility was established at Changhua Christian Hospital, Changhua, Taiwan with a private room with lead shields to maintain area dose rates ≤ 0.5 uSv/hr outside the room, independent sanitary pipelines & tank, and patient isolation until dose rate < 50 uSv/hr at 1 meter. The maximal allowable dose was 300 mCi/infusion. Parents were isolated outside/behind lead shields, trained in radiation safety principles and given real-time radiation monitors. The genomic type of NB was determined by Array-based Chromogenic Genomic Hybridization (CityoChip^{TM} Oligo 8×60k; BlueGnome, Cambridge, UK). *MYCN* status was confirmed by chromogenic *in situ* hybridization or droplet digital PCR.

Results

During 2015–19, four doses of [^{131}I]MIBG were given to three male patients with MIBG-avid NB aged 13, 24, and 3 years, respectively. All patients tolerated the isolation & infusion without complications.

Case #1 was diagnosed with retroperitoneal NB with bony metastases, *MYCN* non-amplified with SCAs (loss at 1q42.2q44 and gains at 1p13q42.12 and Xp) and numerical chromosomal alterations (NCAs; +13, +14, -Y) at 11 years of age (y/o); he was refractory to TPOG-N2002-HR2 (N6 induction) and Topotecan/Cyclophosphamide/Etoposide. [^{131}I]MIBG of 150 mCi (3 mCi/kg) was given 25 months after initial diagnosis. Stable Disease was observed for 1 month.

Case #2 was diagnosed with retroperitoneal NB with marrow & nodal metastases at 12 y/o. After CR to TPOG-N2002-HR2 including high-dose CEM + ASCR and Focal Radiotherapy, he suffered multiple relapses since 6 years after diagnosis. Tumor at relapse showed *MYCN* low-level gain (5 signals/cell) with SCAs (losses at 1p, 3p, 9p, 11q, 14q, 15q, 19q, 22q and gains at 1p, 1q, 2p, 2q, 5p, 5q, 7p, 8p, 8q, 12q, 13q, 17q, 18p) and NCAs (+12, -Y). [^{131}I]MIBG of 300 mCi (5 mCi/kg) × 2 tandem doses were given at 24 y/o. Stable Disease was observed for 4 months before Progression with Liver Metastases. The patient died of tumor progression 17 months after [^{131}I]MIBG.

Case #3 was diagnosed with retroperitoneal NB with bone & marrow metastases at 9 m/o. He achieved Complete Response after TPOG-N2002-HR1 Induction (Cyclophosphamide/Cisplatin/Epirubicin/Etoposide) but relapsed at 10 months after diagnosis. Biopsy at relapse showed *MYCN* non-amplification (2.03×), *MYC* low-level gain (2.7×), and *ALK* F1174L (MAF=51.4%) and *TERT* promoter -124C>T (MAF=49.4%) mutations by targeted next-generation sequencing. After failing ICE & Irinotecan/Temozolomide/Dinutuximab beta, he received Palliative Radiotherapy followed by [^{131}I]MIBG of 100 mCi (9.5 mCi/kg) at 3 y/o. Clinical Response was observed for 3 weeks. Further targeted therapy with an ALK inhibitor was initiated.

Summary/Conclusions

Submyeloablative doses of [^{131}I]MIBG have been successfully administered to Taiwanese patients with relapsed/refractory NB with SCAs, showing short-term clinical benefit. Dose-escalation of [^{131}I]MIBG and/or combination with chemotherapy or other radiosensitizers may further improve treatment response.

Type Clinical

Presentation Preference Rapid Fire Poster Presentation

Abstract nr. 254

MYCN cooperates with HAND2 to control neuroblastoma growth and differentiation

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Topic Gene regulatory networks and epigenetics: from basic science to therapy

Background/Introduction

Neuroblastoma (NB) is a neural crest derived tumor of the developing sympathetic nervous system. Amplification of *MYCN* correlates with high-risk disease. How MYCN alters the transcriptional landscape of presumptive sympathoadrenal progenitors to cause NB remains ill-defined.

Aims

To identify critical nodes regulating MYCN's diverse effects on growth, tumorigenicity and differentiation in NB.

Methods/Materials

The MYCN interactome was defined by co-immunoprecipitation(co-IP) and protein mass spectrometric analyses in IMR32 cells. RNAi, RNA-seq and ChIP-seq were performed to investigate how the novel protein partners affect MYCN oncogenic activity.

Results

Co-IP and protein mass-spectrometry results showed that MYCN interacts with 8 neural crest lineage-transcription factors (TFs) that are essential for sympathoadrenal development. Among these HAND2, PHOX2B, ISL1, TBX2 and GATA3 are components of a core transcriptional regulatory circuitry that maintains NB proliferative state and cell identity. SiRNA silencing of these 8 TFs led to a 30-80% decrease of NB cell proliferation. Realtime PCR showed that most of these TFs regulate the expression of selected MYCN target genes, but only HAND2 completely phenocopies for this set of MYCN target genes. Proximity ligation assays support the interaction between HAND2 and MYCN. Structure/function studies showed a critical region of MYCN that is required for HAND2 interaction shares only ~30% homology with c-Myc. Consistently, antibodies to HAND2 immunoprecipitate MYCN but not c-Myc. Silencing *MYCN* and *HAND2* together inhibited the growth of NB cells to the same extent as silencing either gene alone. However, only the combined decrease of *HAND2* and *MYCN* significantly induced neurite-like extensions. RNA-seq after silencing either *MYCN* or *HAND2* revealed that ~45% genes regulated by *MYCN* (811

out of 1822 genes) were also regulated by *HAND2*. Gene set enrichment analysis after si*HAND2* showed a significant negative enrichment for MYC signature genes (p=0.05, FDR=0.05). For genes commonly regulated by both *MYCN* and *HAND2*, the combined silencing of *MYCN* and *HAND2* resulted in significantly increased changes of mRNA levels compared to silencing of either alone. Ingenuity pathway analysis (IPA) showed that the commonly regulated genes after silencing of both MYCN and HAND2 are positively enriched in NGF and TRKA pathway (neural differentiation, p=0.05) and negatively enriched in STAT3 pathway (proliferation, p=0.05). Chromatin immunoprecipitation sequencing (ChIP-seq) using an anti-HAND2 and MYCN antibodies identified that genes bound by both HAND2 and MYCN are positively enriched in noradrenergic neuron differentiation (p=0.05). Importantly, *HAND2* silenced cells resulted in decreased binding not only of HAND2 but also MYCN on the same genomic locus and decreases in RNA Pol II on the associated gene bodies consistent with decreased transcription elongation.

Summary/Conclusions

Our results suggest a mechanism for NB tumorigenesis in which MYCN interacts with HAND2 and forms a module on genomic DNA to facilitate the upregulation of MYCN signature genes. The HAND2/MYCN interaction may function to raise the threshold which sympathoadrenal progenitors must overcome to implement a differentiation program.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 255

Dissecting neuroblastoma tumor heterogeneity and cell plasticity by single cell RNAseq

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

Half of the patients diagnosed with neuroblastoma have a high-risk metastatic disease, mostly refractory to intensive multi-modal therapies, and associated to a poor prognosis. Despite recurrent genomic alterations (*MYCN* amplification, chromosomal alterations and telomere maintenance aberrations) found in 1/3 of the patients, the biological explanation for their specific aggressiveness is not yet well defined. The lab and others have recently demonstrated non-genetic neuroblastoma heterogeneity based on the analyses of the super-enhancer profiles of human cell lines and patient derived xenografts (PDXs), highlighting the existence of two main tumor identities governed by distinct core regulatory circuitries: a noradrenergic and a mesenchymal phenotype, the last being more resistant to chemotherapies.

Aims

Whether noradrenergic and mesenchymal identities co-exist in patient tumors, their interactions and dependencies between each other’s, and their roles in resistance to treatments remain to be

deeply addressed. We want to decipher intra-tumor cellular diversity of high-risk NB at the single cell level, and study the effects of chemotherapies on this heterogeneity.

Methods/Materials

We are dissecting patient tumor heterogeneity using 10x Genomics single cell RNA sequencing (scRNAseq) on neuroblastoma stage 4 samples. We have access to fresh tumors from surgery at diagnosis and relapse thanks to the French national MAPPYACTS and MICCHADO programs, and to a cohort of 12 PDX models. In parallel, we have recently established a new cell line from a PDX neuroblastoma that allows us to decipher neuroblastoma cell plasticity.

Results

We already sequenced 11 different cases (5 biopsies and 6 PDXs). Using bioinformatics, we demonstrated the co-existence of noradrenergic and mesenchymal tumor cells in some patients, and propose specific gene signatures in good accordance to super-enhancers profiles. Thanks to a couple of PDXs obtained from the same patient at diagnosis and relapse, we could also follow mechanisms of disease evolution.

In parallel, we dissected the heterogeneity of the SK-N-SH cell line and our novel PDX-derived cell line. Using scRNAseq, we observed that these two models are composed of 2 distinct tumor cells identities. We identified a surrogate mesenchymal cell surface marker, which we used to sort the noradrenergic and mesenchymal tumor cells. We proved their trans-differentiation potential *in vitro* and *in vivo*. Finally, we demonstrated that the mesenchymal tumor component support the resistance to chemotherapies observed in patients.

We are now using kinetics of reconstitution and differentiation trajectories within these models to dissect the molecular mechanisms supporting the “**N**oradrenergic to **M**esenchymal **T**ransition” and inversely, either during spontaneous differentiation or in response to chemotherapies, in order to identify new potential therapeutic targets for this poor prognosis disease.

Summary/Conclusions

The single cell dissection of neuroblastoma tumors allows a fine characterization of tumor cell diversity at the transcriptomic level, and proves the existence of a tumor cell population with mesenchymal properties in a subset of cases. This description is accompanied by mechanistic studies of the plasticity of neuroblastoma cells, harboring one noradrenergic or one mesenchymal phenotype depending on the context, and in response to drugs.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 256

Identification of core genes involved in high risk neuroblastoma without MYCN amplification by bioinformatic analysis

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Topic Gene regulatory networks and epigenetics: from basic science to therapy

Background/Introduction
Neuroblastoma (NB) is the most common extracranial malignancy in childhood and with great heterogeneity, high-risk NB usually has dismal long-term survival.

Aims
The aim of this study was to explore the differentially expressed genes(DEGs) of high-risk patients and to provide bioinformatics basis for the occurrence and treatment of neuroblastoma.

Methods/Materials
GSE49710 dataset from the Gene Expression Omnibus (GEO) database was downloaded and differentially expressed genes were analyzed by GEO2R tool. The gene ontology (GO) function analysis and pathway enrichment analysis of DEGs were carried out using the DAVID database and Metascape database3 respectively. Then, the protein-protein interaction (PPI) network was constructed using the STRING database. Hub genes were screened using Cytoscape software and verified using the R2 Genomics Analysis and Visualization Platform. Finally, we obtained the transcription factor (TF) of hub genes using the Network Analyst online tool.

Results
A total of 209 DEGs were identified, which were mainly enriched in cell cycle, DNA replication, and p53 signaling pathway. Six genes (KIF20A, RRM2, CDK1, TOP2A, DLGAP5 and CCNB2) were selected as hub gene based on the degree of centrality. The overall survival reflected that high expression of these six crucial genes significantly predicted poor prognosis. Importantly, using the median of DLGAP5 mRNA expression as the cut-off points, multivariable Cox regression analysis showed that high levels of DLGAP5 mRNA expression was associated with poor patient overall survival and event-free survival, independent of disease stage, age at diagnosis and

MYCN amplification status.TF analysis showed that MYBL2,SOX2 and FOXM1 were regulators of these six hub genes.

Summary/Conclusions

The identification of the crucial genes and pathways would contribute to the development of novel molecular targets and biomarker-driven treatments for high-risk neuroblastoma.

Type Translational

Presentation Preference Traditional poster

Abstract nr. 257

Preclinical evaluation of HDAC inhibitor-mediated modulation of MIBG avidity in neuroblastoma

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

Norepinephrine transporter (NET) is differentially over-expressed in most human neuro-ectodermal tumors, and is a highly specific mechanism for directing uptake of meta-iodobenzylguanidine (MIBG). Phase 2 trials incorporating ^{125}I-MIBG have shown promising responses, but unfortunately 40% of neuroblastoma tumors are ^{125}I-MIBG-non-avid and cannot benefit from this treatment. While this may be due to low surface NET protein expression, histone deacetylase inhibitors (HDACi's) have been shown to increase total and surface NET expression and afford a potential mechanism to positively modulate MIBG avidity in neuroblastoma.

Aims

We hypothesized that NET expression and surface localization could be enhanced pharmacologically with HDAC inhibitors to increase preclinical ^{125}I-MIBG avidity, within human-equivalent dose limits. We sought to characterize the MIBG uptake and cellular cytotoxicity in neuroblastoma cell lines, and modulation of MIBG avidity in neuroblastoma xenograft models, following exposure to vorinostat (SAHA) at preclinical concentrations equivalent to human plasma Cmax at current pediatric maximum-tolerated dose (MTD) of 180mg/m^2.

Methods/Materials

SK-N-SH, SK-N-BE3, and SK-N-BE2C cell lines were exposed to SAHA at 0-4.00μM from 8–24h.

Cell viability was evaluated with MTS assay, and ^{125}I-MIBG uptake was measured normalized to number of viable cells. To verify corresponding modulation of protein expression following 24h of treatment at MTD Cmax-equivalent concentration, total NET protein expression was evaluated by western blot and SLC6A2 (hNET) transcript expression was evaluated by qPCR in lysates of cell lines and xenograft tumors (cell line xenografts and patient-derived xenografts (PDXs)). Finally, to evaluate *in-vivo* modulation of MIBG avidity, orthotopic SK-N-SH cell line xenografts and PDXs were treated with allometrically scaled MTD-equivalent dose of SAHA, and ^{125}I-MIBG and NET biodistribution evaluated in tumor and organs after 24h.

Results

Greatest increase in ^{125}I-MIBG uptake was seen with SK-N-SH cells at higher SAHA concentrations after 24h exposure, however there was concomitant cytotoxicity with decreased viability of SK-N-SH cells under these conditions. At 8 and 16h, across all SAHA concentrations, ^{125}I-MIBG uptake was 2.5 fold higher in SK-N-BE2C cells than SK-N-SH, consistent with higher NET expression as per reported data ; while at 24h, at 0.73–4.00μM SAHA, ^{125}I-MIBG uptake increased by 2-fold in SK-N-SH and decreased by 2-fold in SK-N-BE2C. Correspondingly, following 24h exposure to SAHA at MTD Cmax-equivalent concentration, normalized NET protein expression increased median 5-fold in SK-N-SH cells. However, in xenografts, similar upregulation was only seen in PDX tumors but not SK-N-SH cell line xenograft tumors, with only the former demonstrating increase in normalized NET protein and hNET expression 24h following 50mg/kg per-oral SAHA. Yet, following the same dose of SAHA, biodistribution studies showed no significant increase in ^{125}I-MIBG avidity of PDX or SK-N-SH cell line xenografts.

Summary/Conclusions

NET protein expression and ^{125}I-MIBG uptake increased in neuroblastoma cell lines after 24h exposure to SAHA concentrations equivalent to expected human plasma Cmax at MTD. Following *in-vivo* dosing, NET protein and hNET expression was upregulated only in PDX tumors. However this did not result in increase in ^{125}I-MIBG avidity *in-vivo*. Our findings suggest that at current pediatric MTD, SAHA may have limited clinical utility in positively modulating MIBG avidity of neuroblastoma tumors.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 258

The immunomodulatory role of apoptotic neuroblastoma primed mesenchymal stromal cells on tumor microenvironment

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

Background: One of the contributory factors for treatment refractoriness of neuroblastoma is the unfavorable tumor microenvironment (TME) for immune cells. Within the TME, mesenchymal stromal cells (MSCs) can migrate through blood vessels and exerts immunosuppression and become major hindrance to immunotherapy. However, studies showed MSCs can be manipulated by the environment and change their phenotype.

Aims

Aim: We would like to study the influence of apoptotic cells (AC) on MSCs' function.

Methods/Materials

Results: The AC primed MSCs could induce T cells proliferation and activation. Moreover T cells were induced to differentiate to pro-inflammatory phenotype. It is associated with reduction in regulatory T cells (Treg) differentiation. In contrast to conventional thinking, T cells co-cultured with AC-primed MSC have a greater cytotoxic function. As immunogenic molecules were not found in the supernatant of the dying neuroblastoma cells, we explored the role of extracellular vesicles (EVs). Interestingly apoptotic neuroblastoma derived EVs contained HMGB1 which is associated with immuno-stimulatory function. There was a up-regulation of the HMGB1 receptor, RAGE and TLR2 on the hTMSC. This eventually led to an activation of the NF-kB signalling pathway and increased the secretion of pro-inflammatory cytokines IL-6, IL-8 and IL-33.

Results

Results: The AC primed MSCs could induce T cells proliferation and activation. Moreover T cells were induced to differentiate to pro-inflammatory phenotype. It is associated with reduction in regulatory T cells (Treg) differentiation. In contrast to conventional thinking, T cells co-cultured with AC-primed MSC have a greater cytotoxic function. As immunogenic molecules were not found in the supernatant of the dying neuroblastoma cells, we explored the role of extracellular vesicles (EVs). Interestingly apoptotic neuroblastoma derived EVs contained HMGB1 which is associated

with immuno-stimulatory function. There was a up-regulation of the HMGB1 receptor, RAGE and TLR2 on the hTMSC. This eventually led to an activation of the NF-kB signalling pathway and increased the secretion of pro-inflammatory cytokines IL-6, IL-8 and IL-33.

Summary/Conclusions

Conclusion: After chemotherapy, abundant ACs release and they alter the immune milieu of TME. Apoptotic neuroblastoma can release HMGB1 to convert the MSCs from immunosuppressive to pro-inflammatory phenotype. Our findings suggest a paradigm shift in anti-cancer concept. This can potentially improve the efficacy of immunotherapy by manipulating the TME towards a more favourable condition for immune surveillance.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 259

MYCN and differentiation state alter inflammatory signaling in neuroblastoma

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

While immune checkpoint blockade (ICB) therapies have revolutionized the treatment of some adult cancers, these therapies have been largely ineffective in pediatric solid tumors, including neuroblastoma. In adult cancers, tumors with baseline inflammatory signaling and infiltration with cytotoxic T cells are more likely to respond, while immunologically “cold” tumors are more resistant. Response to ICB can be restored by converting tumors from a cold to a hot immunologic state, which may also enhance response to other immune-mediated therapies. Neuroblastoma, particularly *MYCN*-amplified disease, is a classically cold tumor with a lack of T cell infiltration and very low MHC I expression. To identify pathways that can be used to activate inflammatory signaling in neuroblastoma, we tested the functional responses of neuroblastoma cell lines to inflammatory stimuli and found that responses to some stimuli are consistent and those to others are heterogenous. Exploring the mechanisms behind this heterogeneity demonstrates a role for MYCN and mesenchymal/adrenergic differentiation state in regulating inflammatory signaling in neuroblastoma.

Aims

-Test the ability of exogenous interferon, toll-like receptor agonists, and cytosolic DNA to activate inflammatory signaling in neuroblastoma cell lines
-Determine the role of MYCN in modulating TLR-responsiveness in neuroblastoma cell lines
-Determine the role of mesenchymal-adrenergic differentiation in regulation TLR-responsiveness in neuroblastoma cell lines

Methods/Materials

We treated 19 neuroblastoma cell lines with a variety of inflammatory stimuli including exogenous interferon and agonists of toll-like receptors and other pattern recognition receptors. Response was determined by measuring phosphorylation of STAT1 and IRF3, change in mRNA expression of NF-κB and interferon response genes, and activation of an NF-κB reporter construct. We tested the effect of overexpression and knockdown of MYCN on responses to these inflammatory stimuli.

We then used inducible expression of PRRX1a in adrenergic cell types to convert them to a mesenchymal state and test the impact of this state switch on response.

Results

We found that all cell lines tested generated an inflammatory response to treatment with exogenous interferon and none responded to cytosolic DNA, but responses to TLR agonists were heterogenous. MYCN amplified cell lines were largely unresponsive and acute MYCN activation in non-amplified cell lines attenuated response. Differentiation state accurately classified the cell lines into responsiveness categories, with mesenchymal lines able to respond and adrenergic lines unresponsive. Exogenous expression of PRRX1a converted an unresponsive adrenergic line to a fully responsive state.

Summary/Conclusions

Neuroblastoma cell lines are universally sensitive to interferon and insensitive to cytosolic DNA but have heterogenous responses to toll-like receptor agonists. This responsiveness is partially suppressed by MYCN signaling but more strongly driven by mesenchymal differentiation. This suggests a potential vulnerability of mesenchymal cells to ICB and other immune-mediated therapies and future experiments will test this hypothesis.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 260

DDX3X and infant neuroblastoma: an oncogenic driver or coincidence?

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Topic Neuroblastoma as developmental disease

Background/Introduction

DDX3X syndrome is a recently identified genetic disorder associated with brain malformations and likely accounts for 1-3% of girls with otherwise unexplained intellectual disability (ID), and can also impact boys. Affected individuals present with ID, hypotonia brain anomalies such as dysgenesis of the corpus callosum and polymicrogyria, and systemic malformations including cardiac and ophthalmologic. DDX3X, located on the X-chromosome (Xp11.4), belongs to the DEAD-box RNA helicase family and acts as a key RNA-binding protein to exert its regulatory functions in various cellular processes including transcription, splicing, translation, and transport. DDX3 mutations have been reported to have a role various cancers, including colorectal cancer, breast cancer, lung cancer, hepatocellular carcinoma, Ewing sarcoma and glioblastoma multiforme.

Aims

Methods/Materials

Results

We present three cases of infant females with DDX3X-related neurodevelopmental disorder who were diagnosed with localized neuroblastoma; two of which were found incidentally while undergoing imaging for their neurodevelopmental delays. The first patient was diagnosed at the age of 4 months with mediastinal neuroblastoma. She underwent resection of the mass without recurrence. The second patient was diagnosed at the age of 7 months with unresectable presacral

neuroblastoma invading the foramina. She received 6 courses of chemotherapy and surgery. The third patient was diagnosed at the age of 6 months with an unresectable abdominal mass. She received 4 courses of chemotherapy. She had a recurrence one year later, which was completely resected. All three individuals after treatment were tumor-free at annual follow-ups.

Summary/Conclusions

This is the first report on a possible correlation between individuals with DDX3X-related neurodevelopmental disorder syndrome and neuroblastoma. All patients were female infants with localized disease, completely treated with no evidence of disease. Patients with DDX3X-related neurodevelopmental disorder have not been reported to have other cancers other than infantile neuroblastoma to date. Future studies are planned to explore the pathogenic mechanisms or the oncogenic role of DDX3X in neuroblastoma development.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 261

Therapy with dinutuximab beta in combination with chemotherapy in patients with refractory relapsed neuroblastoma. Initial results.

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

In spite of combined intensive therapy, prognosis in high risk neuroblastoma (NBL) patients with disease relapse or progression remains poor. There are data showing that immunotherapy combined with chemotherapy may be safe and effective treatment option.

Aims

The aim of the study was evaluation of feasibility, safety and effectiveness of combination of immunotherapy with dinutuximab beta (DB) and chemotherapy with irinotecan and temozolomide in patients with NBL relapse or progression.

Methods/Materials

Dinutuximab beta (10 mg/m^2/5 days) with chemotherapy (irinotecan 50 mg/m^2/5 days, temozolomide 100 mg/m^2/5 days) was given as compassionate use in patients with therapy resistant NBL relapse or progression. Patients received 1-7 cycles of therapy. For every patient the acceptation of the Ethical Committee was obtained. Therapy was given in 6 patients, age range 2.3-14.8 years. At diagnosis 5 patients were in stage 4 and 1 in stage 3 with MYCN amplification. MYCN amplification was found in 4/6 patients There were 3 distant, 2 combined and 1 local relapses. Two patients received immunochemotherapy at the 2nd relapse and 4 patients at the 1st relapse. DB was used in previous therapy in 3 patients (1 with interleukin-2). Before immunochemotherapy all patients received treatment with the second line standard chemotherapy with or without 131-MIBG treatment with autologous stem cell rescue, surgery and radiotherapy.

Results

Objective response was observed in 4 patients, including complete remission (CR) in 3 patients (2

with disseminated bone relapse and 1 with bone marrow relapse) and 1 very good partial response (VGPR, relapse in the localization of primary tumor). Three of them had previous immunotherapy. In 2 patients, in whom relapse occurred during the 1st line DB therapy (during cycle 2 and after cycle 1), only mild temporary improvement was observed after the first cycle (decrease of NSE and LDH, slight regression of tumor size, reduction of pain), with rapid progression few days after the cycle. Both patients died of disease. The patient with VGPR also died because of disease progression diagnosed during the fifth scheduled course of immunochemotherapy. Three patients are alive in CR (one during therapy, two – 5 and 6 months after end of therapy). Immunochemotherapy was quite well tolerated, with acceptable side effects. Hematological toxicities (stage 3 and 4 CTC WHO) and diarrhea (stage 2 and 3 CTC WHO) were common, but they did not influence therapy. Patients treated previously with DB had good tolerance of immunochemotherapy.

Summary/Conclusions

Therapy with DB in combination with chemotherapy is feasible, also in patients treated previously with DB. Objective response was observed in 4/6 pts (67%), including 3/6 pts (50%) with CR. It may be an option in therapy resistant patients, however it seems to have no benefit in patients who relapsed during immunotherapy. Clinical trials are needed to confirm its safety and efficacy.

Type Clinical

Presentation Preference Traditional poster

Abstract nr. 262

Evaluation of the role played by chemokines in shaping the immune microenvironment in neuroblastoma associated with opsoclonus-myoclonus

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Topic Other

Background/Introduction

Neuroblastoma (NB) is a highly heterogeneous disease, and *MYCN* amplification has been associated with a T cell-poor immune microenvironment. Among *MYCN* non-amplified tumors, some tumors are associated with Opsoclonus Myoclonus Syndrome (OMS), a rare neurological disorder linked with autoimmune targeting of the nervous system. Chemokines are a family of soluble cytokines that act as chemoattractants to guide the migration of cells, especially immune cells.

Aims

We designed a study to describe and understand the role played by chemokines in shaping the immune microenvironment in neuroblastoma associated with opsclonus-myoclonus.

Methods/Materials

We analyzed chemokine secretion *In vitro* by 4 NB cell lines displaying distinct *MYCN* status and ex vivo with fresh tumor samples obtained from 2 patients with or without OMS, and assessed the ability of tumor supernatants to recruit immune cells from peripheral blood mononuclear cells (PBMC) from healthy volunteers. Chemokine arrays were used to characterize the secretion of 38 chemokines in supernatants from NB cell lines and primary tumors. Transwell migration experiments were performed to analyze immune cell migration driven by these supernatants. The nature of migrating cells in transwell experiments together with the composition of tumor-infiltrating cells from the patients were characterized by multiparametric flow cytometry, using a panel allowing identification of antigen presenting cells (myeloid and plasmacytoid dendritic cells,

monocytes and B lymphocytes), T lymphocytes and their subsets (CD4+, CD8+, Treg, CD56+ T cells), and innate effector cells (NK cells - both CD56 bright and dim, gd T lymphocytes and iNKT).

Results

NB cell lines without *MYCN* amplification secrete higher level of IL-8, CCL2 and CXCL10 compared to *MYCN*-amplified NB cell lines. When assessing the ability of NB cell line supernatants (SN) to recruit immune cells, we observed that SN from NB cell lines without *NMYC* amplification induced active preferential migration of antigen presenting cells (APC=monocytes, myeloid (mDCs) and plasmacytoïd dendritic cells (pDCs)) and of gd T lymphocytes. Blocking experiments clearly demonstrated that this migration was dependent on CCR2/CCL2 axis, CCR2 being expressed at high levels by mDCs, pDCs and monocytes.
Notably, the direct ex vivo analysis of immune cell infiltration by flow cytometry of tumor samples from 2 patients with NB showed an enrichment of mDCs in tumors compared to blood samples, associated with CCL2 secretion observed in tumor supernatants. Interestingly, OMS patient's tumor had higher numbers of effector cells (CD3+CD8+, CD3+CD56+ and γδT lymphocytes), compared to patient's blood and the non-OMS patient's tumor. The chemokines responsible for this preferential recruitment are under investigation, and migration experiments are being performed to decipher the mechanisms involved.

Summary/Conclusions

We provide here for the first time an exhaustive characterization of chemokine expression and immune cell infiltration in NB, highlighting the major role of CCR2/CCL2 in the preferential recruitment of monocytes, mDCs and pDCs by MYCN non-amplified NB tumors. We also demonstrate that tumor from patient with OMS is infiltrated by higher numbers of effector cells of both adaptive and innate immunity compared to non-OMS patient, which might be directly related to the autoimmune activation leading to OMS symptoms.

Type Basic

Presentation Preference Traditional poster

Abstract nr. 263

Meta-[211At]astatobenzylguanidine ([211At]MABG) increases overall survival in a preclinical model of disseminated neuroblastoma

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

The alpha particle emitting targeted radiotherapeutic [^{211}At]MABG has superior radiobiological properties (shorter path length and higher energy to effect double strand breaks) compared to the currently used [^{131}I]MIBG. Responses to [^{131}I]MIBG therapy are typically short-lived, at least in part due to re-emergence of micrometastatic disease within the bone marrow compartment.

Aims

We sought to define the anti-tumor activity of [^{211}At] MABG in the setting of disseminated minimal residual disease (MRD).

Methods/Materials

We used lentiviral transduction in IMR-5 cells to generate *hNET* overexpressing neuroblastoma cell line models and performed *in vitro* cytotoxicity studies using [^{211}At]MABG. Dose finding studies were performed with [^{211}At]MABG (n=10 at each dose, cumulative dose range 1-1.8 mCi/kg) to establish the maximum tolerated dose (MTD) in NOD *scid* gamma (NSG) mice. Disseminated MRD models were established in NSG mice by intravenous tail vein injection of dual GFP/luciferase and *hNET* transfected IMR-5 cells (1x 106 cells per mouse). Mice (n=10) were treated with [^{211}At]MABG intraperitoneally (IP) in four fractionated doses of 300 uCi/kg each

administered twice weekly after adequate thyroid protection and followed subsequently with twice weekly bioluminescence (BLI) imaging and toxicity assessment for 60 days.

Results

NET overexpressing IMR-5 cell lines showed higher uptake of NET ligands and differential superior cytotoxicity compared to parental isogenic IMR-5 cells (EC50's in transfected lines were ~0.001 uCi/ml compared to 0.01 uCi/ml in wild type). In pilot studies, all NSG mice injected with IMR-5-Luc/GFP-*hNET* cells showed tumor engraftment, with bioluminescence arising in liver, spleen, adrenals, ovaries and abdominal cavity at a range of 12-15 days. Thus, intervention trials began at day 4 post injection of tumor cells. Onset of disseminated disease was significantly delayed in the [^{211}At]MABG treated animals (median 40.5±11.5 days versus 15±16.4 days), and at day 60, eight of nine of the [^{211}At]MABG treated mice were alive as compared to 2/10 of the control mice. Therapy was well tolerated with no evidence of significant weight loss or other toxicity.

Summary/Conclusions

[^{211}At]MABG is a safe and effective monotherapy for neuroblastoma MRD, and thus has the potential for synergistic anti-tumor efficacy with [^{131}I]MIBG due to their complementary radiobiologic properties.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 264

High resolution copy number analysis of UK high risk neuroblastomas

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Neuroblastoma (NB) is the commonest childhood extracranial solid tumour with around 100 new cases per year in the UK. In Europe high risk NB (HRNB) comprises patients > 1 year of age with metastatic disease, or < 1 year with metastatic disease and *MYCN* amplification or localised (stage 2 or 3) disease with *MYCN* amplification.

Aims

To perform a comprehensive genetic analysis of HRNB using high-density single nucleotide polymorphism (SNP) array data to identify other genetic abnormalities that may be targeted therapeutically or used to develop a future HRNB risk score.

Methods/Materials

SNP arrays from NB DNA from UK and Ireland patients on the European HRNB trial (HR-

NBL1/SIOPEN) were generated through the national NB reference laboratory at the Northern Genetics Service (Illumina CytoSNP-850k array) (n=195), and AROS (Affymetrix CytoScan HD array) (n=6). SNP array data was analysed using BioDiscovery Nexus copy number software (BioDiscovery, USA). Chi-squared analyses were performed to assess associations using IBM SPSS Statistics Version 25.

Results

Thirty-two DNA samples were removed because their genomic profile was flat (low tumour content) and three were removed for poor quality (n=166). *MYCN* amplification (MNA) was identified in 79 samples (48%). Chromosome 1p deletion was associated with MNA ($p<0.001$) and chromosome 11q deletion was associated with non-MNA ($p<0.001$) as previously reported. MNA tumours had significantly fewer copy number abnormalities (CNA) (mean=7.1) compared to non-*MYCN*-amplified tumours (mean=11.7) ($p<0.001$). 17q gain was the most common SCA, (141 cases, (84.9%)). Intragenic deletions of the *ATRX* gene were identified in 7 cases (4.2%) all in non-*MYCN*-amplified tumours. This study identified 102 amplified regions (amplicons) in addition to the *MYCN* amplicon, with the most frequent amplified genes in tumours being *ALK* (n=9, all MNA), *CDK4* (n=5, 2 MNA) and *MDM2* (n=4, 2 MNA), all of which can currently be targeted therapeutically. *TERT* amplification was identified in three cases (1.8%); two cases with MNA. Focal gain of *TERT* was found in a further five MNA tumours (3.0%). *PTPRD* heterozygous deletion was found in eight cases (4.8%). In two cases *MYCN* and *ALK* amplification were the only CNA found at diagnosis. Novel potentially targetable amplicons including oncogenes such the insulin receptor substrate 1 (*IRS-1)* (n=1) and Baculoviral IAP repeat-containing protein 1 *(BIRC-1, Survivin)* (n=1*)* were also identified. Analysis of structural defects identified the catastrophic event of chromothripsis (chromosome shattering and incorrect re-joining) in 5 tumours (3%), (one with stage 2 *MYCN*-amplified disease and four with stage 4 disease).

Summary/Conclusions

MNA is present in NB with fewer CNA than non-MNA HRNB and in two cases occurred with only one other CNA. Other targetable amplified genes occur in up to 12% of high risk NB and have been reported to lead to an inferior prognosis. The frequency of chromothripsis is low and its clinical significance uncertain. Ongoing work as part of an international SIOPEN Biology Group HRNBL-1 trial collaborative study will combine national CNA datasets with known clinical and genetic risk factors to generate a risk score which can be tested in future clinical trials of high risk NB.

Translational & Clinical Research Institute

Newcastle University

Sam Whiteman

Translational & Clinical Research Institute

Type Translational

Presentation Preference Oral presentation

Abstract nr. 265

Deriving predictive biomarkers for epigenetic drug response in neuroblastoma by supervised machine learning

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Children with high-risk neuroblastoma suffer from the lack of treatment options, and since relapse tumours are almost always resistant to chemotherapy, the disease outcome often is fatal. Recently, several drugs which either directly target neuroblastoma oncoproteins, such as ALK, or indirectly target MYCN and other drivers via epigenetic regulation have been introduced as promising treatment options for multiple cancer indications and are being evaluated in early-phase clinical trials. We and others have demonstrated preclinical efficacy of BET bromodomain protein inhibitors (BETi) and ALK inhibitors in neuroblastoma, and several inhibitors have already entered into clinical trials for adult tumours and paediatric indications.

Aims

With respect to biomarkers, mechanistic insights and potential combination therapies of these inhibitors, clinical trials currently run ahead of preclinical development. Our goal is to facilitate future clinical trials by deriving biomarkers for and insights into the activity of promising candidate drugs.

Methods/Materials

We screen a representative panel of 21 neuroblastoma cell lines for their response to three BET inhibitors and two ALK inhibitors which have a high potential to enter clinical trials for neuroblastoma. Based on cell-viability measurements upon treating the cellls with a wide range of drug concentrations, the dose-response curve for each drug and each cell line combination is derived. Leveraging the histone-modification ChIP-Seq data and RNA-Seq gene expression data

available for these cell lines, we then employ an iterative machine-learning algorithm, based on Random Forests, to identify genes and epigenetic marks which robustly predict treatment outcome for each class of inhibitor drugs. Thus found candidate predictive biomarkers are further evaluated in independent data sets and by small-scale wet-lab perturbation experiments.

Results

For BET inhibitors, the cell lines were reproducibly stratified into good responders, poor responders and non-responders. A small set of genomic and epigenomic markers was found to robustly and accurately predict neuroblastoma response to BET inhibitors. These predictive features, which include features strongly linked to BET protein biology, are currently being validated in the lab and in independent data sets. For ALK inhibitors, the drug-efficacy tests have been completed and features that are predictive for drug response are currently being elucidated using the machine-learning algorithm.

Summary/Conclusions

Validated predictive biomarkers can serve to define inclusion and exclusion criteria as well as to establish cohort sizes for future clinical trials. Moreover, certain predictive biomarkers may provide additional insights into potential resistance mechanisms and suggestions for combination therapies for treating high-risk neuroblastoma.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 266

Human anti- chimeric Ab (HACA) response in patients treated with the anti-GD2 antibody dinutuximab beta (DB): Frequency and effect on outcome and toxicity in high risk relapsed/refractory neuroblastoma patients. Results of a randomized SIOPEN-study

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

Immunotherapy with the human-mouse chimeric anti-GD_2 antibody dinutuximab beta (DB) may be associated with the development of a human anti-chimeric (HACA) immune response in treated patients.

Aims

To determine the frequency and the impact on efficacy and toxicity of HACA following long term infusion (LTI) of DB and oral isotretinoin with or without scIL-2 of DB in patients (pts) with high-risk relapsed/refractory neuroblastoma.

Methods/Materials

The SIOPEN Phase II clinical trial (EudraCT 2009-018077-31) enrolled 284 pts. In the single arm

phase of the trial (2011-2014), pts were treated with up to 5 cycles of 100 mg/m^2 DB-LTI (d8-17) and 160 mg/m^2 oral isotretinoin (d19-32) with 6x10^6IU/m^2 scIL-2 (d1-5; 8-12). From 2014-2017 pts were randomized to either receive IL-2 or not. HACA was determined by validated ELISA methods (Siebert N et al, J Immunol Methods. 2014 May; 407:108-15).

Results

The primary endpoints of the study were an increase of activated NK-cells and a DB level of >1µg/ml in cycle 1, which were met in all evaluable patients. The overall clinical response rate was 45%. In the randomization phase no difference for EFS and OS was observed for patients treated with DB and IL-2 vs DB alone (3y OS 71±6% vs 71±6% p=0.9; 3y EFS 54±7% vs 57±6% p=0.7). We identified 57/284 (20%) of pts with a HACA response (26/124 in the single arm phase; 31/160 in the randomized phase). The majority of HACA responses occurred before cycle 3 (70% of pts) and in the randomized phase the incidence of HACA was the same in pts treated with or without IL2 (15/81 (19%) (DB) vs. 16/79 (20%) (DB and scIL2). HACA resulted in a reduction of DB drug concentration levels and abrogated antibody effector functions (CDC and ADCC).
However, the incidence of any Grade 3&4 toxicity was not statistically different in HACA positive vs negative patients including key inflammatory toxicity parameters such as fever (38% vs 60%; p=0,074), capillary leak syndrome (23% vs 16%; p=0.39), allergic reaction (15% vs 11%; p=0.74) and flu like symptoms (8% vs 4%; p=0.61).
Of particular interest was the absence of difference for Grade 3&4 pain (19% vs 26% p=0.61): Although HACA response was associated with no detectable ADCC and CDC, pts experienced the same level of pain, indicating that there are other mechanisms involved in anti-GD_2 antibody-associated pain toxicity than CDC.
We also evaluated the effect of HACA response on outcome of pts of the single-arm cohort with the longest follow up period. Surprisingly, pts with HACA showed a higher event free survival at 2, 3 and 5 years compared to HACA negative patients (2y EFS 78±9% vs 58±6%; 3y EFS 74±9% vs 55±6%; 5y EFS 74±9% vs 48±7%) (p=0.066).

Summary/Conclusions

The 20% HACA response to dinutuximab beta was independent of IL-2. It reduced DB serum levels, abrogated antibody effector functions, had no influence on toxicity but was associated with improved EFS. Thus it may trigger adaptive immune responses (i.e. anti-idiotypic network), and provide a rationale for further vaccination strategies.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 267

Immunotherapy Initiated Before Surgical Resection Improves Survival and Reduces Metastatic Burden in Neuroblastoma

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

High-risk neuroblastoma portends a poor prognosis. While immunotherapy with anti-disialoganglioside (GD2) monoclonal antibody (dinutuximab) is an important therapeutic adjunct to chemotherapy and surgery, a majority of patients with high-risk disease still succumb to recurrent, metastatic disease. Our previous work demonstrated that the addition of adoptively transferred *ex vivo*-activated human natural killer cells (aNK) to dinutuximab results in improved survival and decreased metastatic burden in a neuroblastoma mouse model of post-resection minimal residual disease. While dinutuximab is currently administered after primary disease resection and post-consolidation, the impact of the timing of immunotherapy treatment relative to surgery has yet to be examined.

Aims

To demonstrate that immunotherapy with dinutuximab combined with adoptively transferred aNK cells initiated before surgical resection improves survival and reduces metastatic burden relative to therapy initiated after resection in a mouse model of metastatic neuroblastoma.

Methods/Materials

One million human neuroblastoma cells (CHLA-255 or KCNR), tagged with luciferase, were implanted beneath the left renal capsule of immunodeficient NOD-scid gamma (NSG) mice. Mice were randomized to three treatment groups: (i) surgical resection alone (“surgery only”), (ii) dinutuximab plus aNK cells initiated two weeks before resection (“early tx + surgery”), or (iii) dinutuximab plus aNK cells initiated after resection (“late tx + surgery”). Gross total resection of the xenograft tumors was performed 17 days after implantation. Immunotherapy and aNK cells were

administered with IL2 and IL15 via tail vein injection twice weekly for a total of four weeks. Weekly bioluminescent imaging was used to measure tumor cell flux (photons/sec) in each mouse as a means of quantifying total metastatic burden. Survival data was collected. Mixed-effects logistic regression was used to model flux data; paired t-test was used to calculate significance. Log-rank test was used to calculate significance for survival data. *P* value < 0.05 was considered significant.

Results

In mice implanted with the neuroblastoma cell lines CHLA-255 and KCNR, the total metastatic disease burden of the mice was decreased when immunotherapy was given before tumor resection compared to given after resection (p=0.06) or compared to surgery alone without immunotherapy (p=0.01). Immunotherapy plus aNK cells initiated prior to surgical resection resulted in significantly longer overall survival following implantation with either cell line (p<0.01) (Fig 1).

Summary/Conclusions

Our data shows that immunotherapy with an anti-GD2 monoclonal antibody plus adoptively transferred aNK cells administered before surgical resection prolongs survival and reduces the metastatic burden over time. These findings support the clinical investigation of immunotherapy in high-risk neuroblastoma during the pre-surgical induction phase of chemotherapy.

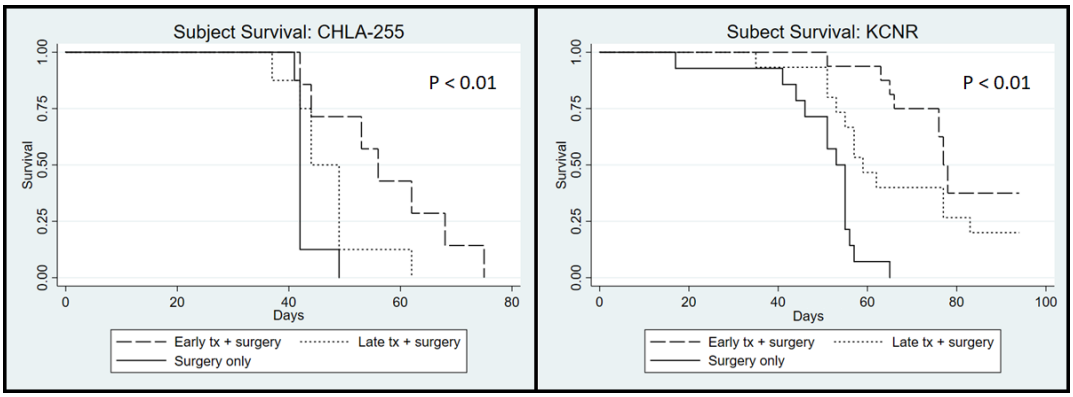

Figure 1. Survival Curves: CHLA-255, KCNR

Type Basic

Presentation Preference Oral presentation

Abstract nr. 268

Longitudinal cell-free DNA analysis in patients with high-risk neuroblastoma is suitable to monitor treatment response and identify actionable targets

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

The invasive nature of surgical biopsies most often prevents their sequential application to monitor disease. Single biopsies also fail to reflect cancer dynamics, intratumor heterogeneity and drug sensitivities that most likely change during clonal evolution and under the selective pressure of therapy.

Aims

Detection and characterization of cell-free DNA (cfDNA) in peripheral blood and bone marrow samples from neuroblastoma patients may serve as a minimally invasive liquid biopsy approach.

Methods/Materials

Duplex digital droplet PCR (ddPCR) assays assessing *MYCN* and *ALK* copy number status as well as ALK^{F1174L} and ALK^{R1275Q} hotspot mutations were applied to longitudinally collected blood and bone marrow plasma samples during or after first-line therapy (≥3 samples per patient) as well as corresponding primary tumor biopsies from 34 patients with high-risk neuroblastoma. Cell-free DNA was purified before ddPCR, and fragmentation of tumor-derived genomic DNA was achieved by direct enzymatic digestion in the ddPCR reaction mixture. Results were compared with data derived from clinical routine diagnostics including histopathological analyses of tumor

samples, bone marrow cytology/immunocytology and imaging in patients (MIBG and MRI scans).

Results

Persistence of markers was observed in patients with mixed responses and progressive disease, while clearance of cfDNA markers from plasma correlated with complete remission and survival in patients. A decline in total cfDNA levels over time corresponded with favorable treatment response. In individual courses, *MYCN* amplifications and *ALK* mutations were detected when analyzing the cfDNA, but not the corresponding primary tumor biopsy, suggesting tumor heterogeneity and underlining the potential of cfDNA analysis for actionable target identification. Reappearance of a cfDNA marker in plasma preceded the diagnosis of a relapse based on established clinical parameters by > 3 months in one patient, supporting a future role for monitoring patient-specific cfDNA markers in clinical routine aftercare programs.

Summary/Conclusions

Our results indicate liquid biopsies in patients with suspected or diagnosed neuroblastoma may be superior to tumor biopsies for detecting all markers in heterogeneous tumors and superior to established clinical parameters for the early detection of relapse. Our data justify the further development and testing of molecular disease characterization using cfDNA in blood and bone marrow plasma samples from patients with neuroblastoma in trials.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 269

A genome-wide study of an unfavourable subgroup of intermediate-risk (Group 8) neuroblastoma: A SIOPEN Biology and LINES group study

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Children with intermediate-risk neuroblastoma (20% of all NB) have an 80% 5-year survival but this is a heterogeneous group including a subgroup aged over 18 months at diagnosis with localised, unresectable disease (L2), without amplification of the *MYCN* oncogene and unfavourable histology according to the International Neuroblastoma Pathology Classification (INPC), which have a poorer outcome Within the SIOPEN current low and intermediate neuroblastoma (LINES) trial these patients termed Group 8 are treated with conventional chemotherapy, surgery, local radiotherapy and 13 cis retinoic acid while other protocols treat them, as high risk.

Aims

To investigate the genetics of Group 8 (G8) tumours and to determine if a subgroup with a poorer prognosis can be identified which in the future could be re-classified as high risk and treated accordingly.

Methods/Materials

Copy number analysis of 48 G8 diagnostic tumours from 7 countries participating in the LINES trial and the UK was performed from single-nucleotide polymorphism array (SNPa), array-CGH, MLPA, and FISH to detect numerical (NCA) and segmental chromosomal aberrations (SCA). Raw SNPa (Illumina CytoSNP-850k array or Affymetrix CytoScan HD array) copy number data was obtained and analysed by Nexus software (BioDiscovery,CA, USA) in 10 cases. Processed data was analysed for the remaining cases. Whole-exome sequencing (WES) (Illumina Truseq Rapid Exome Library Prep Kit) to a read depth of 100x run on an Illumina NextSeq 500 or NovaSeq 6000 flow cell with sequence reads analysed using a GATK pipeline, and SNPa were conducted on 8 paired UK G8 cases for analysis of SCAs and mutations acquired at relapse (1 case 3 relapses). The cohort had a mean age at diagnosis of 51.0 months (range 18.3-204.5).

Results

33/48 (68.8%) profiles contained ‘typical’ SCAs found in neuroblastoma. 13/48 profiles (27.1%) were NCA only, 2/48 (4.1%) profiles only had atypical SCAs. A mean of 2.47 SCAs (range 1-21) were observed with the following SCAs, in order of frequency: 17q gain (43.8%; n=21); 11q loss (41.7%; n=20); 2p gain (27.1%; n=13); 1q gain (20.8%; n=10); 1p loss (14.6%; n=7); 4p loss (14.6%; n=7) and 3p loss (10.4%; n=5). Gene amplifications were identified in 2 patients, both with SCA profiles. One case had 3 amplicons on chromosome 8: 8p(36,53-37,11Mb); 8q(94,84-95,15Mb) and 8q(128,52-128,77Mb including *MYC*. One case had *TERT* amplification with additional 16q(72,83-73,30Mb) amplicon containing *ZFHX3*. In 7/8 paired cases there was a change in copy number profile between diagnosis and relapse. In 5 cases an increase in SCAs at relapse and in 2 cases a decrease. *ALK* gene mutations were found in 4/11 (36%) post chemotherapy or relapsed tumours by WES and Sanger sequencing with one case switching from an F1174L mutation post chemotherapy to an R1275Q mutation at first relapse, before returning to the F1174L mutation in 2 subsequent relapse samples.

Summary/Conclusions

This ongoing SIOPEN Biology study reports a high frequency of SCA and novel amplicons in G8 tumours. WES is underway together with an investigation of telomere maintenance mechanisms to

link with clinical outcome and help guide future allocation to treatment groups.

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Type Clinical

Presentation Preference Oral presentation

Abstract nr. 270

Effect of CDK4 and MDM2 inhibition on cell viability in neuroblastoma

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

High-grade amplification of 12q13.3-14.1 and 12q15 were detected in small subset of tumors in our Swedish cohort of neuroblastoma patients including some with fatal outcome. Genes with an important role in cancer progression that are located in these amplified areas includes *CDK4,* which is involved in the cell cycle and *MDM2,* which is a proto-oncogene involved in p53 regulation.

Aims

The aim of the project is to investigate the effect of CDK4 and/or MDM2 inhibition on cell viability in relation to genetic background to determine in which cases these treatments could be beneficial for the patients. Furthermore, we wanted to study the interaction between CDK4 and MDM2 inhibitors to analyze if there is any possibility of a synergetic interaction between them.

Methods/Materials

For this project ten NB cell lines were used including two 12q-amplified cell lines. The cell lines were treated with Ribocilib (CDK4 inhibitor), Nutlin-3a (MDM2 inhibitor) or a combination of Ribociclib and Nutlin-3a after 24h seeding in 96 well plates. The viability assays were done with PrestoBlue at different time points (24-48-78h). IC50 analysis was calculated by Prism 8 while the synergy analysis was performed using SynergyFinder. Western blot was used to evaluate the downstream effects of the inhibitors.

Results

The viability analyses showed that nine out of ten cell lines response to CDK4 inhibition with Ribociclib, resulting in a 50% cell viability reduction (IC50) after 72h treatment. The resistance of the remaining cell line to Ribociclib treatment, SK-N-FI, could possibly be explained by its high expression of MDR1 (Multidrug Resistance Protein 1).
In the case of MDM2 inhibition through Nutlin-3a, five out of ten cell lines were sensitive to the

treatment where 50% cell viability reduction occurred after 24h; the other five cell lines were resistant to Nutlin-3a even after 72h of treatment.
The response of Nutlin-3a was dependent of *TP53* mutation status where all cell lines resistant to the treatment are *TP53* mutated. On the other hand, the sensitive cell lines were *TP53* wild type (wt), except for NGP which is *MDM2* amplified. Further studies of NGP are needed in order to identify if the sensitivity to the treatment is related with the amplification of *MDM2*, if NGP is heterozygous for *TP53* or if the mutant retains some wt functionality.
No significant synergy could be detected with combined Ribociclib and Nutlin-3a treatment.

Summary/Conclusions

This study indicates that CDK4 inhibition could provide an efficient treatment for NB, possibly also in patients with *CDK4* amplified tumors and that MDM2 inhibition is a potential treatment option for *TP53* wt NB. Interestingly, combined CDK4 and MDM2 inhibition does not provide any synergistic effects on the NB cell lines that have been used.

Type Basic

Presentation Preference Traditional poster

Abstract nr. 271

Strong induction of regulatory T cells by subcutaneous interleukin-2 is associated with no survival benefit in relapsed and refractory high risk neuroblastoma patients treated with long-term infusion of anti-GD2 antibody dinutuximab beta, a randomized SOIPEN-study.

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

We previously evaluated efficacy and toxicity of long term infusion (LTI) of dinutuximab beta (DB) in combination with subcutaneous interleukin 2 (scIL-2) and oral isotretinoin in single arm clinical studies conducted from 2009-2013 in patients with high-risk relapsed/refractory neuroblastoma. Immune modulation by DB and isotretinoin without scIL-2 is unknown.

Aims

To evaluate immune modulation in patients receiving DB and oral isotretinoin therapy with or without IL-2 in a prospective randomized trial.

Methods/Materials

160 patients from 11 countries were enrolled between 07/2014 and 07/2017 into an open label

Phase II clinical trial (EudraCT 2009-018077-31). Patients were randomly assigned to receive up to 5 cycles of 100 mg/m^2 DB LTI (d8-17) and 160 mg/m^2 oral isotretinoin (d19-32) without (81 patients) and with 6x10^{6}IU/m^2 scIL-2 (d1-5; 8-12) (79 patients). Toxicity (CTCAE), response rates (INRC) and 2yrs-event free and overall survival were evaluated.
Regulatory T cells (Tregs, CD4+/CD25+/CD127-) were assessed by flow cytometry in every treatment cycle on d1 (prior to start of treatment), d8 (5 days of scIL-2 treatment, prior to start of DB LTI), d15 (3 days after end of scIL-2 treatment, 8 days of DB-LTI) and d18 (last day of DB-LTI).

Results

Disease stage, patient age, MYCN amplification, relapse and remission status were balanced between the treatment arms (median follow-up 2.6 y). The 2yrs-EFS and -OS for DB (81 patients) vs. DB combined with scIL-2 (79 patients) was 59%±6% vs. 65%±6% (p=0.721) and 79%±5% vs. 84%±4% (p=0.904). In 97 patients with evaluable disease, response rates of 49% (9% CR, 40% PR) vs. 52% (26% CR, 26% PR) after treatment with DB vs. DB and scIL-2 were observed. Grade 3&4 fever (16% vs. 46%, p=0.000), allergic reaction (1% vs. 14%, p=0.004), hematological toxicity (46% vs. 66%, p=0.013) and neurotoxicity (0% vs. 8%, p=0.003) were significantly worse in the combination arm.
Prior to the treatment start, Treg levels were 47±20 cells/µl. Five days of scIL-2 treatment resulted in a 6-fold increase of Tregs (305±51 cells/µl) on d8. Following the combined treatment a slight reduction of Tregs counts on d15 and d18 compared to d8 was observed (219±33 and 154±48 cells/µl, respectively). Moreover, we found an increase of the peak levels in subsequent cycles compared to the first cycle except for cycle 4 (385±48, 493±126, 342±51 and 517±68 cells/µl for cycle 2, 3, 4 and 5, respectively), indicating Tregs accumulation during the whole treatment period of DB and scIL-2.
In contrast, patients receiving DB without scIL-2, showed similar Tregs levels on d8 compared to the baseline (85±52, 84±45, 112±70 and 93±47 cells/µl for cycle 2, 3, 4 and 5, respectively), and Tregs remained low during DB-LTI on d15 and d18, confirming that Tregs induction was mediated by scIL-2.

Summary/Conclusions

No significant difference in efficacy of DB and oral isotretinoin compared to DB and oral isotretinoin combined with scIL-2 was observed. The IL-2-dependent induction of Tregs may provide an explanation for lack of beneficial effects of IL-2 co-treatment.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 272

INDIA NEUROBLASTOMA REGISTRATION AND BIOLOGY STUDY (INPOG-NB-18-01): IMPROVING OUTCOME FOR PATIENTS WITH NEUROBLASTOMA IN A LOWER-MIDDLE INCOME COUNTRY

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Neuroblastoma (NB) is a common childhood cancer, but India has an annual incidence rate one-third that of expected (4.3/million children 0-14 yrs age per year), with 75-90% of patients presenting at an advanced stage. It is unclear whether this represents selection bias. Outcome data for patients with NB who undergo therapy, or have associated complications, are lacking in the absence of a national neuroblastoma registry. Risk stratification of NB in India and clinical management is affected by the lack of genetic testing for N-MYC amplification and other validated markers, and India lacks an effort to systematically analyse NB using next generation sequencing (NGS), such as utilized by the MAPPYACTS study in France and the NANT consortium in the USA, to allow for translational research. This prospective trial is an effort to generate all of these data.

Aims

The aims of this study are two-fold. An online registry will be used to collect data on the incidence and stage of patients with NB in India and their clinical outcomes, including complications such as opsoclonus-myoclonus syndrome and spinal cord compression. Genetic testing on NB tumors using NGS will help risk stratify patients for clinical management, as well as for soon-to-be-opened InPOG NB clinical trials, and document the genomic landscape of NB in India, creating a biobank of NB tumour DNA to allow meaningful translational research.

Methods/Materials

The study was designed by a pediatric oncology committee within India, peer-reviewed within and outside India, and registered with the Indian Pediatric Oncology Group (InPOG). A custom designed online database housed in the Resonance platform, will allow collection of clinical and staging data of patients with NB (www.Resonanceoncology.org). Formalin-fixed paraffin

embedded tumor slides (10 unstained, 2 H&E) are couriered to Neuberg Supratech in Ahmedabad where NGS is performed with the Oncomine Childhood Cancer Assay. N-MYC amplification and ploidy status are returned to institutions within 3 weeks. The report includes fluorescent in situ hybridization (FISH) for 1p and 11q deletions, to provide meaningful data for risk stratification. The total cost of analysis is approximately USD $25 per patient. Variant genomic data on all NB samples is stored at Neuberg in cbioportal for further analysis. The goal is to enroll 300 patients with NB over 3 years, and 20 institutions in India have expressed interest. Funding support was gratefully received from Cankids…kidscan, (www.cankidsindia.org), a childhood cancer support organization.

Results

Five institutions already have ethics committee approval and have begun enrollment, and despite delays in opening the study due to lack of investigator protected time, sufficient research staff, or a centralized ethics review board, we expect to meet our accrual target by 2023.

Summary/Conclusions

This study demonstrates it is possible to bring together organizations and individuals in a lower-middle income country such as India, to create a clinical trial network to register patients with NB to better define outcomes, and to analyze NB tumor biology to allow improved risk stratification, with an overall goal of improving NB outcomes.

InPOG NB-18-01 trial sites India
Type Clinical

Presentation Preference Oral presentation

Abstract nr. 273

Distinct roles of noradrenergic core regulatory circuitry transcription factors in neuroblastoma cell identity

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Topic Gene regulatory networks and epigenetics: from basic science to therapy

Background/Introduction

The non-genetic heterogeneity of neuroblastoma cell identity has been recently revealed with the characterization of noradrenergic and NCC (Neural Crest Cell)-like/mesenchymal cells, each cell type being governed by a Core Regulatory Circuitry (CRC) of specific transcription factors (Boeva et al, 2017; van Groningen et al, 2017). Whereas the noradrenergic CRC includes the PHOX2B, HAND2, and GATA3 transcription factors, the CRC associated to the NCC-like/mesenchymal identity is composed of AP-1 transcription factors among others.

Aims

The present study aims at deciphering how the PHOX2B, PHOX2A and GATA3 transcription factors shape the noradrenergic identity of neuroblastoma cells.

Methods/Materials

Using the CRISPR-Cas9 gene editing approach, we performed the full knock-out of either *PHOX2B, PHOX2A* or *GATA3* genes in the SH-SY5Y neuroblastoma cell line. The obtained clones were characterized in terms of growth, phenotype and identity, using in particular transcriptomic (RNA-seq) and epigenomic (H3K27ac ChIP-seq for super-enhancer detection) analysis.

Results

PHOX2B or *PHOX2A* KO clones showed a morphology close to that of the parental SH-SY5Y cells with neurite-like processes. In contrast, *GATA3* KO cells presented with a different morphology, exhibiting a more abundant cytoplasm and many actin stress fibers, reminiscent of a mesenchymal phenotype. A clustering analysis using RNA-seq data, based on a gene expression signature that discriminates the noradrenergic and mesenchymal neuroblastoma cell identities, confirmed that the *PHOX2B* and *PHOX2A KO* clones harbored transcriptomic profiles highly similar to that of the parental noradrenergic SH-SY5Y cell line whereas those of the *GATA3* KO clones were close to that of the mesenchymal SH-EP cell line. We further documented that *GATA3* KO clones exhibited mesenchymal features including increased migration and invasion capacities and higher resistance to etoposide and doxorubicin *in vitro*. *PHOX2B* and *GATA3* KO cells displayed a decreased proliferation both *in vitro* and *in* vivo in xenograft experiments. In a principal component analysis based on neuroblastoma and human neural crest cells super-enhancer log scores, *GATA3* KO clones closely resembled the group II of mesenchymal cell lines, comprising the SH-EP, GIMEN and GICAN cell lines. In addition, DNA binding motif analysis using I-cis Target on the *GATA3* KO cells super-enhancers revealed an enrichment for AP-1 transcription factors, consistent with a mesenchymal identity and associated CRC. Strikingly, we observed that these *GATA* KO cells shifted towards a noradrenergic identity when injected in mouse.

Summary/Conclusions

Our data demonstrate that PHOX2B and GATA3 play different roles in the maintenance of the noradrenergic identity in neuroblastoma cells *in vitro*. Whereas the KO of either *PHOX2A* or *PHOX2B* did not affect the noradrenergic identity of the SH-SY5Y cells, the KO of *GATA3* induced a global reprogramming from a noradrenergic to a mesenchymal identity. However, a noradrenergic identity was recovered under *in vivo* selective pressure in the mouse, indicating that the noradrenergic CRC is functional in the absence of GATA3 in these conditions.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 274

Pretreatment 18F-FDG PET/CT May Have Prognostic Value in Neuroblastoma

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Topic Other

Background/Introduction

Despite advances in therapy, survival for patients presenting with metastatic neuroblastoma remains poor. While ^{123}I-metaiodobenzylguanidine (MIBG) remains the dominant scintigraphic imaging modality for staging and follow-up of neuroblastoma, ^{18}F-FDG PET/CT plays a role in patients with MIBG negative disease. The role of ^{18}F-FDG PET/CT in patients with MIBG-avid disease continues to be explored.

Aims

The objective of this study was to report quantitative FDG PET findings in a cohort of children with neuroblastoma (irrespective of MIBG avidity) and to assess for associations with MYCN amplification status, as a prognostic marker, and with survival.

Methods/Materials

This was a single institution retrospective review of patients with newly diagnosed neuroblastoma for whom a pre-therapy ^{18}F-FDG PET/CT was obtained between July 2006 and July 2019. FDG-PET examinations had been obtained with 0.1-0.14 mCi/kg of FDG with imaging at 1 hour after radiopharmaceutical administration. Using the PET-edge tool in MIM (MIM Software; Cleveland, OH), a single observer drew regions of interest around the primary tumor to measure SUVmax, SUVmean, tumor volume (TV), and total lesional glycolysis (TLG, SUVmean x tumor volume). Event free survival (EFS) was defined as the time from diagnosis to tumor recurrence or death. Overall survival (OS) was defined as the time from diagnosis to death. Univariate and multivariate (age-adjusted) analysis via a Cox proportional hazards regression analysis was used to assess the predictive performance of PET indices for EFS and OS. Optimal thresholds for the PET indices were determined using receiver operating characteristic (ROC) curve analysis, and the survival significance of these thresholds were determined using a log-rank test.

Results

A total of 55 patients were identified, 45 of whom had MYCN FISH performed. The median age at diagnosis was 2.92 years (range 0.011-10.4 years). Average follow-up was 1654 days (range 27-

4433 days). Nine patients (16%) experienced disease recurrence, and eleven patients (20%) expired during the study period. Thirteen patients (29%) had MYCN amplification. SUVmax ranged from 1.1 to 11.2 (mean 4.38 ± 2.23). SUVmean ranged from 0.78 to 4.67 (mean 2.05 ± 0.84). TV ranged from 0.80 to 1039 mL (mean 179 ± 232 mL). TLG ranged from 0.77 to 2681 (mean 418 ± 601 mL). Patients with MYCN amplification were older than those without MYCN amplification (3.6 ± 2.3 vs 2.4 ± 1.6 years, $p = 0.04$). Mean SUVmax (5.9 ± 2.4 vs 4.2 ± 2.1, $p = 0.028$), tumor volume (438 ± 335 mL vs 95 ± 96mL, $p<0.0001$), and TLG (1056 ± 845 vs 226 ± 329, $p<0.0001$) were significantly higher in MYCN amplified tumors. SUVmax ($p = 0.028$) and SUVmean ($p = 0.045$) were significantly associated with overall survival. An SUVmax threshold of 4.77 ($p = 0.028$) best predicted OS with a median OS of 2604 days for patients with SUVmax above this threshold versus >2957 days for patients with SUVmax below this threshold.

Summary/Conclusions

On average, MYCN amplified neuroblastomas are larger and more metabolically active than MYCN non-amplified tumors. Additionally, higher pretreatment SUVmax and SUVmean are associated with shorter overall survival.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 275

Pattern and predictors of sites of relapse in neuroblastoma: a report from the International Neuroblastoma Risk Group (INRG) project

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Topic Other

Background/Introduction

As relapse remains the major obstacle to cure for patients with high-risk neuroblastoma, it is imperative to assess the clinical and biologic predictors of patterns of failure.

Aims

To perform a detailed analysis of the biologic, clinical, and prognostic differences according to the pattern of failures at the time of first relapse.

Methods/Materials

Children < 21 years of age diagnosed with neuroblastoma or ganglioneuroblastoma between 1989-2017 with available data regarding site of first relapse (isolated local vs. isolated distant vs. combined local and distant sites) were identified from the INRG database. Data were compared between sites of relapse according to clinical features (age, tumor type, INSS stage, serum ferritin, and LDH); biologic features (*MYCN* status, histology, pooled segmental chromosomal aberrations); initial treatment; and overall survival from time of first relapse. Univariate analyses evaluating potential differences in any of the above variables according to the pattern of relapse were performed using ANOVA and chi-square tests. A competing risk regression was used to evaluate predictors of specific patterns of failure.

Results

The pattern of first relapse among 1,833 children was: 19% isolated local; 65% isolated distant;

and 16% combined failure sites. All evaluated clinical and biologic variables differed statistically by relapse site, with exception of LDH and INPC diagnostic category. High-risk features and receipt of initial intensive therapy were more frequent in patients with isolated distant and combined relapse sites ($p<0.05$). Patients with INSS stage 3 disease were more likely to have isolated local failures (88/181 relapses, 49%) compared to patients with all other stages (258/1,627 relapses, 16%; $p<0.001$). The overall survival from time of first relapse was significantly different by site of relapse (5-year OS +/- standard error): isolated local, 64% +/- 3%; isolated distant, 23 +/- 2%; and combined sites, 26 +/- 4% ($p<0.001$).

Summary/Conclusions

Patients with isolated distant and combined failure sites have a higher proportion of unfavorable clinical and biological features. Understanding differences based upon sites of relapse may inform our understanding of the failure of therapy at these sites.

Type Clinical

Presentation Preference Traditional poster

Abstract nr. 276

ß3-adrenoreceptor modulates the immune-checkpoint PD-L1 expression and impairs tumor growth in a murine syngeneic neuroblastoma model

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

Neuroblastoma (NB) is the most frequently observed among extracranial pediatric solid tumors. Despite the availability of numerous therapies, high risk NB is still difficult to cure, therefore, new therapeutic strategies are desperately needed. Immune therapies are the most promising anti-tumor strategy; moreover, several literature data support the concept that the PD1/PD-L1 immune checkpoint axis, among others, is crucial for allowing NB immune-evasion. β3-adrenoreceptor (β3-AR) has been recently identified as a crucial regulator of different tumor-related processes, including immune reactivity. Moreover, we recently demonstrated that β3-AR-blockade, via sphingosine 1-phosphate (S1P) signaling modulation, is able to affect NB tumor progression using *in vivo* and *in vitro* models.

Aims

The present project aims to investigate the relationship between the β3-AR pathway, the S1P signaling and the expression of the PD1/PD-L1 immune-checkpoint in NB. Given that, recently, our group demonstrated that β3-AR is involved in regulating the immune reactivity of melanoma, and that its blockade is also able to impair NB tumor growth, the goal of our study was to investigate whether β3-AR modulation affects the immune reactivity in NB through S1P signaling. Specifically, we evaluated the expression of PD1/PD-L1 immune-checkpoint in a murine syngeneic model of NB following β3-AR antagonism.

Methods/Materials

Female NCI A/J mice 4-weeks-old were bought from Charles River Laboratories (Frederick). Murine NB Neuro-2A cells were subcutaneously implanted in A/J recipient mice by injecting $1x10^6$ cells in 100 µl of PBS in the right flank. Tumor growth rate was evaluated by measuring tumor mass with a caliber, and tumor mass volume was calculated as Volume=[(length x width)^2/2]. Treatments with β3-AR antagonist (SR59230A) and $S1P_2$ receptor selective agonist (CYM5520) started when Neuro-2A cells formed a palpable tumor. Mice were sacrificed after 8 days of treatment. Tumor masses were then excised and divided to perform western blot, immunofluorescence, and citofluorimetric analyses, in order to evaluate immune-checkpoint expression in tumor cells and tumor infiltrating lymphocytes (TILs). TILs were isolated by tumor mass using anti-CD45 micro-beads and the AutoMACS Separator Pro (Miltenyi Biotec).

Results

β3-AR pharmacological blockade, induced with SR59230A, strongly reduced NB tumor growth in A/J mice through the involvement of the sphingosine-1-phosphate receptor 2 ($S1P_2$). The expression of the immune-checkpoint PD-L1 decreased in tumour masses excised from SR59230A-treated mice compared to control group. Moreover, this decrease was partially reverted by using a selective $S1P_2$ receptor agonist (CYM5520). These results suggest that β3-AR is able to regulate the PD1/PD-L1 expression in NB tumor, through the S1P signalling involvement.

Summary/Conclusions

Even though cancer immune therapy has given great chances of good prognosis to several oncological patients, some of them are still not responsive to these therapies. Thus, the understanding of the molecular mechanisms that regulate the expression of the PD1/PD-L1 and other immune checkpoints during tumor progression could open new therapeutic strategies to overcome tumor resistance of this inauspicious pediatric tumor.

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β3-adrenoreceptor modulates the immune-checkpoint PD-L1 expression and impairs tumor growth in a murine syngeneic neuroblastoma model

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Type Translational

Presentation Preference Traditional poster

Abstract nr. 277

New strategies to reprogram dysfunctional tumor-infiltrating T cells in high-risk neuroblastoma

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

The tumor microenvironment (TME) plays a crucial role in cancer progression. The presence of tumor-infiltrating T cells (TILs) and the absence of immunosuppressive elements have been associated with favorable prognosis in high-risk NB patients. Recently, a subset of intratumoral dendritic cells (DC) has been found crucial for anthracycline-induced anticancer immune responses, suggesting that they could be exploited to improve NB therapy.

Aims

Herein, we investigated the modulation of the immune infiltrate in tumors derived from the TH-MYCN transgenic mice, both *in vivo* and *ex vivo* models and in patient-derived organotypic tumor spheroids (PDOTS) collected from high-risk NB tumors, following treatment with chemotherapeutic drugs in combination with antibodies blocking the immune checkpoints.

Methods/Materials

Luciferase-expressing NB cell lines derived from spontaneous tumors of TH-MYCN transgenic mice were injected subcutaneously or orthotopically into syngeneic mice. Mice bearing established tumors were sacrificed and the functional status of the TILs was evaluated by flow cytometry. The crosstalk between TILs and tumor cells was evaluated in murine-derived organotypic tumor spheroids (MDOTS), culture systems resembling to native three-dimensional (3D) TME. PDOTS from high-risk NB tumors were also collected and analyzed. Specifically, MDOTS and PDOTS were treated with chemotherapeutic drugs, alone or in combination with immune checkpoint inhibitors (ICI) and then co-cultured with TILs. The ability to recall immune cells to tumor spheroids and the functional status of TILs have been evaluated with microfluidic devices and flow cytometry, respectively.

Results

The orthotopic tumor model showed a more immunosuppressive TME than the subcutaneous

ones, predominantly infiltrated by macrophages, myeloid-derived suppressor cells and T regulatory cells. *Ex vivo* experiments showed that treatment with chemotherapeutic drugs in combination with ICI caused a decreased in the diameter of MDOTS and an increase in TIL recruitment. Flow cytometry analysis revealed that $CD8^+$ T cells co-cultured with MDOTS expressed higher levels of IFN and TNFα, and reduced levels of Tbet and Tigit. Remarkably, drug treatment suppressed tumor growth *in vivo* and most interestingly induced a strong recruitment of IFN-expressing $CD8^+$ T cells in drug-treated PDOTS microfluidic devices.

Summary/Conclusions

Overall, our findings provide insights for investigating novel immunotherapeutic approaches in NB.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 278

Discovery and Immunotherapeutic Targeting of Lineage-restricted Major Histocompatibility Complex (MHC) Antigens in Neuroblastoma

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

The MHC presents a snapshot of the intracellular proteome for surveillance by T cells, including peptides from mutated oncoproteins (neoantigens) and nonmutated but aberrantly expressed proteins. Neuroblastoma is characterized by low mutational burden and low MHC expression, thus presenting a unique set of challenges in identifying and targeting tumor-specific antigens.

Aims

1) Discover antigens derived from lineage-restricted oncoproteins in high-risk neuroblastomas. 2) Engineer T cell receptors (CARs and TCRs) targeting neuroblastoma antigens and show anti-tumor efficacy.

Methods/Materials

We first performed LC/MS/MS proteomics on peptides eluted from purified MHC class I and II from 8 high-risk patient derived xenograft (PDX) and 8 primary archived patient tumors. We then compared antigens eluted from neuroblastoma tumors to healthy tissue ligandomes, and expression of parent genes between tumor and normal transcriptomes. We validated putative antigens by synthesizing peptides and performing LC/MS/MS, and validated binding to predicted HLA by refolding the pMHC complex. We then performed phage display to generate CARs directed at prioritized antigens in the proper MHC context, using decoy pMHC to remove non-specific binders. For any candidate binder with cross reactivity to MHC, we applied mutagenesis screens across the CDR loops to characterize the binding of each residue to MHC to abrogate non-specificity. TCRs were generated by enriching antigen-specific CD8 cells and performing single-cell sequencing on functionally expanded cells. CARs and TCRs were screened for anti-

tumor efficacy in preclinical models through imaging of cleaved caspase.

Results

We identified 265 novel antigens presented on MHC, and prioritized six (PHOX2B, IGFBPL1, HMX1, TH, GFRA2, and CHRNA3) as lead candidates based on binding affinity (<500nM), level of differential gene expression, lack of MHC presentation in healthy tissue, relative abundance (>95th percentile), biological relevance to neuroblastoma, recurrence across multiple tumors, and presentation by HLA-A2 or HLA-A24. LC/MS/MS of synthetic tumor peptides matched spectra of peptides eluted from tumors and peptides properly refolded with predicted MHC binders, resulting in crystal structures of both PHOX2B/HLA-A24 and IGFBPL1/HLA-A2 complexes. We engineered CAR and TCR receptors targeting PHOX2B and IGFBPL1 pMHC and find that the CAR targeting IGFBPL1 is specific for tumor peptide and does not cross-react with MHC, whereas the PHOX2B-directed CAR cross-reacted with MHC. We developed a 2nd generation PHOX2B pMHC CAR in which MHC cross-reactivity is ablated through mutation of two residues in the CDR binding loops. We demonstrate complete ablation of cancer cells using CARs at 2:1 E:T ratio following 24 hours of co-culture with T cells.

Summary/Conclusions

Neuroblastoma cells present a unique ligandome, including a significant number of MHC antigens derived from lineage-restricted oncoproteins. We prioritized six antigens for preclinical development, demonstrating initial proof-of-concept with CARs showing potent cytolytic activity against PHOX2B and IGFBPL1 tumor antigens on common HLA alleles. These data demonstrate that lineage-restricted oncoproteins can provide a source of tumor-specific self-antigens that can be targeted using CARs, and support targeting these antigens in neuroblastoma and other low mutational solid tumors. Additionally, we present methods for engineering and optimizing CARs that can be applied to tumor neo/self-antigens, and activity of the pMHC CARs in PDX models of neuroblastoma.

very and Immunotherapeutic Targeting of Lineage-restricted Major Histocompatibility Cor (MHC) Antigens in Neuroblastoma

Type Translational

Presentation Preference Oral presentation

Abstract nr. 279

Bim protein-protein interactions provide predictive biomarkers for Bcl2-family inhibitor responses in neuroblastoma

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Chemotherapy induced cell death is governed by the Bcl2-protein family. Effective therapies activate death-domain proteins (like Bim) that engage Bak or Bax, causing cell death. In cancers, these death-domain proteins are often sequestered by survival proteins like Bcl2 or Mcl1 instead. This preserves viability but makes such cells continually dependent on this function. Drugs that competitively displace death-proteins from these survival proteins can restore apoptosis. Such Bcl2-family inhibitors show great promise to impact cancer outcomes. Venetoclax, a Bcl2 inhibitor, is FDA-approved and many other drugs in this class are advancing in the clinic. However, no predictive biomarkers exist to guide their development.

Aims

We sought to identify the mechanisms underlying neuroblastoma survival dependencies, and develop predictive biomarkers to guide the clinical use of emerging Bcl2-family inhibitors.

Methods/Materials

Neuroblastoma cell lines and PDXs were studied, including paired samples from the same patients at different times in therapy and/or different anatomic sites. Tumor models were studied using: mitochondrial profiling (probing tumor-isolated mitochondria with diverse stress proteins) to infer their survival dependency; cytotoxicity assays after exposure to ABT199 (BCL2-inhibitor, aka BCL2i), ABT737 (BCL2/BCLXi), A1155463 (BCLXi), and S63845 (MCL1i) alone and in combination; and coIP to identify key Bcl2-family protein:protein interactions (PPIs) that correlate with inhibitor sensitivity.

Results

We identified two patterns of mitochondrial profiles: the first predicts a dependency on Bcl2, and all such tumors are sensitive to BLC2i's in vitro (IC50<1uM) and in vivo, but not other inhibitors. In each, we found Bim bound by Bcl2 using coIP (not Mcl1). The mechanism of BCL2i activity in "Bim:Bcl2" tumors is displacement of Bim, freeing it to activate Bak/Bax. The second mitochondrial profile predicts an Mcl1 dependency, and in each Bim was bound by Mcl1 (not Bcl2). Surprisingly, "Bim:Mcl1" tumors are insensitive to MCL1i's (IC50>2uM). Our coIPs show Bim is displaced from Mcl1 by the drug, but re-sequestered by Bclx, and these tumors are exquisitely sensitive to inhibition of both Mcl1 and Bclx (IC50<50nM), fully predicted by Bim PPIs. Seven sets of patient-matched tumors from distinct sites and times had identical Bim PPIs, supporting this as a stable actionable tumor feature. Neither *MYCN* nor *ALK* mutation status correlated with Bcl2 or Mcl1 survival dependency, yet all tumors harboring *MAPK* mutations (*PTPN11, RAS, RAF, NF1*) were in the Bim:Mcl1 class. Genetically-activating *RAS* (via NF1 knock-down) drives Bim from Bcl2 to Mcl1, with the predicted consequences on inhibitor sensitivity.

Summary/Conclusions

We show that neuroblastomas sequester Bim to stay alive, and drugs that selectively displace Bim provide a lethal stress. Our work decodes these survival interactions to provide numerous opportunities to develop predictive biomarkers, and to optimize the clinical efficacy of these agents, particularly as our Bim PPI-defined survival dependency appears stable and cell intrinsic. Our work also defines a hierarchical Mcl1-Bclx dependency and links *MAPK* activation to this class, and are testing *MAPK* inhibitors for their potential to synergize with Bclx inhibition, as predicted by our data.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 280

Immunology of neuroblastoma. Beyond the immunotherapy

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

Nowadays immunotherapy is becoming the standard-of-care in treatment of high-risk neuroblastoma, while molecular and cellular mechanisms underlying tumor-host interaction are still mostly unraveled.

Aims

Investigation of mechanisms of anti-tumor immune response against neuroblastoma and immune-mediated paraneoplastic opsoclonus-myoclonus syndrome (OMS).

Methods/Materials

39 tumor specimens from primary chemotherapy and immunotherapy naïve patients with neuroblastoma were subjected to mRNA analysis with Nanostring PanCancer Immune Profiling Panel. This type of the assay implies bulk investigation of mRNA both from tumor cells and cells of microenvironment. All patients were treated or observed according to NB2004 protocol. 9 patients had neuroblastic tumor accompanying by paraneoplastic OMS. All these patients belonged to the observation group. 15 patients with matching clinical characteristics were used as a control. 13 patients belonged to high-risk group (5 harbored MYCN amplification) and 2 – to intermediate-risk group.

Results

Group of patients enriched with OMS cases (6/9) was characterized by significant prevalence of T (CD3D,E,G) and B cells in the tumor microenvironment. Cytotoxic cell population was present

mostly by CD56dim NK cells. The major pathway of cellular cytotoxicity was perforin-granzime mediated, comparing to FAS-dependent. Also population of tumor-infiltrating cells was enriched in MHCII-positive antigen-presenting cells demonstrating marks of activation (CD80/86 expression) and FOXP3-positive T-reg cells (median Log2 fold change is 2.14SE0.50, adjusted p=0.020). However, 3 patients with clinically confirmed OMS did not fell into this group and vice versa 6 patients without OMS demonstrated similar molecular profile.
Tumor cells were characterized by retained expression of MHCI mRNA, significant gain of expression of integrins (2.93SE0.53, p=0.005), activation of SMAD and inhibition of PIK3CA pathways. Microenvironment cells actively produced mRNA of factors of the innate immunity: complement components (3.00SE0.52, p=0.002), TLRs (2.25SE0.44, p=0.005), pro-inflammatory cytokines and chemokines (mostly CCLs, CXCLs, TNF-superfamily, IL6 with corresponding receptors, 2.45SE0.51, p=0.009).
The analysis of entire patient's cohort let to formation of two independent groups, which were characterized by completely different profile of the expression of genes involved in both innate and adoptive immune response. First group (n=21) contained mostly favorable cases with or without OMS and 8 high-risk patients, while in the second group (n=18) patients from all risk groups were present and prone to have the adverse events. Interestingly, that the expression signature discriminating these groups was identical to the signature depicting OMS positive and negative cases. Similar to the cohort enriched in OMS cases, first group was characterized by upregulation of the mediators of innate and adoptive immunity. The slight differences were observed in balanced FAS-dependent and perforin-granzime cytotoxicity, and extensive repertoire of upregulated pro-inflammatory cytokines (2.38SE0.53, p=0.003). Among very few genes, which were upregulated in the second group were cancer-testis antigens PBK and TTK (2.28SE0.58, p=0.002). But expression of these antigens was not enough to induce anti-tumor immune response.

Summary/Conclusions

Thus, based on mRNA profiling of tumor cells and microenvironment two types of neuroblastoma were recognized – immunological "hot" and "cold" neoplasms. These findings have no direct impact on the prognosis of patients with neuroblastoma but could be useful to predict the efficacy of the immunotherapy and clinical course of immunological paraneoplastic syndromes, such as OMS.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 281

CircARID1A is an oncogenic circular RNA and regulates neuroblastoma differentiation states

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Circular RNAs (circRNAs), a noncoding RNA class originating from alternative splicing, are highly abundant in neural tissues and were shown to regulate gene expression e.g. by interacting with microRNAs and RNA-binding proteins. Neuroblastoma is an embryonal neoplasia, which arises from undifferentiated neural-crest cells. Recent data suggest that neuroblastoma is composed of a mesenchymal and an adrenergic differentiation state. Previously, we have sequenced whole transcriptomes of 104 primary neuroblastoma and identified 26 neuroblastoma-specific circRNAs, including a circRNA derived from the *ARID1A* tumor suppressor gene.

Aims

Here, we aimed to elucidate the role of circARID1A in neuroblastoma biology and the functional mechanism behind it.

Methods/Materials

The abundance of circARID1A was investigated in a neuroblastoma cell line panel by specific qRT-PCR and Northern blot. Overexpression and knockdown models were established to explore the impact of circARID1A gain or loss on cell viability, proliferation, apoptosis and differentiation. To further investigate the molecular changes upon circARID1A knockdown, RNA sequencing was performed. The subcellular localization of circARID1A was assessed by RNA FISH. An RNA pulldown assay and bioinformatics motif analysis were performed to identify interaction partners of circARID1A.

Results

CircARID1A was validated and quantified in 10 neuroblastoma cell lines of different genetic background. It was found to be less abundant than the linear *ARID1A* mRNA, but had a longer half-life after blocking transcription with actinomycin D. Moreover, circARID1A proved resistant against exonuclease degradation, thus confirming circularity of the molecule. Cell fractionation and RNA FISH revealed a cytoplasmatic localization of circARID1A. We detected on average 2-5 transcripts per cell, whereas for the linear *ARID1A* mRNA the number was at least 2-3 fold higher. Specific circARID1A knockdown resulted in reduced proliferation, cell numbers and viability, and prompted apoptosis. The outgrowth of neurite-like extensions and up-regulation of differentiation markers were observed. Neither knockdown nor overexpression of circARID1A affected *ARID1A* mRNA and protein levels. RNA sequencing after knockdown confirmed the specificity of the knockdown, as circARID1A was the only circRNA significantly down-regulated and the linear *ARID1A* mRNA expression was unchanged. Moreover, among the genes, which showed significant expression changes upon circARID1A knockdown, there was an upregulation of adrenergic (e.g. *DLK1*,*ALK*) and a suppression of mesenchymal (e.g. *PRRX1*, *SEMA3C*) marker genes. We computationally identified several binding sites of microRNAs and RNA-binding proteins enriched in the circARID1A sequence. An RNA pulldown assay strongly and specifically enriched circARID1A in lysates of the neuroblastoma cell line IMR-5. Mass spectrometry and RNA sequencing are currently being performed to identify RNA and protein interaction partners of circARID1A in order to validate *in silico* predictions.

Summary/Conclusions

We demonstrated that circARID1A, a circRNA that is derived from the *ARID1A* tumor suppressor gene is specifically upregulated in neuroblastoma tumor tissues and acts in a tumor-promoting manner in neuroblastoma cell lines, independent of linear mRNA transcripts. We showed that a knockdown of circARID1A induces differentiation and expression of adrenergic markers, indicating a potential role of circARID1A in maintaining the mesenchymal neuroblastoma state. This study highlights an important role of circRNAs in neuroblastoma biology and may establish this RNA class as future therapeutic targets.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 282

ALK mutations in primary and relapsed neuroblastoma in a GPOH cohort

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

In sporadic neuroblastoma, *ALK* mutations are found in 8-10% of all cases. Previous studies suggested a higher prevalence of *ALK* mutations in the high- and intermediate-risk group whereas others reported an almost even distribution among subgroups. Reliable information on the prevalence of *ALK* mutations and their association with clinical phenotypes is of increasing relevance to neuroblastoma diagnostics as ALK inhibitors are on their way to first-line treatment.

Aims

We here aimed to investigate *ALK* mutations frequencies in a large cohort of neuroblastoma samples at diagnosis and relapse and determine their associations with clinical variables.

Methods/Materials

A cohort of 841 neuroblastoma samples (683 at diagnosis; high-risk, n=325, non-high-risk, n=358; 158 at relapse; high-risk, n=117, non-high-risk, n=41) was analyzed by dideoxy-sequencing, whole-exome sequencing, or panel sequencing for the presence of *ALK* mutations. For 49 patients, paired samples from diagnosis and relapse were available, which were analyzed by panel sequencing with a minimum sequencing depth of 200x and a minor allele frequency of 5%.

Mutations found by next generation sequencing within this cohort were validated by digital droplet PCR (ddPCR) or dideoxy-sequencing.

Results

ALK mutations were found in 10.5% (72/683) of neuroblastoma samples at diagnosis. In the relapse cohort, *ALK* mutations were detected in 17.1% (27/158). Analyses of paired samples revealed *ALK* mutations in 6 patients both at diagnosis and at relapse (12.2%). In two other paired samples, mutations were detected only at relapse, which were confirmed as *de novo* mutations by ddPCR. The *ALK* mutation frequency of initial high-risk and non-high-risk tumors did not differ (10.8% vs. 10.3%, p=0.90). In the entire cohort, *ALK* mutations were not associated with *MYCN* amplification status or age at diagnosis. In the non-high risk cohort, however, patients whose tumors harbored *ALK* mutations were significantly older than patients with *ALK* wild-type tumors (15.8 vs. 10.5 months, p=0.032). Three-year overall survival of patients with *ALK*-mutated tumors was slightly poorer than that of patients with *ALK* wild-type tumors in the entire cohort (0.72±0.05 vs. 0.84±0.02, p=0.055). This difference in patient survival was due to worse outcome of high-risk patients with *ALK*-mutated tumors as compared to high-risk patients with *ALK* wild-type tumors (0.53±0.09 vs. 0.67±0.03, p=0.036), whereas survival of non-high-risk patients was not associated with the *ALK* mutation status (0.92±0.05 vs. 0.98±0.01, p=0.278).

Summary/Conclusions

No differences were observed for *ALK* mutations frequencies in high- and non-high risk neuroblastoma in this cohort, whereas the prevalence of *ALK* mutations is higher at relapse, emphasizing the need of re-sequencing of tumors at relapse. Association of *ALK* mutations with poor overall survival was observed only in high-risk patients, but not in non-high-risk patients.

Type Translational

Presentation Preference Rapid Fire Poster Presentation

Abstract nr. 283

Detection of Alternative Lengthening of Telomeres Phenotype via C-Circle Assay in EDTA and Heparinized Plasma

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

The alternative lengthening of telomeres (ALT) mechanism is present in approximately 20% of high-risk neuroblastoma tumors, conferring an indolent, but often lethal prognosis. C-circles, self-primed circular telomeric DNA repeats, are a sensitive and specific biomarker for ALT+ cancers. The C-circle assay (CCA) identifies a subset of neuroblastoma patients with a uniformly poor clinical outcome. Methods for identifying ALT+ neuroblastoma patients will be useful for identifying patients for participation in clinical trials of drugs with high activity against ALT+ neuroblastoma. Plasma-derived cell-free DNA (cfDNA) has been shown to be a potentially useful biomarker in many cancers, including neuroblastoma. We hypothesized that C-circles could be detected in cfDNA collected in both EDTA and heparinized tubes to identify ALT+ neuroblastoma patients.

Aims

Methods/Materials

Plasma was collected in EDTA and lithium heparin BD Vacutainer tubes, and cfDNA was extracted using the QIAamp Circulating Nucleic Acid Kit. The CCA employs phi29 DNA polymerase rolling cycle amplification (RCA), which selectively enriches the telomeric C-circles. Subsequently, real-time PCR amplification of telomere content was normalized to the VAV2 single copy gene and compared to non-RCA controls.

Results

ALT+ DNA was seeded into 2 ml of fresh plasma using serial dilutions (1000, 100, 10, and 1 ng/ml). C-circles were readily detected down to 10 ng/ml in plasma using EDTA as an anticoagulant. The CCA failed in heparinized samples, likely, as heparin is a polymerase inhibitor. C-circles, however, could be detected in heparinized samples that were pre-treated with ecteola

cellulose (50-55mg) to remove heparin. Use of Bacteroides Heparinase I (24 units) did not overcome heparin inhibition of CCA. Results are shown in Table 1. The CCA was positive in bone marrow plasma with metastatic neuroblastoma that grew a CCA+ neuroblastoma cell line and in peripheral blood plasma from a patient with an *ATRX*-mutant neuroblastoma.

Summary/Conclusions

The CCA was positive with as low as 10 ng/ml of ALT+ DNA in plasma cfDNA obtained in EDTA and ecteola cellulose pre-treated heparinized samples. Heparin depletion diminished sensitivity of the assay and required larger CCA reaction volumes. Detection of C-circles in plasma provides an alternative to obtaining tumor tissue in order to identify ALT+ neuroblastoma patients. Use of EDTA as an anticoagulant is optimal, but heparin depletion enables use of the CCA in heparinized plasma, though with diminished sensitivity.

Serial Dilution (ng/ml)	EDTA 10ul CCA	Heparinized 10ul CCA	Heparinized Cellulose 10ul CCA	Heparinized Cellulose 25ul CCA	Heparinized Heparinase 10ul CCA
1000	+	-	+	+	-
100	+	N/A	+	+	N/A
10	+	N/A	-	+	N/A
1	-	N/A	-	-	N/A

TABLE 1: C-Circle Detection of cfDNA in EDTA and Heparinized Plasma

Type Translational

Presentation Preference Rapid Fire Poster Presentation

Abstract nr. 284

A Novel Near-infrared Fluorescent Protein, iRFP720, Facilitates Transcriptional Profiling of Neuroblastoma Metastasis in Mice

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

Metastatic relapse is a major cause of high risk neuroblastoma mortality and improved approaches to study in vivo metastasis will advance evaluation of novel therapeutics.

Aims

Here we describe a novel system for tracking metastasis by using a dual marker (bioluminescence and near infrared fluorescent protein 720 - iRFP 720) to label human neuroblastoma cell lines.

Methods/Materials

Tumor growth and metastasis was evaluated in vivo using orthotopic (renal capsule) or tail vein injection-derived xenografts in NOD scid gamma (NSG) mice.

Results

We compared the performance of this dual marker in whole-body imaging experiments using combined CT scans with bioluminescent and fluorescent imaging. Tumors from a panel of NB cells (CHLA20, SH-SY5Y, NGP and GIMEN) stably expressing the construct for luciferase and iRFP720 were evaluated. The high sensitivity of bioluminescence and iRFP720 as probes for in vivo imaging enabled detection of tumors at initial stages and the precise isolation of malignant cell subpopulations from metastatic sites. We monitored the growth of the tumors by epifluorescent whole-body imaging for up to six weeks after cell injection. The brightness of the tumors expressing iRFP720 correlated with the brightness levels of the bioluminescent levels for whole body imaging. In the tail vein injection model, the Lung and liver are the primary sites for NB metastasis. In the orthotopic model, human NB cancer cells reliably metastasize to distant organs from primary tumors grown within kidney renal capsule.
We evaluate and quantify the brightness of bioluminence and iRFP720 fluorescence for ex vivo

organ images to obtain an accurate picture of the overall distribution of the primary and metastatic NB cells in each organ. We also used iRFP720 for flow cytometry to quantify metastatic propensity for NB cell lines and FACS to isolate metastatic cell populations.

Summary/Conclusions

Overall, this approach permits us to precisely localize and continuously monitor the growth of the tumor by dual labeling - luciferase and iRFP 720 markers, and rapidly isolate district metastatic populations from different locations. Ongoing transcriptomic analysis comparing bone marrow to lung and liver metastasis will provide addition insight into mechanisms regulating metastasis in NB and the mechanisms of action for novel therapeutics targeting metastasis.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 285

Generation and characterization of PDL1 knock NB cell lines

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

Immune check points are central to host tumor interactions and blockade of PD-1 / PD-L1 interactions may be an important adjuvant immunotherapeutic approach to neuroblastoma and other solid tumors. PD-L1 expression varies widely across NB cell lines. Multiple tumor and host derived cytokines modulate surface expression of tumor checkpoint ligands to alter the tumor/immune synapse. In addition to its ligand/receptor function, additional cell autonomous functions for PD-L1 modulating tumor cell physiology are under investigation.
PD-L1 is typically upregulated by IFNγ although this is highly cell line dependent inpart due to epigenetic silencing of the locus.

Aims

Methods/Materials

In order to better understand the functions of PD-L1 in neuroblastoma we are generating a panel of NB cells with variable PD-L1 expression using CRISPR-Cas12a based ribonucleoprotein approaches to modify expression of endogenous PD-L1 for in vitro and in vivo phenotypic studies.

Results

We examined the expression of PD-L1 by qPCR and flow cytometry in SH-SY5Y, SHEP, CHLA20, GIMEN, BE2, NGP and LAN5 cell lines. Surface expression ranges from almost undetectable (NGP) to 97% positive (GIMEN). In addition, response to INFγ varied significantly (eg. SH-SY5y 5 % untreated increased to 95%-97% positive treated, CHLA-20 2% untreated to 95%-97% positive treated).
Next, we used electroporation of Cas12a-gRNAs to target the first and second exons of PD-L1 and isolated single cell derived subclones. Clones are evaluated by qPCR, flow cytometry and responsiveness to INFγ.
For CHLA-20 clone 1, flow cytometry demonstrated no surface expression or response to INFγ. DNA sequence confirmed bi-allelic frameshift deletions by DNA sequencing. Interestingly, initial

evaluation suggests that prolonged culture induces a senescent phenotype is a small fraction of these cells based on senescence associated β-gal staining and morphology.
Subsequent characterization of this clone and others is ongoing, including evaluation cell proliferation kinetics, differentiation and neurosphere formation relative to control PD-L1 +/+ and +/- cell lines. We are also evaluating the in vivo tumorigenicity and cytokine responsiveness of these cells compared to wild type CHLA-20 using a novel ‘humanized’ murine xenograft approach. This permits us to assess engraftment in the presence of human CD4 and CD8 T-cells as well as monocytes, macrophages and other fully human immune components.

Summary/Conclusions

Conclusions: We have developed an efficient approach to isolating and characterizing PD-L1 knockout cell lines for functional evaluation of PD-L1. Both cell intrinsic and ligand/receptor mediated functions of will be evaluated using in vitro and novel humanized in vivo murine models. We hope to elucidate novel mechanistic insights and novel alternative therapeutic approaches to modulate PD-L1 functions in the clinical setting.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 286

Personalised xenograft modelling and high-throughput drug screening to support the PRISM clinical trial

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

Long-term survival rates for children with relapsed and refractory high-risk neuroblastoma (HR-NB) remain around 10%. Personalised medicine clinical trials aim to improve survival rates by matching targeted agents with vulnerabilities identified for individual patients. The Zero Childhood Cancer Program (ZERO) provides a comprehensive precision medicine platform for Australian high-risk paediatric cancer patients, including those with HR-NB, through the nationwide PRISM clinical trial. ZERO combines genomics, high-throughput drug screening (HTS) and personalized patient-derived xenograft (PDX) modelling to increase the percentage of high-risk cancer patients for whom a therapeutic recommendation can be made.

Aims

Our primary objective was to identify therapeutic recommendations for relapsed and refractory HR-NB patients using the ZERO platform. We also aimed to establish a cohort of high-risk neuroblastoma PDX models, to expand our database of *ex vivo* drug responses, and to assess techniques to accelerate xenograft development.

Methods/Materials

The ZERO molecular profiling platform combines WGS of tumour and germline DNA, deep sequencing of a panel of cancer associated genes, and whole transcriptome RNASeq. HTS is

conducted against a 125 oncology drug library enriched for drugs with paediatric safety data. PDX modelling is attempted for each patient where possible and enables *in vivo* efficacy testing for drugs identified by molecular profiling or HTS. Regimes representing early phase trials that a patient may be eligible for are also tested where possible. Results are reviewed by a Multidisciplinary Tumour Board to assign recommendations. Parallel to the ZERO program, we established and expanded additional HR-NB PDX models and subjected them to HTS to expand our drug response database. To improve engraftment times and success rates, we directly compared engraftment rates for patient tumours at subcutaneous, intramuscular and orthotopic implantation sites.

Results

To date, 26 high-risk neuroblastoma patients have been enrolled on the ZERO Program which encompass the TARGET Pilot Study (n=5) and the ongoing PRISM Trial (n=21). Actionable molecular aberrations were identified for 16 of 26 patients. PDX models were generated for 57% (12/21) of attempts, with engraftment times of 33–415 days. A further 29% are pending engraftment. HTS has been conducted for 11 patients, either from primary patient material or PDX, with 45% of screens identifying reportable drug hits. PDX-based drug efficacy studies have been completed for 6 patients to date. Within the PRISM study, a recommendation has been issued for 67% (14/21) of patients with clinical follow-up currently ongoing. Our successful PDX model program has resulted in significant representation of HR-NB samples within our paediatric drug response database (27% or 19/70), despite only 8% of patients in ZERO being diagnosed with HR-NB. Orthotopic engraftment was more efficient that subcutaneous or intramuscular engraftment, shortening engraftment times by up to 40%.

Summary/Conclusions

The ZERO program demonstrates the feasibility of a comprehensive personalised medicine platform to identify treatment recommendations for HR-NB patients. Our growing drug response database and PDX model cohort are valuable resources for future research. Orthotopic implantation allows more rapid PDX model development, increasing the likelihood of developing an avatar model within a clinically useful timeframe.

ACRF Drug Discovery Centre for Childhood Cancer

Children's Cancer Institute, Lowy Cancer Research Centre,

Personalised xenograft modelling and high-throughput drug screening to support the PRISM clinical trial

ACRF Drug Discovery Centre for Childhood Cancer, Lowy Cancer Research Centre

Type Translational

Presentation Preference Oral presentation

Abstract nr. 287

The histone demethylase KDM6B modulates Neuroblastoma stemness and cancer stem cell survival

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Topic Gene regulatory networks and epigenetics: from basic science to therapy

Background/Introduction

We have previously characterized a cancer stem cell (CSC) subpopulation in neuroblastoma based on expression of the G-CSF receptor (CD114). These CSCs are present in all neuroblastoma cell lines, PDXs, and primary tumor specimens typically representing less than 1% of the total tumor cell population. FACS purified CD114+ NB cells self-renew, differentiate and recapitulate heterogenous tumors in vivo. They are approximately 10 fold more tumorigenic than CD114 negative tumor cells based on limiting dilution assays. Previously we demonstrated the KDM6B (aka JMJD3 – Jumonji D3 protein)is over expressed in CD114+ CSCs and we and others have shown that KDM6B inhibition a) dramatically reduces CD114+ subpopulation b) induces tumor regression in vivo. This histone demethylase specifically targets the repressive chromatin modification of H3K27me3 formed by PRC2 activity.

Aims

We therefore sought to evaluate how gain of function of KDM6B impacts cancer stem cell survival and stemness phenoptypes in NB cell lines and xenografts.

Methods/Materials

We used the dCAS9-VPR system to specifically up-regulate the endogenous KDM6B locus. Stable SHSY5Y and NGP NB cell lines expressing dCAS9 fused to VP64-p65-Rta (dCAS9-VPR) were generated by lentiviral transduction. Subsequent introduction of sgRNA guides targeting the KDM6B promoter or control guides allowed us to isolate stable lines expressing control or elevated levels of endogenous KDM6B. Western Blot and qPCR assays demonstrated a modest 2-fold increase in JMJD3 expression.

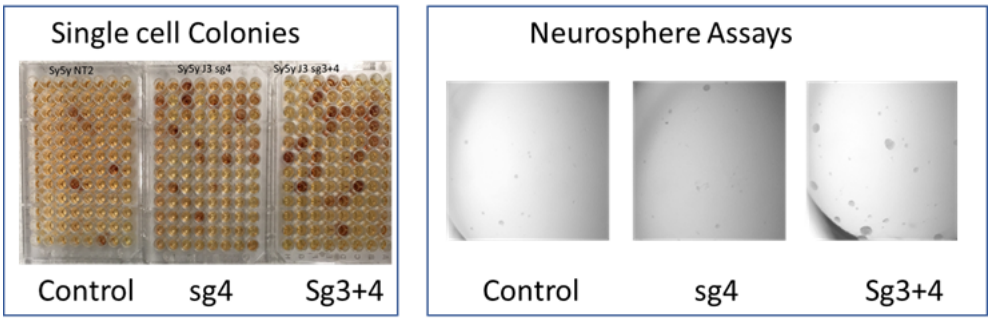
Results

To determine if KDM6b expression altered the CD114+ subpopulation, we assessed CD114 expression for flow cytometry in JMJD3 high and control NB cell lines. We found that increased

KDM6B expression consistently increased the proportion of CD114+ cells in SH-SY5Y to 0.8% from 0.3-0.4% for control SH-SY5Y cells, and in NGP cells to 1.1% from 0.7% for control NGP cells.
Next, we evaluated the ability of the KDM6B high and low cell lines to establish colonies from single cells under standard culture conditions and from tumor spheres in stem cell media at limiting dilution. We found that the modest increase in KDM6B expression dramatically enhanced both these in vitro surrogate assays for stemness. Single cell Colony formation on 96 well plate increased from 20 to 31 ($p < 0.05$) and tumor sphere formation increased 2.5 folds ($p<0.001$). a representative illustration is below.

Summary/Conclusions

Up-regulation of endogenous KDM6B increases the in vitro stemness phenotypes of NGP and SHY5Y as assayed by colony formation and sphere formation. Ongoing in vivo tumorigenesis assays by orthotopic injection and tail vein injection models are testing the relative tumorigenicity of the KDM6B-high and control cell lines. We also demonstrate the utility of dCAS-VPR for sgRNA mediated target gene regulation in neuroblastoma cell lines.



Type Basic

Presentation Preference Oral presentation

Abstract nr. 288

The evolution of MDM2 and MYCN amplifications in neuroblastoma

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Amplification of genomic regions containing proto-oncogenes is a frequent transforming event in neuroblastoma and other cancers. The complexity of such amplifications is highly variable, ranging from simple tandem duplications to highly complex amplified patterns comprising multiple rearrangements. While the development of simple copy number gains is well understood today, the evolution of highly complex amplifications has remained largely unclear.

Aims

We here set out to elucidate the evolution of complex high-level amplifications in neuroblastoma and other cancers during tumorigenesis.

Methods/Materials

We applied whole-genome sequencing, optical mapping, FISH, m-FISH, and RNA sequencing to neuroblastoma tumor samples and cell lines bearing high-level amplifications, and complemented our experimental data with computational modeling of these alterations. In addition, we investigated a large pan-cancer cohort of 2677 samples across 37 entities to validate our hypothesis on the evolution of complex amplifications in cancer.

Results

In a cohort of 71 primary neuroblastomas and cell lines, we identified highly amplified (≥20 copies) genomic regions in 19 cases (26.8%). We noticed that 14 of these amplifications comprised multiple rearrangements with an almost uniform distribution of inverted and non-inverted rearrangements, which did not match to known categories of complex amplifications, such as chromothripsis or the breakage-fusion-bridge mechanism. We defined this new category of complex high-level amplification as “firestorm amplification” based on copy numbers and the rearrangement profile. Firestorm amplifications occurred most frequently on chromosomes 12q (9/71) and 2p (8/71), covering the oncogenes *MDM2*, *CDK4 and MYCN*, respectively. In a large pan-cancer cohort, we detected firestorm amplifications in 3.9% of the cases, accounting for one third (105/315) of all high-level amplifications. Detailed reconstruction of the rearrangements and copy numbers revealed a stepwise evolution behind firestorm amplification (Figure 1): The first step in the development of these alterations was a singular event of chromothripsis (1), followed by formation of extrachromosomal circular DNAs (2) that subsequently underwent repetitive rounds of circular recombinogenic events (3). The resulting amplified regions were finally stabilized (4) by formation of double minutes (DM), reintegration into the genome as homogeneously staining regions (HSR) or neochromosomes (NC). We validated our evolutionary model of stepwise amplification by computational modeling of firestorm amplifications. We also observed that amplified regions detected at diagnosis did not substantially change at relapse, suggesting that recombination of unstable circular DNA fragments had occurred relatively early in tumor development, and that resulting amplifications in manifest tumors are stable events, independent of their occurrence as DM, HSR or NC.

Summary/Conclusions

We demonstrate that high-level amplification of proto-oncogenes, such as *MYCN* and *MDM2* in neuroblastoma, occur regularly as exceedingly complex genomic firestorms. Despite their variable presentation as DM, HSR or NC, these alterations have a common evolutionary path during tumorigenesis, involving an initial chromothripsis event and subsequent recombination of extrachromosomal circular DNAs.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 289

Combined inhibition of EZH2 and EGFR results in neuroblastoma cell death

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

The outcome of high-risk neuroblastoma (HR-NB), despite multimodal therapy, remains unsatisfactory at 50% at best. Thus, there is an urgent need to develop novel therapeutic strategies to increase the survival of these patients. Enhancer of zeste homolog 2 (EZH2), the catalytic subunit of Polycomb repressive complex 2 (PRC2), silences transcription through trimethylation of histone H3 lysine 27 (H3K27me3). EZH2 is frequently overexpressed in HR-NB and predicts clinical outcome. Moreover, EZH2 mediates epigenetic silencing of NB suppressor genes and inhibition of histone H3K27 demethylation is effective against HR-NB. Preclinical EZH2 inhibition or depletion has been reported effective but *in vivo* studies suggest that EZH2 inhibition alone may not be sufficient. Expression of human epidermal growth factor receptor (EGFR) has been correlated in a variety of tumors including NB with cancer progression, poor survival, response to therapy and resistance to cytotoxic agents. Due to the vital roles in tumor biology and progression, many EGFR inhibitors have been developed. However, monotherapy of EGFR inhibitors rapidly produces resistance.

Aims

We explored the putative synergistic effect of targeting EZH2 with GSK126 and EGFR with Canertinib in NB, both *in vitro* and *in vivo*.

Methods/Materials

The anti-proliferative effect induced by GSK126, Canertinib, and their combination was evaluated

by MTS cell proliferation and clonogenic assays with crystal violet staining. The apoptotic effect of these drugs was evaluated by Western blot and annexin V staining by flow cytometry. Xenograft mouse models were generated to evaluate the *in vivo* effect.

Results

Treatment with either GSK126 or Canertinib alone in two NB cell lines caused a decrease in cell viability, after 8 days of treatment. The combined treatment induced a more pronounced effect. Accordingly, the combined treatment also decreased the ability to form colonies more effectively. Moreover, after 48h of treatment, the combination of GSK126 and Canertinib synergistically induced cleavage of PARP, strongly suggesting induction of apoptosis. The apoptotic effect was confirmed by annexin V staining. *In vivo*, although the inhibition of EZH2 or EGFR alone did not induce a significant delay of tumor growth, the combined treatment synergistically induced tumor growth inhibition.

Summary/Conclusions

Our findings suggest that the combined use of GSK126 and Canertinib exerts a synergistic effect on tumor growth inhibition both *in vitro* and *in vivo* by inducing apoptosis. This data provides a novel therapeutic opportunity in neuroblastoma.

Goncalves-Alves

Type Translational

Presentation Preference Traditional poster

Advances in Neuroblastoma Research (ANR) Meeting
ANR 2020
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May
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Abstract nr. 290

124I-MIBG PET/CT to monitor metastatic disease in children with relapsed neuroblastoma

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Topic Other

Background/Introduction

Iodine-123 metaiodobenzylguanidine (MIBG) scan is one of the most sensitive noninvasive lesion detection modalities for neuroblastoma. Instead of I-123, I-124 for MIBG scan offers high-resolution positron emission tomography (PET).

Aims

We have compiled the cumulative data of ^{124}I-MIBG PET/CT we have acquired at our institution to evaluate its diagnostic value over that of ^{123}I-MIBG planar and single photon emission computer tomography (SPECT)/CT as well as to estimate radiation dose of ^{124}I-MIBG.

Methods/Materials

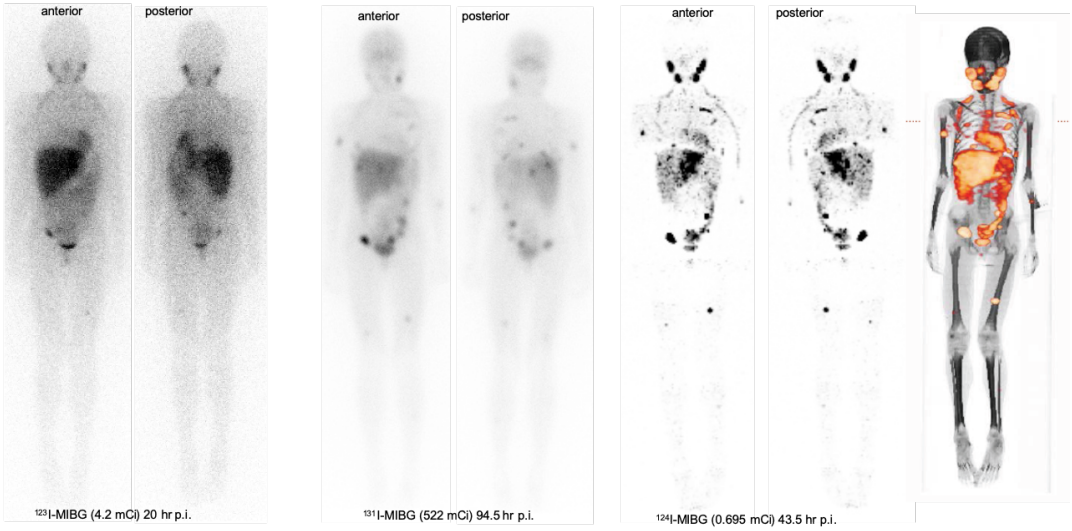
Conventional ^{123}I-MIBG scans (5.2 MBq/kg) that included whole-body anterior-posterior planar scans and a single field-of-view (FOV) SPECT/CT as well as whole-body ^{124}I-MIBG PET/CT (1.05 MBq/kg) were performed in 8 patients before ^{131}I-MIBG therapy. After therapy, 2 out 8 patients also completed ^{124}I-MIBG PET/CT as well as paired ^{123}I-MIBG planar and SPECT/CT scans. For direct comparisons, locations of lesions identified, the number of total lesions detected, and the Curie scores were recorded for all paired imaging datasets by two highly experienced nuclear medicine physicians. In five patients, ^{124}I-MIBG PET/CT scans were performed at multiple time points for radiation dose estimation. Using patient-specific CT as voxelized phantoms for Monte Carlo simulation and ICRP103 tissue weighting factors, effective doses were calculated for ^{124}I-MIBG.

Results

In all paired datasets we reviewed, ^{123}I-MIBG whole-body planar scans, single FOV SPECT/CT, and whole-body ^{124}I-MIBG PET/CT detected 25, 32, and 87 lesions, respectively. There were 63 lesions clearly visualized by ^{124}I-MIBG PET/CT, but not visualized by ^{123}I-MIBG planar scans, and 59 lesions clearly visualized by ^{124}I-MIBG PET/CT, but not visualized by ^{123}I-MIBG SPECT/CT. However, there were 1 lesion seen by ^{123}I-MIBG planar scan and 4 lesions seen by ^{123}I-MIBG SPECT/CT, which were not clearly visualized on ^{124}I-MIBG PET/CT. These observations indicated that there were significantly more lesions identified on ^{124}I-MIBG PET/CT than those on ^{123}I-MIBG whole-body planar scans ($p<0.0001$) and ^{123}I-MIBG SPECT/CT ($p<0.0001$). Lesion identification assessment also resulted in changes in the Curie scores. The Curie scores by ^{124}I-MIBG PET/CT were higher than those by ^{123}I-MIBG planar and SPECT/CT scans in 7 out of 8 patients. Finally, the estimated effective doses were, 0.161 mSv/MBq for 122-kg female, 0.235 mSv/MBq for 63-kg male, 0.339 mSv/MBq for 40-kg male, 0.706 mSv/MBq for 29-kg female, and 0.795 mSv/MBq for 23-kg female patients. These values are approximately 10 times higher those of ^{123}I-MIBG (0.019 mSv/MBq) for 73.7-kg male). Hence, the effective dose for ^{124}I-MIBG scan was only twice the dose for ^{123}I-MIBG scan (5.2 MBq/kg) because of the low-dose protocol (1.05 MBq/kg).

Summary/Conclusions

The first in human use of low dose ^{124}I-MIBG PET for monitoring response demonstrated superior tumor detection capability as compared to ^{123}I-MIBG planar and SPECT/CT scans.



Representative 123I-MIBG, 131I-MIBG, and 124I-MIBG images

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 291

Functional genomic screens identify the nuclear export factor NXT1 as a therapeutic target in MYCN-amplified neuroblastoma

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Neuroblastoma, like most pediatric cancers, is notable for its relatively low mutation rate relative to adult cancers. This has made identifying new therapeutic targets for this disease a challenge, particularly as several of the known drivers, such as *MYCN*, are transcription factors which cannot be readily drugged. Therefore, an alternative approach to target discovery is required for neuroblastoma.

Aims

We aim to use functional genomic CRISPR-Cas9 screens to identify and prioritize new therapeutic targets in *MYCN*-amplified neuroblastoma.

Methods/Materials

We mined genome-scale CRISPR-Cas9 dependency data to identify 197 putative genetic dependencies that are selective to neuroblastoma relative to other cancers. We then generated a focused sgRNA library targeting these genes and performed CRISPR and CRISPRi time-course dropout screens, cell death positive selection screens using Annexin-V purification, and an *in vivo* screen in a subcutaneous xenograft model. Validation studies of the top hit were performed using CRISPR-Cas9 as well as a conditional degron system in multiple cellular models of *MYCN*-amplified neuroblastoma.

Results

At the intersection of these screens, we identified the nuclear export factor NXT1 as a top dependency in *MYCN*-amplified neuroblastoma. We validated that *NXT1* loss impairs viability and induces apoptosis in *MYCN*-amplified neuroblastoma cell lines. NXT1 forms a heterodimer with NXF1 and together they mediate export of mRNA from the nucleus. While many cancer cell lines can grow normally after *NXT1* loss, with neuroblastoma as an exception, *NXF1* is essential in all settings. Interestingly, NXF1 protein levels decrease when *NXT1* is lost in *NXT1*-dependent cells. However, in non-dependent settings loss of *NXT1* does not reduce *NXF1* levels, suggesting that *NXT1* deletion eliminates an essential protein in a context-dependent manner. We integrated RNA-seq expression data with CRISPR dependency data, and identified low expression of *NXT2*, a paralog of *NXT1*, as the feature most associated with *NXT1* dependency. Using overexpression of *NXT2*, we show that low *NXT2* is necessary for dependency on *NXT1*. Finally, we discovered several other pediatric cancers, including rhabdomyosarcoma and medulloblastoma, have low expression of *NXT2* and show exquisite sensitivity to *NXT1* loss, demonstrating that these findings have relevance beyond neuroblastoma.

Summary/Conclusions

Here, we use a series of functional genomic screens to identify the nuclear export factor NXT1 as a key dependency in *MYCN*-amplified neuroblastoma. We further find that NXT1 loss leads to selective depletion of the essential protein NXF1 in the context of low NXT2 expression, and that NXT2 expression is a biomarker of NXT1 dependency. Further, we demonstrate that CRISPR-Cas9 functional screens can be used to identify new therapeutic targets, particularly in diseases like neuroblastoma that lack “druggable” oncogenic driver mutations.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 292

MYCN negatively regulates starvation-induced autophagy through upregulating miR19

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

MYCN is known to regulate expression of many microRNAs. However, how MYCN regulates cell growth and survival through microRNAs remains unclear. Autophagy is a key pathway for cell survival under starvation and other stressors. Here we reported that MYCN negatively regulates expression of a large group of autophagy genes and of starvation-induced autophagy, through miR-17 family member miR-19.

Aims

Aim 1: To dissect the function of MYCN in regulating autophagy.
Aim 2: To understand how miR-19 links MYCN and autophagy.
Aim 3: To design new therapeutics targeting the MYCN-miR-19-autophagy cascade.

Methods/Materials

SHEP-Control, SHEP-MYCN, Kelly, IMR32, SMS-KCN-R, SY5Y, CHLA20 and SKNSH cells were used in this study. Trypan blue, cell titer glo and flow cytometry assays were performed to measure cell growth and survival. Western blotting and qRT-PCR were performed to determine protein and mRNA levels. Neuroblastoma patient survival and mRNA expression levels in tumors were obtained from R2. For mouse experiments, *TH-MYCN* mice were treated twice a week, for 2 weeks with low dose anti-miR19 (10mg/kg) or vehicle. C57BL/6 mice bearing orthotopic *MYCN*-driven xenografts were treated every 3 days with low does anti-miR19 LNA (1.5mg/kg) or vehicle for 5 doses.

Results

Compared to SHEP-Control cells, SHEP-MYCN cells were more sensitive to starvation-induced

apoptosis, more resistant to starvation-induced autophagy, and hypersensitive to inhibition of autophagy regulator ULK1. The results were confirmed in additional *MYCN*-amplified and non-amplified neuroblastoma cell lines. In neuroblastoma patient samples in the R2 database, expression levels of a large group of autophagy genes correlated inversely with those of MYCN and positively with survival. Moreover, cells with amplification or overexpression of MYCN expressed significantly lower levels of autophagy proteins. We found binding sequences for miR-17 family microRNAs miR-19 on the 3'-UTRs of those autophagy genes. In addition, miR-19 was transcriptionally upregulated by MYCN. Anti-miR-19 treatment prolonged survival of *TH-MYCN* mice and C57BL/6 mice bearing orthotopic *MYCN*-driven xenografts, and induced autophagy in the tumors.

Summary/Conclusions

Our findings suggest that autophagy, commonly held as a survival pathway, plays a surprising and critical role in inhibiting the growth and survival of *MYCN*-amplified neuroblastoma. Our study also clarifies a role for MYCN in regulating autophagy, and suggests both miR19 and the autophagy pathway as therapeutic pathways/targets for patients with *MYCN* amplified high-risk neuroblastoma.

Type Basic

Presentation Preference Traditional poster

Abstract nr. 293

Considering new anti-GD2 immunotherapeutic approaches to high-risk neuroblastoma.

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

Neuroblastoma (NB) is the most common extracranial tumor of childhood. Dinutuximab, a therapeutic antibody against ganglioside GD2, was approved for treatment of high-risk neuroblastoma patients. NK cells are indicated as the main effector cells for dinutuximab-mediated antibody dependent cellular cytotoxicity (ADCC). Next to ADCC, complement dependent cytotoxicity (CDC) is another effector mechanism of dinutuximab. Unfortunately, complement activation is also an important cause of neuropathic pain, related to the expression of GD2 on peripheral nerves. In addition to this severe toxicity, a third of patient experiences treatment failure. Additional strategies are thus needed to improve outcomes for this malignancy.We have found recently that mAb 8B6 specific for O-acetyl GD2 (OAcGD2) show a strong anti-tumor effect via ADCC and CDC. In addition, mAb 8B6 does not cross-reacts with peripheral nerves. Importantly, macrophages are often found in high numbers within neuroblastoma tumors, contrary to NK cells. In fact, the contribution of macrophages has been marginalized in the past : their complex relationship with tumor cells underscores the potential that they act as immune effector cells. Nonetheless, macrophages may perform antibody-dependent phagocytosis (ADP) of tumor cells.

Aims

On these grounds, we hypothesized that macrophages play a significant role in the efficacy of anti-OAcGD2 monoclonal antibodies.

Methods/Materials

We first evaluated 8B6 ADP endowment and further correlated this faculty to innate immune checkpoint expression in vitro. We went on studying the efficacy of 8B6 mAb in vivo, alone or in combination with proof of concept innate immune checkpoint inhibitors (ICI). We collected tumor samples from these mice to further characterize the immune infiltrate using flow cytometry and gene expression profiling (nCounter analysis).

Results

We observed that neuroblastoma cells express the "don't eat me" CD47 immune checkpoint inhibitor that limits macrophages responses to mAb 8B6. Yet, we were able to redirect macrophages to kill mAb 8B6 bearing neuroblastoma targets using ICI reagents.

Summary/Conclusions

Collectively, these data highlight that macrophages are important mediators of the efficacy of anti-OAcGD2 mAb combined with ICI therapy. Thus, targeting macrophages represents an attractive option to achieve durable responses in a greater number of patients with NB.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 294

Zero Childhood Cancer (ZERO): A comprehensive precision medicine platform for high-risk cancer including relapsed/refractory high-risk neuroblastoma

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

Children with high-risk cancer, particularly those with relapsed/refractory high-risk neuroblastoma (HR-NB), have poor survival with conventional salvage approaches including enrolment on early phase clinical trials. The majority of precision medicine programs have focused on identifying targetable vulnerabilities by matching genomics with molecularly targeted agents. In contrast to adult cancer, children’s cancers display distinct molecular profiles, encompassing aberrations in different pathways, lower rates of single nucleotide mutations, and a prevalence of structural rearrangements. As a result, isolated genomic analysis may not effectively identify therapeutic options for children with high-risk cancer.

Aims

For the Zero Childhood Cancer Program (ZERO), we hypothesized that a multifaceted precision

medicine platform combining comprehensive genomics, personalized cancer modelling and high-throughput drug screening (HTS) would increase the percentage of high-risk cancer patients for whom a therapeutic recommendation could be made.

Methods/Materials

Eligibility for ZERO participation is any newly diagnosed, relapsed or refractory cancer with expected overall survival <30%, which includes patients with relapsed/refractory HR-NB. The ZERO platform combines whole genome sequencing (tumor, germline DNA), whole transcriptome RNA sequencing, methylation profiling (brain tumors and sarcomas), *in vitro* single agent HTS (125 compound library) and patient-derived xenograft (PDX) drug efficacy testing. Results are curated and recommendations (targeted therapy, change of diagnosis or genetics referral for a germline cancer predisposition gene alteration) are made by a national Multidisciplinary Tumor Board (MTB).

Results

TARGET, a 2-year feasibility pilot clinical trial of the ZERO platform was completed in 2017 and a national, multicenter, prospective clinical trial, PRISM (PRecISion Medicine for Children with Cancer) has been open in all 8 Australian children's cancer centers since September 2017. PRISM has enrolled 274 patients across the broad spectrum of HR cancers including central nervous system tumors (39%), sarcoma (27%), leukemias/lymphomas (15%), neuroblastoma (8%) and other rare tumors (11%).

Twenty-six participants with relapsed/refractory HR-NB have been enrolled on the TARGET/PRISM trials. The median age of HR-NB participants is 7y (range 1-31y) with a slight predominance of females (15) to males (11). Actionable somatic variants were identified in 12 (46%) and actionable germline alterations in 4 (15%, *CHEK2*, *BRCA2*, *HOXB13*), one of which had been previously identified. At least one therapeutic recommendation has been made by the MTB for 16 participants (62%) based on genomic data and/or HTS. The most common therapeutic recommendations included ALKi (n=4), MEKi (n=4), ERRBi (n=3), mTORi (n=2), and PARPi (n=2). So far 4 of 16 participants (25%) have received MTB recommended therapy (all ALKi). Currently 17 (65%) patients with HR-NB enrolled on TARGET/PRISM are alive.

Summary/Conclusions

ZERO demonstrates the feasibility of a comprehensive precision medicine platform to identify actionable treatment & germline recommendations in the majority of relapsed/refractory HR-NB patients. ZERO is partnering nationally and internationally to expand datasets and conduct parallel research studies in immunoprofiling, liquid biopsy, psychosocial impact of pediatric precision medicine, health economics and health implementation. The PRISM trial is expected to recruit ~400 patients by the end of 2020 and plans for enrolment expansion are underway due to the high engagement and clinical demands of the Australian pediatric oncology.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 295

Adrenergic and Mesenchymal neuroblastoma cells have opposite resistance to ALK inhibitors and TRAIL, allowing dual therapy to impede resistance and relapse development.

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

One of the most devastating features of neuroblastoma is that initially therapy-responsive tumours often relapse as therapy-resistant disease. Neuroblastoma displays intra-tumour heterogeneity and includes a majority of lineage-committed adrenergic (ADRN) tumour cells and a minor population of immature mesenchymal (MES) tumour cells. Both cell types can transdifferentiate into one another. MES-type cells are resistant to chemotherapy and are enriched in post-treatment samples, suggesting that they are selected by therapy and may essentially contribute to development of resistant relapses. Identification of drugs that are MES specific and drugs that are

ADRN-specific would allow to test whether combination therapy limits the emergence of relapses in neuroblastoma.

Aims

To identify drugs that specifically kill ADRN cells or MES cells and test whether combination treatment attenuates relapse development.

Methods/Materials

Gene expression and ChIP-seq studies were analyzed of 36 NB cell lines, including 4 pairs of isogenic MES- and ADRN-type cell lines and our isogenic model of ADRN-to-MES reprogramming. Lorlatinib and/or TRAIL sensitivity was analyzed in neuroblastoma cell lines and xenografts, including our reprogramming model.

Results

MES-type Neuroblastoma cells were found to lack ALK expression, even when an oncogenic mutation was present. Consequently, MES cells were resistant to the ALK inhibitor Lorlatinib. H3K27ac ChIPseq analysis showed that ALK has a strong super enhancer in all analyzed ADRN cell lines. MES-type cells lack this super enhancer, underscoring that ALK belongs to a core set of ADRN-specific regulatory genes. Reprogramming of ADRN cells into MES cells by NOTCH3 induction eradicated the ALK super-enhancer, silenced ALK expression and rendered the cells resistant to Lorlatinib. This was also observed in a xenograft model, revealing a potential route for escape from therapy targeting ALK. In search for MES-specific drugs, we found that CASP8, a mediator of the extrinsic apoptosis pathway, was exclusively expressed by MES cells. Activation of this route by TRAIL killed MES cells, but did not harm ADRN cell lines. TRAIL treatment of ADRN-type xenografts had no effect on tumor outgrowth, while TRAIL induced complete regression of MES-type SK-N-AS neuroblastoma xenografts.
Treatment of in vivo xenograft models of ALK-mutant neuroblastoma cell lines with Lorlatinib induced rapid tumor regression. This was followed by re-outgrowth of the tumor several weeks after cessation of treatment. This provided us with a model to test the effects of dual therapy on relapse development. Dual treatment of this model with Lorlatinib and TRAIL significantly delayed the onset of tumor regrowth as compared to Lorlatinib alone ($p<0.001$; ANOVA).

Summary/Conclusions

Our results show that MES- and ADRN-type neuroblastoma cells have complementary drug-sensitivity profiles. Lorlatinib inhibited only the ADRN cells and not the resistant MES cells, revealing an important potential route for escape from targeted therapy. Combining Lorlatinib with specific targeting of MES cells delayed outgrowth of tumors in a pseudo-relapse model, suggesting that targeting these cells can abate development of therapy-resistant relapses in neuroblastoma.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 296

Preclinical assessment of MEK inhibitors in combination with chemotherapy in neuroblastoma

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

Treatment of patients with high-risk neuroblastoma remains clinically challenging. The use of novel small molecule inhibitors targeting oncogenic pathways perturbed in neuroblastoma offers a non-DNA damaging, potentially more effective and less toxic treatment strategy. Aberrations in the RAS/MAPK occur frequently in relapsed neuroblastoma and support the use of MEK inhibitors as a potential novel therapy in high-risk neuroblastoma.

Aims

To determine the sensitivity of preclinical models of neuroblastoma to MEK inhibitors alone and in combination with cytotoxic chemotherapies used in the treatment of neuroblastoma.

Methods/Materials

Using XTT assays, 72h GI_{50} values of MEK inhibitors, trametinib, MEK-162 and cobimetinib were determined in a large panel of neuroblastoma cell lines of varying MAPK pathway status. Synergistic interactions between MEK inhibitors with topotecan or temozolomide were determined in a *KRAS* mutant neuroblastoma cell line using median-effect analysis together with Calcusyn software. Induction of apoptosis was assessed using Caspase-Glo 3/7 assays. *In vivo* efficacy studies were conducted using a subcutaneous xenograft model of *NRAS* mutant neuroblastoma.

Results

Sensitivity to trametinib, MEK-162 and cobimetinib correlated with the presence of a MAPK pathway mutation with cell lines with *RAF*/*RAS* aberrations being more sensitive than those with *NF1* aberrations. Combinations of MEK inhibitors with topotecan or temozolomide were shown to be synergistic with average CI values at ED_{50}, ED_{75} and ED_{90} within the synergistic range (CI value 0.3-0.7). In support of the latter, all combination treatments led to increased levels of apoptosis as evident by higher levels of caspase 3/7 activity compared to single agent treatment. Consistent with *in vitro* data, assessment *in vivo* demonstrated anti-tumour efficacy of cobimetinib alone and in combinations with cyclophosphamide and topotecan or irinotecan and temozolomide, with combination treatments leading to the greatest tumour growth inhibition. All treatments were well tolerated with no toxicities observed.

Summary/Conclusions

These data support the use of MEK inhibitors in combination with chemotherapy as a potential therapeutic option for neuroblastoma patients with aberrations in the MAPK pathway and could be a therapeutic arm of the forthcoming BEACON2 trial.

Type Translational

Presentation Preference Traditional poster

Abstract nr. 297

Inhibition of Exportin-1 as part of combinatorial treatment for patients with high-risk neuroblastoma

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

In many adult cancers, upregulation of the nuclear-to-cytoplasmic transport protein Exportin-1 (XPO1) correlates with poor outcome and responsiveness to selinexor, an FDA-approved XPO1 inhibitor. Similar data are emerging in childhood cancers, for which selinexor is being evaluated in early phase clinical studies. We hypothesize that selinexor and the proteasome inhibitor, bortezomib, act synergistically to decrease NF-kB-mediated proliferation by halting excessive exportation and proteasome degradation of IkB, an XPO1 cargo protein.

Aims

Our aims are to identify patients most likely to benefit from selinexor combination therapy and define the cellular mechanism responsible this effect.

Methods/Materials

We performed ion intensity-based label-free semiquantitative proteomic profiling on primary tumor material from fifty patients with uniformly treated high-risk NB, half of whom were long term survivors and half of whom died <18 months from diagnosis from disease progression. We cross-referenced differentially abundant proteins with gene expression profiling data available through the R2 database. A panel of neuroblastoma cell lines was screened for XPO1 expression using Western blotting and sensitivity to selinexor and bortezomib as single agents and in combination using cellular viability assays. Localization and quantification of IkB after vehicle or selinexor treatment was done by confocal imaging, using immunofluorescent labeling and Volocity software. Flow cytometry was used to define treatment effect on cell cycle arrest and apoptosis using BrdU and Annexin-V labeling, respectively. siRNA-mediated knockdown of IkB was used to investigate the relationship between presence of IkB and drug effectiveness. The downstream effect on NF-kB activity was measured with a luciferase assay.

Results

High tumor protein and gene expression of XPO1 correlates with poor outcome. Neuroblastoma

cell lines demonstrate variably high XPO1 expression compared to normal tissue, as well sensitivity to selinexor (IC50 range 4-300nM). When selinexor is used in combination with bortezomib the effect on cell proliferation is synergistic. Flow cytometry demonstrates that selinexor contributes to G1-0 arrest while combination treatment results in significantly enhanced apoptosis. IkB is retained in the nuclear compartment after treatment with selinexor, and siRNA against IkB diminishes the effect of single and combination treatment. Measurement of NF-kB transcriptional activity is in progress.

Summary/Conclusions

This work demonstrates that XPO1 expression is a biomarker of highly aggressive neuroblastoma and may define patients most likely to be responsive to treatment with selinexor-based combinatorial therapies. Synergistic treatment effect with selinexor and bortezomib is mediated, in part, through IkB and independent of *MYCN*copy number and *TP53*status.

Type Basic

Presentation Preference Traditional poster

Abstract nr. 298

Single-cell RNA-seq identifies neuroblastoma cell line subpopulations with enhanced invasive potential

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

The major cause of death in neuroblastoma (NB) is metastatic relapse, and most therapies do not specifically target metastases. We previously developed a metastatic mouse model using intracardiac injection of human SK-N-AS NB cells followed by multiple rounds of *in vivo* selection from bone and brain metastatic sites. Gene expression profiles identified novel genes and pathways dysregulated in metastatic NB cells that when genetically or pharmacologically manipulated affected metastasis.

Aims

We now ask whether there is a transcriptionally-distinct subpopulation of cells within the parental population that has enhanced invasiveness and metastatic potential.

Methods/Materials

We used 10X Genomics single cell RNA sequencing (scRNA-seq) to transcriptionally profile between 2000 and 2500 cells from the parental cell line and two *in vivo*-selected cell lines that showed enhanced metastatic spread to bone/bone marrow (B5) and brain (BR2).

Results

Analysis of the merged dataset revealed that the B2 and BR5 cells were transcriptionally similar to each other occupying the same clusters. The parental cells show two predominant populations: population 1 (~40% of parental cells) resembled the parental gene bulk expression profiles, and population 2 (~60% of the parental cells) resembled the B5 and BR2 gene expression profile that we previously reported drove migration/invasion *in vitro* and/or metastasis *in vivo* including elevated expression of sphingosine kinase 1 (*SPHK1*).
These results suggest that subpopulations of the parental cells with enhanced metastatic gene expression are selected *in vivo* for honing to metastatic sites. To examine this, we identified a cell surface protein, TM4SF1, enriched in the parental populations transcriptionally resembling the B5

and BR2 cells. Using fluorescence activated cell sorting we generated both TM4SF1^{+} and TM4SF1 lines from parental cells. TM4SF1^{+} cells had increased *in vitro* migration and invasion and a gene expression profile enriched in cell motility and locomotion gene sets. Ongoing work involves measuring the *in vivo* metastatic potential of the TM4SF1^{+} cell populations and investigating functional roles of TM4SF1 in promoting invasion.
Enrichment analysis of the increased metastatic populations and the TM4SF1+ parental cells in the scRNA-seq data highlighted gene sets involved with 1) mesenchymal gene signatures, 2) migration and invasion, and 3) extracellular matrix and collagens. Using AUCell to compute gene set overlaps onto scRNA-seq data we showed that the increased metastatic B5, BR2 and TM4SF1^{+} parental cells overlap with gene sets generated from the mesenchymal-subgroup of neuroblastoma. This analysis identifies potential gene programs and molecular pathways involved in enhancing invasion.

Summary/Conclusions

The use of scRNA-seq to dissect the heterogeneity of NB cells enabled the identification of enhanced metastatic subpopulations in NB and identified genes such as TM4SF1 that mark and/or drive invasive populations. This analysis contributes to our understanding of the molecular mechanisms governing metastases and aims to identify novel targetable pathways that were not detected by profiling bulk tumor populations.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 299

Loss of function of ATRX associates with an inflammatory phenotype in neuroblastoma isogenic cell lines and xenografts

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Neuroblastoma patients carrying loss of function (LoF) mutations of *ATRX* present with slowly-growing disease that often does not respond to conventional chemotherapy. *ATRX* mutant neuroblastoma cells exploit a strategy called alternative lengthening of telomeres (ALT) to regularly extend the telomeric regions of their chromosomes thus escaping senescence and/or apoptosis through mechanisms largely uncharacterised. Elucidating the mechanisms that are responsible for growth and maintenance of *ATRX* mutant neuroblastoma is urgently needed to identify new therapeutic strategies for this subgroup of high-risk patients.

Aims

To improve our understanding about the effects of ATRX LoF in neuroblastoma in order to identify new therapeutic strategies and improve survival of these patients who frequently relapse following conventional chemotherapy.

Methods/Materials

CRISPR-Cas9 genetic engineering was used to induce frame-shift alterations in *ATRX* in SK-N-SH and NBL-S cell lines (non-MYCN amplified, non-ALT activated). Single cells transiently transfected were flow-sorted and a total of ten *ATRX* mutant cell lines were obtained. Loss of *ATRX* expression was validated by western blot and qRT-PCR. Activation of ALT, found in neuroblastoma carrying LoF mutations of *ATRX*, was analysed in the isogenic cell lines by c-circle assay.
Next, RNA sequencing and bioinformatic analysis of the isogenic cell lines was performed to identify common deregulated pathways that were shared among all *ATRX* mutant cell lines. Resulting deregulated pathways were validated *in vitro* and further confirmed *in vivo* by

immunohistochemistry in both xenografts generated from the isogenic cell lines and in patient derived xenograft models.
Lastly, publicly available RNA sequencing data of primary neuroblastomas were utilised to further confirm our results.

Results

Three *ATRX* knock-out SK-N-SH and seven *ATRX* knock-out NBL-S cell lines were obtained by using CRISPR-Cas9, following inactivation of TP53. LoF of ATRX is sufficient to activate ALT in NBL-S isogenic cell lines but not in SK-N-SH isogenic cells.
By RNA sequencing and gene set enrichment analysis (GSEA) of *ATRX* mutant cell lines, we found that ATRX LoF associates with up-regulation of inflammatory response pathways in isogenic cell lines derived from both SK-N-SH and NBL-S. In xenograft models of the isogenic cell lines and patient-derived material, inactivation of ATRX mediates an increased macrophage infiltration in immunocompromised mice.
Interestingly, bioinformatic analysis of publicly available human RNA sequencing data from primary neuroblastoma samples showed up-regulation of inflammation-related pathways in patients with low or null expression of *ATRX* in direct concordance with our findings.

Summary/Conclusions

Our results suggest that LoF of ATRX may mediate an inflammatory phenotype in neuroblastoma by mechanisms currently under investigation. Understanding the role of the immune system in *ATRX* mutant neuroblastoma represents an important opportunity to take into consideration novel combinational therapies.

Type Basic

Presentation Preference Traditional poster

Abstract nr. 300

PRMT5 Inhibition Reduced Neuroblastoma Metastasis Independent of MYC Status

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Introduction: Protein arginine methyltransferase 5 (PRMT5), a type II arginine methyltransferase, is the primary methyltransferase generating symmetric dimethylarginine (SDMA). PRMT5 regulates gene expression via dimethylation of arginine residues on histones H4R3, H3R8, and H2AR3 as well as non-histone proteins. Deregulated expression of PRMT5 is found in many cancers and high levels of PRMT5 is linked to cancer progression and poor prognosis. Of note, several PRMT5 inhibitors are currently under evaluation in early phase clinical trials (NCT02783300, NCT03573310). Evaluation of PRMT5 in neuroblastoma multiple patient cohorts reveals both a strong correlation with advanced disease (p=2.2e-16) and with worse overall survival (p<1.7e-15). Based on these observations, we evaluated the impact of PRMT5 inhibition using *in vitro* and *in vivo* models of neuroblastoma.

Aims

Aims: 1) Test the efficacy of PRMT5 inhibitor in NB cell lines; 2) Validate PRMT5 inhibitor in a mouse xenograft model; 3) Investigate the mechanisms how PRMT5 regulates NB metastasis

Methods/Materials

Methods and Materials: 1) analyzing PRMT5 expression using R2 and TARGET databases evaluated; 2) testing PRMT5 inhibitor and PRMT5 knockdown by shRNA in three NB cell lines in cell proliferation and apoptosis assays; 3) testingPRMT5 inhibitor in a mouse renal capsule xenograft model in two NB cell lines; 4) RNA-seq analysis and 5) targeted proteomic approach to interrogate the major signaling proteins in these two NB cell lines treated with either vehicle or PRMT5 inhibitor.

Results

Results: PRMT5 inhibition by a small molecule inhibitor (GSK3203591, a sister compound of GSK3326595 for *in vitro* study) or shRNA targeting PRMT5 reduced cell proliferation and induced apoptosis in both MYCN non-amplified (CHLA20) and amplified neuroblastoma cell lines (NGP, SK-N-BE2). In orthotopic xenograft models, GSK3326595 treatment attenuated primary tumor growth and strikingly reduced liver metastases as determined by bioluminescent and fluorescent imaging of livers from mice treated with vehicle or GSK3326595 in CHLA20 and NGP. Transcriptional profiling by RNA-seq in these two cell lines treated with vehicle or GSK3203591 revealed that PRMT5 transcriptional regulates EGFR. Neuroblastoma cells treated with GSK3203591 failed to activate tyrosine phosphorylation in response to EGF stimulation. Targeted signaling pathway analysis by proteomics in CHLA20 and NGP revealed that PRMT5 inhibition drastically decreased phosphor-AKT levels. We demonstrated that PRMT5 methylated AKT1 at arginine 15 residue and this methylation event is required for AKT activation. Further, we found that EGFR and AKT signaling pathways converge on regulating the expression of master transcription factors TWIST and SNAIL that are responsible for the epithelial-mesenchymal transition and tumor metastasis.

Summary/Conclusions

Conclusions: These results illuminate a critical role for PRMT5 regulating EGFR and AKT activation in neuroblastoma. We are pursuing further to define the role of PRMT5 in neuroblastoma pathogenesis with genetic approach in the neurocrest cells from which the neuroblastoma arises. Together our findings, as well as the ongoing clinical testing of PRMT5 inhibitors in other solid tumors, support further preclinical evaluation of anti-PRMT5 strategies in neuroblastoma for potential translation into the clinic.

Figure 4. PRMT5 modulates AKT activation via methylation

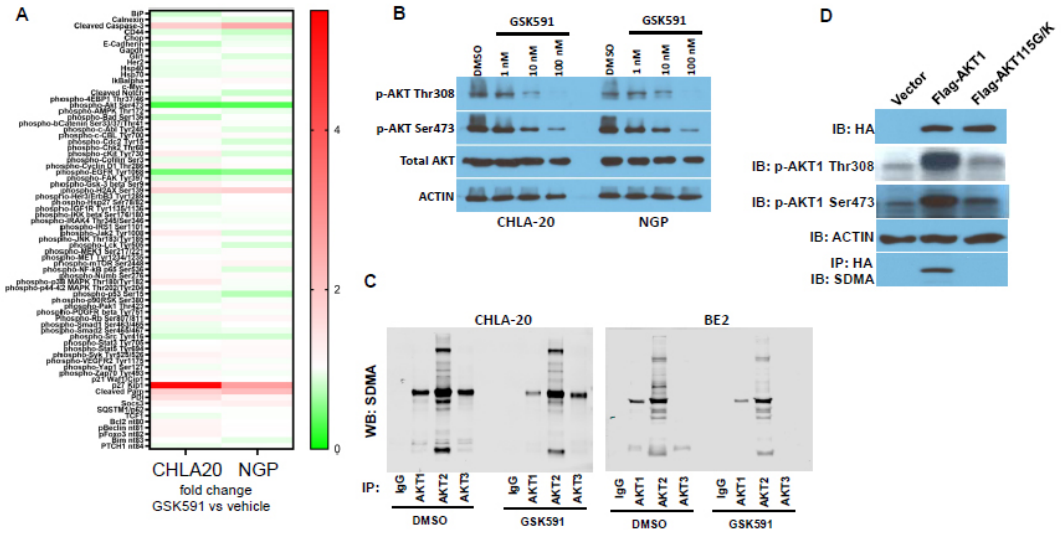


Figure 2. GSK595 Treatment Reduced Primary Tumor Growth and Metastasis

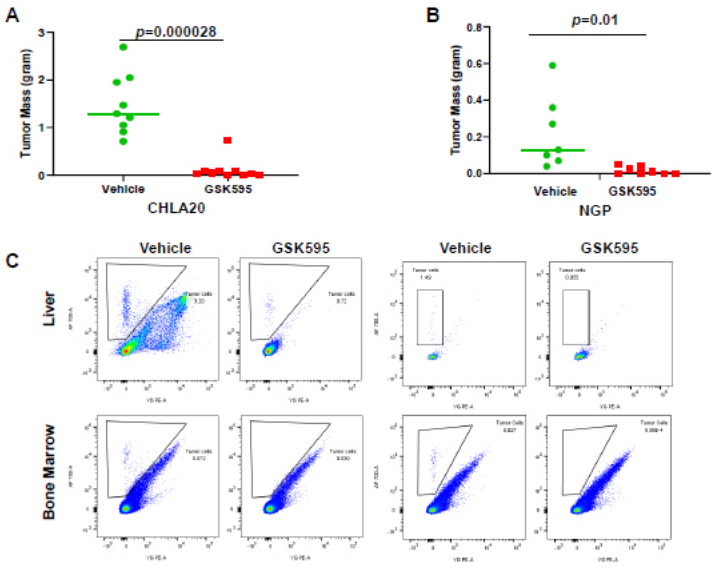


Figure 1. PRMT5 Inhibition Reduced Cell Proliferation & Induces Apoptosis

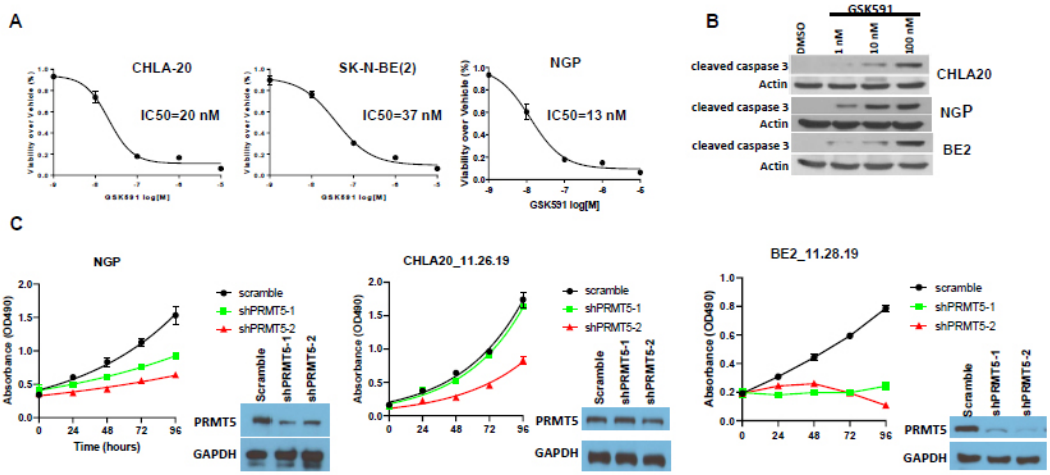
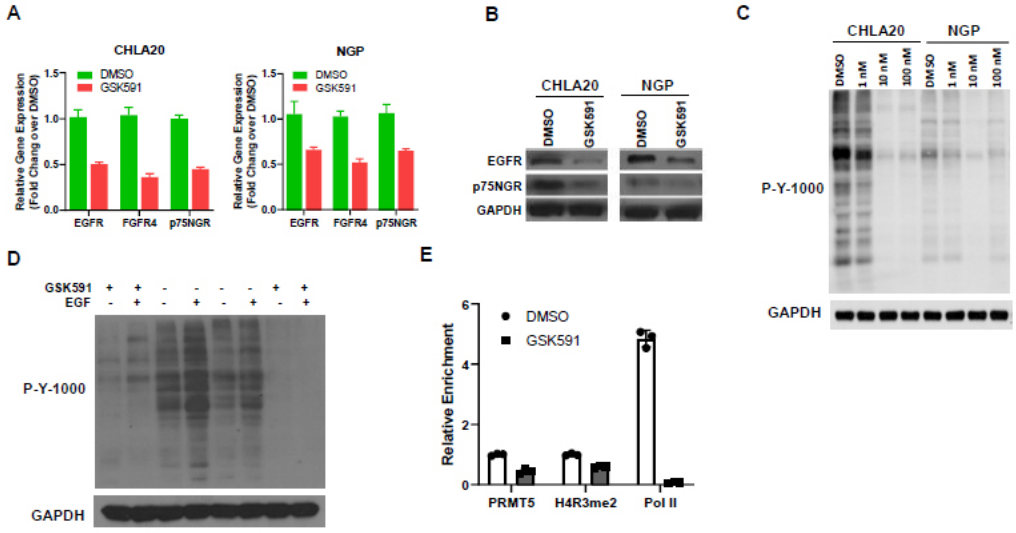


Figure 3. PRMT5 Transcriptionally Regulates EGFR Signaling



Type Translational

Presentation Preference Oral presentation

Abstract nr. 301

RUNX1T1: a transcriptional co-repressor that is essential for neuroblastoma development and progression

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Changes in epigenetic regulation are believed to be a major contributing factor to neuroblastoma development. Using a large-scale *in vivo* mutagenesis screen, we have shown that a single point mutation in *Runx1t1* can block MYCN-driven neuroblastoma growth in *Th-MYCN* mice (Murray et al, ANR 2020). Furthermore, crossing *Th-MYCN* mice with *Runx1t1* knockout mice, demonstrated that loss of only one functional allele of *Runx1t1* is sufficient to prevent neuroblastoma development. Runx1t1 thus represents an entirely novel target not previously described in neuroblastoma.

Aims

To elucidate the mechanism by which RUNX1T1 contributes to neuroblastoma development.

Methods/Materials

Ganglia dissection and H&E staining were performed on mice at 0-4 weeks of age. MYCN modulation was achieved using TET21N cells or overexpressing human flagged MYCN. A tissue microarray including 66 primary neuroblastomas, 5 ganglioneuroblastomas and 12 ganglioneuromas was stained for MYCN and RUNX1T1. *RUNX1T1* gene silencing was performed using doxycycline inducible shRNA constructs. ChIP-seq and ATAC-seq were undertaken for RUNX1T1, and co-immunoprecipitation and mass-spectrometry following transfection with wild-type or mutant RUNX1T1-FLAG.

Results

A prerequisite for neuroblastoma initiation involves MYCN-driven neuroblast hyperplasia within the first few weeks following birth in *Th-MYCN* mice (Hansford, PNAS, 2014). Analysis of murine ganglia collected from 0-4 weeks showed that loss of Runx1t1, through either mutation or gene knockout, completely reverses this hyperplasia to levels observed in non-transgenic mice. In both human neuroblastoma cell lines and primary patient samples, we showed that MYCN does not transcriptionally regulate *RUNX1T1*, but rather drives increased RUNX1T1 protein translation. Importantly, silencing of *RUNX1T1* using shRNA inducible constructs in Kelly and BE(2)-C cells resulted in decreased colony formation *in vitro*, and a significant inhibition of tumour growth ($P<0.0001$) *in vivo*, highlighting a role for this gene in progression of human neuroblastoma. ChIP-seq analysis showed that RUNX1T1 binds primarily within intergenic regions, while ATAC-seq following RUNX1T1 silencing in KELLY cells, revealed that RUNX1T1 increased the accessibility of chromatin regions located in proximity of H3K27me3, a histone mark associated with gene downregulation. Transcriptional profiling and GSEA on both human neuroblastoma and murine coeliac ganglia at two weeks of age, revealed that loss of RUNX1T1 correlated with decreased expression of MYC pathways and increased expression of H3K27me3-associated genes. Since RUNX1T1 co-repressor is known to interact with chromatin modifying proteins, we performed co-immunoprecipitation of either wild-type or mutant RUNX1T1 followed by mass-spectrometry. Wild-type RUNX1T1 was found to interact with the neuroblastoma-specific bHLH transcription factor HAND2, confirming a similar finding from the RUNX1T1 ChIP-seq. Moreover, wild-type RUNX1T1 was found to be a component of a BHC-like complex, a complex mediating repression of neuron-specific genes, whereas mutant RUNX1T1 demonstrated decreased binding to a range of these binding partners including KDM1A, HDACs, GSE1, TBL1XR1, RCORs, HMG20A.

Summary/Conclusions

Our results demonstrate that RUNX1T1 is essential for development and progression of MYCN-driven murine and human neuroblastoma. It acts by recruiting chromatin modifiers that influence the epigenome landscape as well as chromatin accessibility, to repress neuron-specific pathway genes and maintain a de-differentiated state, and as such is an exciting new molecular target in this disease.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 302

ARID1A and a mesenchymal program in neuroblastoma.

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Topic Neuroblastoma as developmental disease

Background/Introduction

NBL arises from immature cells during commitment of neural crest cells (NCCs) to a sympathoadrenal lineage. During development, NCCs undergo an epithelial-mesenchymal transition (EMT) and migrate throughout the embryo before terminally differentiating. EMT is a known pro-metastatic program in adult cancers that is part of a stem cell signature and likely contributes to aggressive metastasis in NBL. N-Myc amplified NBL is a high-risk subset of NBL that frequently associates with loss of heterozygosity (LOH) at 1p36. ARID1A is a subunit of the SWI/SNF chromatin remodeling complex which regulates fate decisions during development. In multiple cancers, ARID1A mutations associate with a mesenchymal state that increases cancer aggressiveness. We recently reported that ARID1A is a critical 1p36 tumor suppressor that collaborates with oncogenic N-Myc in NBL, but the mechanism of action is unknown.

Aims

To determine if loss of *Arid1a* facilitates N-MYC transformation in NBL by retention of a mesenchymal program and to identify therapeutic vulnerabilities based on *Arid1a* status.

Methods/Materials

We innovated an approach for accurately modeling human neuroblastoma that involves N-Myc transformation of primary murine neural crest cells (NCCs). Using NCCs with floxed *Arid1a*, we generated NCCs that are wild-type, have loss of one copy or loss of both copies of *Arid1a*. These NCCs were transfected with N-Myc and injected into mice for tumor formation. The resulting

tumors were analyzed by RNA-seq and immunohistochemistry. Tumor derived cell lines were also generated to identify, base on Arid1a status, sensitivity to small molecule inhibitors and they were whole exome sequenced to find mutations that are selected for during tumorigenesis.

Results

N-Myc/ Arid1a$^{+/-}$ NCCs generated tumors on average 40 days earlier than N-Myc/ Arid1a$^{+/+}$ NCCs, while N-Myc/ Arid1a$^{-/-}$ NCCs did not show reduce tumor latency. Analysis of these tumor by RNA-seq showed a mesenchymal signature in the *Arid1a* het tumors that was not seen in the wild-type or null tumors. Both the wild-type and null tumors were more adrenergic and glial. These differences were confirmed by immunohistochemistry.
Our preliminary data demonstrates that deletion of ARID1A increases expression of the EMT master transcriptional factors TWIST1, SOX9 and SNAI1 expression in our NBL model. While het tumors show a 5-fold increase in TWIST1, null tumors show a 60-fold increase in TWIST1. It is known that TWIST1 dosage dictates different lineage commitments since TWIST1 functions as a homodimer at low levels of expression but interacts with other bHLH transcription factors at higher expression. Further, our small molecule screening has identified increased sensitivity to inhibitors of mesenchymal growth factor receptors and the PI3K pathway when Arid1a is lost. We also observe selection for mutations in the PI3K pathway with loss of ARID1A.

Summary/Conclusions

NCC undergo specification during differentiation by multiple bivalent decisions including an autonomic/mesenchymal fate decision. Our preliminary findings support ARID1A as a repressor of a mesenchymal program found to increase cancer aggressiveness in multiple cancers. We are currently investigating if ARID1A loss maintains a mesenchymal program via overexpression of EMT factors in NBL and if TWIST1 dosage is critical for transformation by MYCN.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 303

Outcome of patients with MIBG-positivity following multi-modality therapy for metastatic neuroblastoma

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

Metaiodobenzylguanidine (MIBG) scans are used to detect neuroblastoma (NBL) metastatic lesions at diagnosis and during post-treatment surveillance. MIBG-positivity following induction chemotherapy for high-risk NBL correlates with poor outcome. However, there are reports of patients with progression-free survival despite MIBG-positivity. Factors distinguishing these survivors from patients who experience progression or relapse remain unclear.. Although some publications have compared the sensitivity and specificity of PET and MIBG scans at diagnosis the roles for these scans during post-therapy surveillance are less well understood. We hypothesized that PET scans may help specifically identify less “active”, potentially differentiated, MIBG-avid lesions.

Aims

Our primary goal was to determine whether PET scan activity can distinguish which MIBG-positive lesions may remain quiescent and thus, can potentially be spared additional therapies. Our secondary aim was to determine whether any known clinical or biological factors at diagnosis are associated with end of therapy MIBG positivity and long-term survival. Understanding how to recognize these rare patients will allow tailoring of therapy and impact surveillance.

Methods/Materials

We conducted a multi-center retrospective analysis of pediatric patients diagnosed with INSS stage-4 NBL between 2002-2018. We identified patients with persistent MIBG positivity on their end-of-therapy scans without evidence of progression during treatment. We recorded patient characteristics including age, histopathology, biomarkers, treatment, and disease response.

Results

Ten patients (median age at diagnosis, 28.6 months) met inclusion criteria. All remain disease-free with a median time-off-therapy of 5 years. Six patients continued to show MIBG uptake up to 6.5 years following active therapy. All patients at diagnosis had MIBG-avid distant metastases and tumors that lacked *MYCN* amplification. Although all patients had confirmed INSS 4 disease at diagnosis, 4/10 of the patients had tumors with INPC favorable histology. Based on the COG risk classification criteria 3/10 patients were classified as intermediate and 7/10 as high risk at diagnosis. 8/10 patients had histologic evidence of pre-treatment marrow involvement. Six underwent at least one course of myeloablative chemotherapy with stem cell transplant. Seven patients had at least one PET scan performed and 7/7 PET scans were negative. Three patients had repeat biopsies of a positive MIBG lesion at some point following the end of upfront therapy. Differentiated NBL was reported for 2/3 biopsies and the third showed only fibrotic changes.

Summary/Conclusions

Our preliminary analysis suggests that persistent positive MIBG scans in a subset of INSS stage 4 patients in first response may not always lead to progression. In our cohort no patients had tumors that harboured *MYCN* amplification. Studies are needed to determine whether *MYCN* status together with other clinical or biological characteristics, and /or PET scans, may identify patients with residual “inactive” MIBG lesions that do not require further therapy.

Outcome of patients with MIBG-positivity following multi-modality therapy for metastatic neuroblastoma

Morgenstern

Type Clinical

Presentation Preference Traditional poster

Abstract nr. 304

Arginine depletion therapy enhances efficacy of chemotherapy in delaying tumour development and increasing survival in the Th-MYCN mouse model of neuroblastoma

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

Arginine is a semi-essential amino acid that is metabolised into ornithine and urea by the expression of cytoplasmic arginase 1 (ARG1) and mitochondrial arginase 2 (ARG2). Neuroblastoma cells have high levels of arginase activity and staining compared to normal cells, and high arginase 2 expression in primary neuroblastomas correlates with worse overall survival. Since arginase is known to consume arginine from the microenvironment, this suggests that neuroblastomas have a high metabolic requirement for arginine and therefore represent an excellent target for arginine depletion therapy. Additionally, neuroblastomas have low levels of arginosuccinate synthase and absent expression of ornithine transcarbamylase, both of which are involved in arginine resynthesis, thus making them potentially more vulnerable to arginine depletion therapy. We have recently shown that pegylated arginase (BCT-100) given twice weekly as a single agent can significantly delay tumour development and prolong survival of neuroblastoma-prone *Th-MYCN* mice (Fultang *et al*, *Cancer Research*, 2019).

Aims

To investigate the effects of arginine depletion therapy in combination with clinically relevant chemotherapy in *Th-MYCN*-transgenic mice.

Methods/Materials

Homozygous *Th-MYCN* mice (n= 8-10/group) were used to assess arginine depletion therapy, involving 60mg/kg BCT-100 as a single agent delivered 2x/week or 4x/week from the time of weaning. Homozygous *Th-MYCN* mice with established tumours (5mm) were treated with 60mg/kg BCT-100 4x/week either alone or in combination with irinotecan (2mg/kg)/ temozolomide (5mg/kg). Targeted metabolite profiling of plasma and tumour samples was carried out by ultra-performance liquid chromatography-mass spectrometry (LC-MS).

Results

Neuroblastoma development was significantly delayed following treatment with BCT-100 as a single agent either 2x/week or 4x/week, prior to tumour development ($P<0.0004$ and $P<0.0001$, respectively, compared to vehicle control). However, the more frequent treatment resulted in significantly longer survival than twice-weekly treatment ($P<0.0012$ for the comparison between treatment 2x/week (median survival from birth, 63 days) versus 4x/week (median survival, 113 days), without any increased toxicity. Treatment with BCT-100 4x/week, but not 2x per week, was associated with significantly decreased intra-tumoral arginine levels compared to vehicle control ($P= 0.0023$). Single agent treatment of *Th-MYCN* mice with BCT-100 4x/week following establishment of small palpable tumours (5mm), resulted in a highly significant increase in survival (median survival 59.5 days from commencement of treatment) compared with vehicle-treated-mice (median survival 4 days, $P<0.0001$). Addition of BCT-100 4x/week to irinotecan/ temozolomide treatment resulted in significantly improved survival (median survival from commencement of treatment >70 days) by comparison with either BCT-100 ($P=0.0016$) or chemotherapy alone (median survival 25 days, $P<0.0001$), with two thirds of mice remaining alive and tumor-free at the end of the combination treatment by comparison with either of the single agent treatments, where no mice remained alive. Importantly, the combination of 4x/week BCT-100 combined with chemotherapy showed an excellent toxicity profile with no adverse effects observed in the mice.

Summary/Conclusions

A phase II study of BCT-100 as a single agent in children with relapsed/refractory solid, CNS and liquid tumours is currently underway (NCT03455140). These data support further trials involving increased dosing of BCT-100 combined with chemotherapy as a potentially exciting new approach for treating high-risk neuroblastoma.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 305

A multi-omic surfaceome study identifies DLK1 as an epigenetically regulated protein and immunotherapeutic target in neuroblastoma

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

The cell surface landscape of neuroblastoma is poorly defined. An unbiased survey of plasma membrane proteins from high-risk neuroblastoma cases would greatly facilitate the identification of candidate immunotherapeutic targets.

Aims

(1) Define the mass spectrometry-based surfaceome of neuroblastoma and prioritize proteins as candidate immunotherapeutic targets using an integrative multi-omic approach; (2) Validate and assess biological relevance of a top prioritized protein; and (3) Develop and test the efficacy of immunotherapy targeting the prioritized protein.

Methods/Materials

We performed plasma membrane protein extraction using sucrose gradient ultracentrifugation coupled to mass spectrometry (nLC-MS/MS) in neuroblastoma cell lines (n=12) and patient derived xenografts (PDX; n=12). These data were integrated with RNA-sequencing (neuroblastoma=153; normal (GTEx)=7859) and chromatin immunoprecipitation (ChIP)-sequencing data from ten neuroblastoma cell lines to evaluate extracellular proteins differentially expressed in neuroblastoma compared to normal tissues. Candidate targets were validated by immunohistochemistry on neuroblastoma tumor and normal tissue microarrays (TMAs), flow cytometry and immunofluorescence. Functional studies were performed following genetic manipulation of candidate targets to assess cell proliferation, differentiation and viability. Finally, we tested ADCT-701 (DLK1-directed antibody drug conjugate [ADC] with Pyrrolobenzodiazepine warhead) in thirteen PDX models with varying levels of DLK1 expression. At enrollment, two mice were each treated with a single dose of ADCT-701 at 1mg/kg body weight, or 1 mg/kg of B12-PL1601 (non-targeting ADC), or saline. Mice were evaluated for 100 days or until tumor reached $2.0cm^3$.

Results

We yielded on average 66% (range:60-68%) membrane protein enrichment with high reproducibility between biological replicates (80%; range:78-84%) and identified 4826 unique membrane proteins. We confirmed abundant representation of peptides from known cell surface proteins such as ALK, GPC2, NCAM1, DLL3 and CD276. Here, we prioritized DLK1 for further evaluation due to it being the top candidate with expression directly associated with a super enhancer element ($P=6.09X10^{-5}$). RNA-sequencing and tissue microarray analysis of neuroblastoma and normal tissues showed DLK1 to be overexpressed in a large subset of high-risk neuroblastoma with minimal expression in normal tissues, excepting adrenal medulla and pituitary. Flow cytometry and immunofluorescence confirmed cell surface expression of DLK1 in a panel of neuroblastoma cell lines. Genetic depletion of DLK1 using shRNA resulted in neurite outgrowth ($P=7.26x10^{-5}$) and terminal differentiation. Full proteome analysis of DLK1 knockdown and control cell lines using MS showed DLK1 regulates proteins that control outgrowth of neurites ($P=3.37x10^{-3}$) and development of neurons ($P=3.76x10^{-3}$). A single dose of ADCT-701 resulted in maintained complete response (N=5), complete response (N=3) and stable disease (N=1) in models with high DLK1 expression, while those with low/no expression showed disease progression (N=3).

Summary/Conclusions

Integrated epigenomics with plasma membrane proteomics has yielded a prioritized list of proteins that may be developed as immunotherapeutic strategies, including multi-specific targeting. DLK1 is an epigenetically regulated immunotherapeutic target in neuroblastoma. ADCT-701 shows potent anti-tumor activity in preclinical models of neuroblastoma and will be prioritized

for clinical development.

Martinez

Delaidelli

A multi-omic surfaceome study identifies DLK1 as an epigenetically regulated protein and immunotherapeutic target in neuroblastoma

Radaoui

Type Translational

Presentation Preference Oral presentation

Abstract nr. 306

DISCRUPTED ENDOPLASMIC RETICULUM-MITOCHONDRIA INTERACTIONS PROMOTES NEUROBLASTOMA MULTIDRUG RESISTANCE

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Many neuroblastoma patients die from progression of multidrug resistant disease, the etiology of which remains poorly understood. Mitochondria integrate diverse stress and survival signals to determine whether a cell lives or dies. We have used functional mitochondrial profiling to show that multidrug resistant neuroblastoma mitochondria are markedly reduced in apoptotic responses to stress. Importantly, apoptotic sensitivity is modulated at mitochondria by interactions with endoplasmic reticulum (ER) at specialized contact sites essential for calcium and lipid transfer between the organelles. ER-mito contacts are enriched for protein complexes, including MFN2 and PACS2, that bridge the organelles. Here we define a novel mechanism for chemotherapy resistance caused by reductions in ER-mito tethers, and provide functional validation for this relationship.

Aims

To determine the extent to which reduced ER-mitochondria tethering contributes to therapy resistance and determine whether altered calcium or lipid transfer is causal.

Methods/Materials

We studied neuroblastomas from diagnosis (DX, largely chemosensitive) and relapse (REL, chemoresistant) obtained from the same patients. Mitochondrial biomass (citrate synthase activity) and mtDNA content (qPCR and MitoChip) were evaluated. Isolated mitochondria were probed with tBid or Bim peptide to define their responsiveness to apoptotic signals, as measured by

cytochrome c release. ER-mito contacts were assessed by physiochemical separation, and by Electron Microscopy (EM). Functional tests of ER-mito tethering involved reducing tethers physically by limited protelolysis, genetically by shRNA silencing (MFN2, PACS2), and chemically by Cyclosporine-A. Mitochondrial calcium levels and transfer were measured using a mitochondrially-targeted calcium reporter and calcium indicator dyes.

Results

REL neuroblastoma mitochondria are markedly attenuated in apoptotic responses to tBid and Bim, and this correlates with chemoresistance across drug classes. These differences are highly reproducible and were not caused by changes in mito biomass or mtDNA content. Instead, we find REL cells have reduced ER-mito contact number and/or increased gap-distance compared with patient-matched DX cells using EM, and confirmed by IB for organelle-specific proteins. The impact of reduced ER-mito connectivity was confirmed using multiple orthogonal methods. MFN2 or PACS2 shRNA in DX cells attenuated mitochondrial responses, phenocopied resistance and reduced ER-mitochondria connectivity. ER-mito calcium transfer was decreased in REL cells, as a consequence, while enhancing ER-mito connectivity using synthetic linkers restored calcium transfer.

Summary/Conclusions

ER-mito contacts serve as physiologic regulators of apoptosis, and we show in patient-matched tumor models that these contacts are markedly reduced in therapy resistant cells. Some resistant cells had reduced numbers of ER-mito tethers (and measurably reduced calcium transer), while other resistant cells had preserved numbers of tethers (and calcium transfer was preserved), but the gap distance was abnormally increased. Ongoing studies are investigating the role of altered lipid transfer as a unifying mechanism for this resistance phenotype. Our work will facilitate the development of clinically useful tools to measure ER-mito interactions and provide a novel framework for testing interventions to prevent emergent therapy resistance or restore mitochondrial competence to resistant cancers.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 307

Neuroblastoma Patient-Derived Cell Lines and Xenografts in the COG/ALSF Childhood Cancer Repository

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

The Children’s Oncology Group (COG) panel of patient-derived cell lines (PCLs) and xenografts (PDXs) from high-risk neuroblastoma was established from patients pre-therapy at diagnosis (DX), at progressive disease (PD), and post-mortem (PD-PM) to enable biological and preclinical therapeutic studies.

Aims

To develop and characterize a panel of neuroblastoma patient-derived cell lines and xenografts.

Methods/Materials

From 2001-2019 COG institutions collected viable samples from 1393 high-risk neuroblastoma patients (63 stage 3, 1330 stage 4): 1334 bone marrow, 972 tumor, and 132 blood samples (33/132 post-mortem); 1596 were pre-therapy and 842 from progressive disease. Samples were

cultured in hypoxia and standard (atmospheric) conditions and/or directly xenografted in NOD SCID gamma (NSG) mice. PCLs and PDXs matched to patient samples by short tandem repeat and were EBV and mycoplasma free. Gene expression used RT-qPCR and/or RNAseq. ALK mutations, *MYCN* amplification, and alternative lengthening of telomeres (ALT) (C-circles) were assessed. Clinical annotation includes event free (EFS) and overall survival (OS). Response to cyclophosphamide + topotecan was assessed for 23 PDXs and RNAseq for 65 PCLs and 52 PDXs.

Results

To date, 260 PCLs and since 2009, 56 PDXs were established, 42 with matching PCL. Human content of PDXs was 99.3±1.2%. PCL take rates: 21% DX, 15% PD, and 78% PD-PM. PDX take rates: 15% DX, 13% PD, and 68% PD-PM. Of 226 samples cultured in multiple conditions, 30% established PCLs only in hypoxia, 1% only in neurobasal-A medium. Table 1 shows PCLs and PDXs with high expression of selected genes.

High MYCN and c-MYC expression were mutually exclusive; 5/137 PCLs and 5/52 PDXs were ALT+; 9/65 PCLs (most non-continuous) were TERT & ALT negative. *ATRX* genomic alterations were detected in 3/3 ALT PCLs and 1/3 ALT PDXs. Pre/Post-therapy pairs of PCLs were established from 16 patients, PDX pairs from one patient. ALK mutations were found in 27% of 71 PCLs and 25% of 26 PDXs. *MYCN* genomic amplification was observed in 27% of patient samples and 68% of PCLs/PDXs. Growth of viable tumor or marrow containing tumor as PCL or PDX was associated with inferior 5-year EFS and OS (Table 2).

Resistance to cyclophosphamide + topotecan was common in PDXs established at PD but also observed in PDXs established pre-therapy.

Summary/Conclusions

The COG panel of neuroblastoma PCLs and PDXs is representative of the different biological subtypes of high-risk neuroblastoma and is freely available at www.CCcells.org. Factors promoting successfully establishing PCL or PDX include *MYCN* amplification and end-stage tumor progression. Hypoxic culture conditions significantly increased the ability to establish neuroblastoma PCLs. Continuous neuroblastoma PCLs and PDXs represent very high-risk neuroblastoma patients. These models have been sent to 212 investigators in 25 countries and have enabled preclinical studies informing clinical trials.

Table 1	**TH**	**%**	**MYCN**	**%**	**c-MYC**	**%**	**TERT**	**%**
PCL	235/260	90.4	51/65	78.5	11/65	16.9	54/65	83.1
PDX	47/50	94.0	31/52	59.6	6/52	11.5	42/52	80.8

Table 2	**912 Patients with Outcome Data**		**606 Pre-therapy Samples Only**	
	EFS	**OS**	**EFS**	**OS**
PCL or PDX +	20.8 ± 4.1%	25 ± 4.5%	31.1 ± 5.6%	39.2 ± 6%
No Growth	56.8 ± 1.9%	68.8 ± 1.8%	67.8 ± 2.1%	74.3 ± 2%

Tables
Type Translational

Presentation Preference Oral presentation

Abstract nr. 308

A polycomb group protein RING1A preferentially mediates H2AK119 ubiquitination with supporting neuroblastoma cell proliferation.

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Topic Gene regulatory networks and epigenetics: from basic science to therapy

Background/Introduction

Polycomb repressive complex (PRC) mediates histone modification and subsequent target gene silencing in various types of cancers as well as in early development. PRC2 encompasses EZH1/2 histone methyltransferases and involves H3K27 methylation. Recent studies have revealed that EZH2 contributes to malignant status of neuroblastoma (NB), dampening expression of tumor suppressor genes. On the other hand, the core complex of PRC1 is composed by RING1A/B E3 ligases and PCGF family proteins, which mono-ubiquitinates H2AK119. PRC1 is classified into several subtypes according to the PCGF family proteins (ex. with PCGF1 → PRC1.1), and they potentially control distinct target genes. Since PCGF4, also referred as BMI1, accelerates NB cell proliferation, PRC1 is expected to be oncogenic and druggable in NB. However, further investigations are required to understand behavior of the divergent PRC1 subtypes and to clarify the druggability.

Aims

In this study we have perturbed RING1A/B expression and challenged to uncover PRC1-mediated gene regulatory network as well as phenotypes in NB.

Methods/Materials

We employed shRNAs to suppress the expression of RING1A/B in NGP and SK-N-BE cells. The cell viability was quantified by WST-8 and flat colony formation assays. The RNA samples were subjected to SurePrint G3 Human GE microarray ver.3. Raw signals were normalized in GeneSpring GX. Differentially expressed genes were defined by one-way AVOVA test's q-value < 0.1 and fold change > 1.5.

Results

The knockdown of RING1A or RING1B impaired NB cell growth. Intriguingly, the RING1A

knockdown remarkably attenuated H2AK119ub, whereas the RING1B knockdown did not. The double knockdown of RING1A/B resulted in H2AK119ub reduction, which was comparable to the RING1A single knockdown. These data indicate that RING1A preferentially mediates H2AK119ub in NB cells with a distinct mechanism from RING1B. To reveal the specific target genes of RING1A/B, we performed microarray analysis and then selected genes up-regulated in the double knockdown, specifically in the RING1A and RING1B knockdown (RING1A/B_DKD_up, RING1A_KD_up and RING1B_KD_up genes). Gene ontology analysis revealed that the RING1A/B_DKD_up genes were exclusively associated with organ morphogenesis and development. Moreover, ChIP-X enrichment analysis (ChEA) showed that the promoter regions of the RING1A/B_DKD_up genes were exclusively occupied by polycomb group proteins. These results suggest that physiological PRC1 function is remained in NB. Besides, we identified potential tumor suppressors in the RING1A_KD_up genes. Of note, ChEA displayed that KDM2B binding was exclusively enriched in the RING1A_KD_up genes promoter but not in that of the RING1B_KD_up genes. Since KDM2B is a component of PRC1.1, this result implies preferential association of RING1A to PRC1.1 to repress unique target gene expression in NB.

Summary/Conclusions

In our study, RING1A involves cell proliferation and preferentially mediated H2AK119ub with unique target gene silencing in NB. Moreover, according to ChEA, RING1A_KD_up genes were expected to be regulated by PRC1.1, that encompasses RING1A and KDM2B. Although no compound is available for its inhibition currently, PRC1.1 is to be a potential therapeutic target in NB.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 309

TAp63; A Molecular Switch between Spontaneous Regression and Aggressive Growth in Neuroblastoma

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Topic Neuroblastoma as developmental disease

Background/Introduction

The clinical feature of neuroblastoma (NB) is very variable and enigmatic because it spans from developmentally programmed spontaneous regression (s-regression) in infants to aggressive growth in children. The nerve growth factor (NGF)/TrkA signaling pathway may be the major target for both the s-regression and the malignant transformation. To unveil the molecular mechanism, we have long worked to identify many novel genes and their products by reverse translational research and genomics using primary NBs and cell lines. They included TrkA, KIF1Bb, TAp63, UNC5D, BMCC1 and NLRR3 as the major regulators for inducing s-regression, most of which were upregulated by NGF depletion, and TrkB, N-CYM (a *de novo*evolved *cis*-antisense gene product of *MYCN* which stabilizes MYCN through GSK3b), NLRR1, ALK and *ncRAN* as those for promoting the aggressiveness in NB. However, the mechanism how s-regression and aggressiveness are molecularly linked remains elusive.

Aims

We aimed to identify the molecule(s) to connect or switch the two poles in NB.

Methods/Materials

Total RNA was prepared from 106 primary NB tissues. Expression levels of mRNA were measured by quantitative real-time RT-PCR. To block the p53 binding site, a CRISPR-dCas9 vector was constructed. To measure the *MYCN* promoter activity, chromatin immunoprecipitation assay was used.

Results

Among the p53 family, high expression of only *TAp63* mRNA was significantly correlated with favorable outcome in NB (n=106; P=0.00696). Furthermore, expression of *TAp63* was inversely correlated with that of *MYCN* (P=0.0144, R=-0.239) and *NCYM* (P=0.000418; R=-0.344). Overexpression of *TAp63* suppressed *MYCN* expression, whereas knockdown of *TAp63* had the opposite effect. By binding to exon 1 of *MYCN* gene, TAp63 suppressed the promoter activities of *MYCN* and *NCYM*. All-*trans*-retinoic acid (ATRA) treatment of CHP134 cells induced *TAp63* and

reduced *p53* expression, accompanied by downregulation of *MYCN*/*NCYM* expressions. Meanwhile, *TAp63* knockdown inhibited ATRA-induced repression of *NCYM* gene expression. Blocking the p53 family binding site by CRISPER-dCas9 system in CHP134 cells induced *MYCN*/*NCYM* expression and promoted apoptosis. TAp63 also repressed transcription of c-*MYC* in glioblastoma. Thus, TAp63 repressed *MYCN*/*NCYM* bidirectional transcription, contributing to inhibition of NB growth.

Summary/Conclusions

p63 was originally cloned as *p51* by our group (*Nat. Med*., 1998). TAp63, the major isoform of p63, is induced during programmed cell death of developing normal sympathetic neurons in mice. Our previous and present studies revealed that, *TAp63* as well as *KIF1Bb*, *TAp63*, *UNC5D* and *BMCC1* is upregulated after NGF depletion in favorable NB cells and primary NBs. The induced TAp63 further targets *UNC5D,* a second dependence receptor gene downstream of TrkA, for its transcriptional activation to enhance expression of pro-apoptotic genes. Thus, these and other evidences suggest that TAp63 is one of the key regulators to induce s-regression downstream of TrkA. On the other hand, our present study also clearly showed that TAp63 is down-regulated in aggressive NBs and that TAp63 directly inhibits transcription of both *MYCN* and *NCYM,* a 100% co-amplified gene with *MYCN*, to further enhance degradation of MYCN. Therefore, TAp63 may be a molecular switch to regulate the cellular machinery of s-regression and aggressive growth in NB.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 310

Tumor-associated macrophages (TAM) and mesenchymal stromal cells (MSC) augment MYC expression in neuroblastoma (NB) cells by affecting its phosphorylation and stability in a STAT3 dependent manner.

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

The presence of TAM in NB tumors is known to be an indicator of aggressivity and poor outcome in particular in NB without amplification of the MYCN oncogene (MYC-NA). MYC overexpression in MYCN-NA tumors is also a marker of very poor outcome.

Aims

Here were have examined whether TAM affect the expression of MYC in MYC-NA NB tumors.

Methods/Materials

Human monocytes from healthy donors were driven toward TAM by being incubated in the presence of NB cells for 4 days. TAM were then harvested and co-cultured (in Transwells) with MYCN-NA NB cells (CHLA255) in the presence or absence of human MSC. After 3 days NB cells were harvested and examined for the expression of MYC and its phosphorylated forms (Ser62 and Thr58) by Western blot analysis. Ruxolitinib (2.5 uM), a JAK2/STAT3 inhibitor was added to the co-cultures as indicated.

Results

Exposure of NB cells to TAM but not to untreated human monocytes or to the conditioned medium of co-cultures of TAM and NB cells increased the expression of MYC in MYCN-NA cells but not in MYCN-A (SK-N-BE(2)) cells. MSC had a similar effect and enhanced the effect of TAM. The increase in protein expression was observed within 30 min. of exposure and was sustained

for more than 72 hours. It was not associated with changes in mRNA expression. Increase in MYC expression corresponded to an increase in phosphorylation at Ser62 and a decrease at Thr58. A similar effect was observed in the presence of IL-6 and its agonistic receptor sIL-6R and was blocked in the presence of ruxolitinib.

Summary/Conclusions

The data provide a mechanism explaining the well know contribution of TAM and MSC in the TME of MYC-NA NB tumors by demonstrating that these cells enhance MYC expression by preventing its degradation and suggest that this effect is mediated by IL-6 released by TAM and MSC in the presence of NB cells. In view of the recently identified contribution of MYC to immune escape, the data also suggest that the upregulation of MYC in NB tumors by TAM and MSC could be a mechanism promoting immune escape

Type Basic

Presentation Preference Traditional poster

Abstract nr. 311

Long-term follow-up of a Phase III Study of ch14.18 (Dinutuximab) Plus Cytokines for Patients with High-risk Neuroblastoma: Children's Oncology Group Study ANBL0032

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

Previously reported results of our randomized Phase III trial COG ANBL0032 demonstrated that immunotherapy including ch14.18 (dinutuximab), a chimeric human/murine anti-GD2 monoclonal antibody, GM-CSF, and interleukin-2 (IL-2) improved survival for children with high-risk

neuroblastoma that had responded to standard induction and consolidation therapy. These results served as the basis for FDA approval of dinutuximab.

Aims

To present extended follow-up results of the randomized COG ANBL0032 cohort to provide insights into the long-term benefits of dinutuximab-based immunotherapy. In addition, this study sought to identify potential biomarkers that may correlate with patient outcome.

Methods/Materials

Patients recieved 6 cycles of isotretinoin with or without 5 cycles of immunotherapy. Dinutuximab was given with GM-CSF in cycles 1, 3, and 5 and with IL-2 in cycles 2 and 4. Accrual was discontinued early due to meeting the protocol-defined stopping rule for efficacy, as assessed by analysis of event-free survival (EFS). Survival curves were compared by intention to treatment group using a one-sided log-rank test. Plasma levels of dinutuximab, soluble IL2 receptor (sIL2R) and human anti-chimeric antibody (HACA) were assessed by ELISA. Fcg receptor 2A and 3A genotypes were determined by PCR amplification and direct sequencing. The FcR SNP genotypes were correlated with EFS of patients based on actual treatment received with a log-rank test.

Results

For 226 eligible randomized patients (out of 230 randomized, median follow-up 9.97 years), 5-year EFS was 56.6±4.7% for patients randomized to immunotherapy (n=114) versus 46.1±5.1% for those randomized to isotretinoin only (n=112) (p=0.0417). Five-year overall survival (OS) was 73.2±4.2% for immunotherapy patients compared to 56.6±5.1% for isotretinoin only patients (p=0.0445). For 179 stage 4, ≥18 months old patients, the EFS (5-year: 51.3±5.3% vs. 41.1±5.6%; p=0.0401) and OS (71.4±4.9% vs. 51.7±5.7%; p=0.0326) were superior for immunotherapy compared to isotretinoin. Thirteen of 122 HACA-evaluable patients receiving dinutuximab developed HACA. HACA positivity was not associated with EFS, OS, or with occurrence of DLT (p≥0.36 for all 3 parameters). Similarly, plasma levels of dinutuximab and of sIL2R did not correlate with recurrence or DLT. Fcg receptor 2A and 3A genotypes matched reported frequencies, but did not correlate with clinical outcome.

Summary/Conclusions

The randomized cohort of 226 patients with a median follow-up of 10 years confirmed better EFS and OS for those that received immunotherapy compared with those treated with isotrentinoin only. Validated biomarkers of response have yet to be identified.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 312

The degree of chromosomal instability in circulating cell-free DNA is associated with clinical features in neuroblastoma

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Chromosomal instability (CIN) is thought to take part in neuroblastoma development and aggressiveness. Circulating cell-free DNA (cfDNA), a promising biomarker for various cancers, is detectable in most patients with neuroblastoma.

Aims

The present study investigated the degree of CIN in cfDNA of neuroblastoma patients and its association with clinical features.

Methods/Materials

Among the patients diagnosed with neuroblastoma at Samsung Medical Center between November 2016 and August 2019, patients who had stored serum samples at diagnosis were enrolled. Low depth whole-genome sequencing of cfDNA was performed to identify genome-wide copy number alteration, and the I-score was used to evaluate CIN. The I-score was defined as the sum of absolute Z-scores of sequenced reads on each chromosome.

Results

A total of 48 patients (22 girls and 26 boys) were evaluated for CIN using serum at diagnosis. Whole-genome sequencing of cfDNA was successful in all patients, with a median I-score of 8396 (range, 0 – 28260). Abdominal primary site, advanced stage, less differentiated pathology (poorly differentiated, undifferentiated), bone marrow involvement by neuroblastoma, high-risk tumor, 1p deletion, 11q deletion and higher NSE values were associated with a higher I-score. Three

patients experienced relapse/progression, the I-scores of these patients were 9203, 26510, and 14047.

Summary/Conclusions

A higher I-score was associated with high-risk features at diagnosis of neuroblastoma. We demonstrated that the I-score using cfDNA is a potential biomarker predicting the aggressiveness of neuroblastoma.

Type Clinical

Presentation Preference Traditional poster

Abstract nr. 313

Cross-talk between neuroblastoma cells and their microenvironment in the metastatic bone marrow niche

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Neuroblastoma (NB) is a childhood malignancy originating from the sympathetic nervous system, prone to metastasize to bone marrow (BM). Persistence of micrometastases in BM is a likely key factor in relapse, which is unacceptably high for NB. The BM microenvironment appears to possess unique features that protect tumor cells. BM-residing Mesenchymal Stromal Cells (MSCs), for example, induce therapy resistance and promote tumor cell survival in several cancer types. MSCs can also exert pro-tumorigenic actions on NB-cells, as demonstrated by *in vitro* studies. We hypothesize that MSCs in BM are modified by NB-cells and involved in bidirectional interactions; thus, supporting NB-cells. Targeting MSCs within the BM microenvironment may offer a new avenue to combat metastatic-NB; hence, a better understanding of this milieu is of great importance.

Aims

To unravel NB-induced effects on MSCs within the BM microenvironment by isolating and characterizing MSCs from BM of NB-patients with and without metastases.

Methods/Materials

Diagnostic BM aspirates from patients with metastatic-NB were compared to BM from patients with localized-NB. The extent of BM infiltration was determined by reverse transcriptase quantitative PCR (RT-qPCR), using NB mRNA markers as previously described (Stutterheim, 2009). We analyzed fresh diagnostic BM of a cohort of 48 patients, and included adult and fetal BM as controls. We also examined follow-up BM samples of metastatic-NB patients: during and after treatment and at relapse. For phenotypic characterization flow cytometry was performed, using the classical MSC marker panel: $CD34^-$, $CD45^-$, $CD90^+$ and $CD105^+$, with addition of CD146 and CD271 to explore subpopulations. Colony-forming unit-fibroblast (CFU-F) assays were used as a functional method to quantify the frequency of stromal progenitors in BM samples. Moreover, MSCs were isolated from BM and their differentiation capacity towards osteogenic and adipogenic lineages was examined.

Results

We found that BM with metastases contains a higher number of MSC. We identified a MSC subtype that is present in BM of NB-patients with metastases, but not in BM of patients with localized-NB or ganglioneuroma. Interestingly, the MSC subtype disappeared after treatment, when no detectable amounts of tumor cells are present, and was found again at relapse. This subtype was also present in fetal BM, suggesting that it might represent an immature MSC population. qPCR testing on FACS-sorted MSC from BM of metastatic-NB patients proved the absence of tumor cells in the MSC population. MSCs isolated from BM of metastatic-NB patients were more prone to differentiate towards osteoblasts compared to localized-NB-MSC; emphasizing that MSCs from metastatic-NB patients not only differ phenotypically, but also functionally.

Summary/Conclusions

We found that the MSC compartment in BM of NB-patients is modified when NB-cells are present. MSCs of patients suffering from metastatic-NB differ from ‘normal’ MSCs. Together, our findings point toward the existence of an active crosstalk between NB and MSC. Ultimately, increasing our understanding of MSC-NB crosstalk could provide targets with exciting potential for a promising novel approach to eradicate incurable metastatic disease: targeting the BM microenvironment.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 314

WIP1 inhibitor SL-176 suppresses neuroblastoma growth and acts synergistically in combination with an H3K27 demethylase Inhibitor

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

The phosphatase WIP1, encoded by *PPM1D* on chromosome 17q, is a putative oncoprotein overexpressed in many malignancies including neuroblastoma. It halts DNA damage response by directly dephosphorylating p53, inhibiting p53 kinases, stabilizing the p53 inhibitor MDM2 and by promoting apoptosis through dephosphorylation of BAX. Knockdown of WIP1 reduces neuroblastoma cell viability. Several WIP1 inhibitors have been preclinically evaluated, among others SL-176 and GSK2830371, but to date, none has reached clinical testing.

Aims

We aim to study the effect of WIP1 inhibitors as single treatment and in drug combinations on neuroblastoma growth *in vitro* and *in vivo*.

Methods/Materials

Eleven neuroblastoma cell lines were exposed to different concentrations of SL-176; three neuroblastoma cell lines were treated with GSK2830371. After 72 h, cell viability was assessed with a formazan-based colorimetric assay. For the xenograft experiment, mice were injected subcutaneously with SK-N-BE(2) neuroblastoma cells and treated once daily with intraperitoneal SL-176 injections after establishment of macroscopic tumors.
In a semi-automated drug combination screening experiment, the WIP1 inhibitors SL-176 and GSK2830371 were each tested in combination with 528 different clinical and experimental compounds. For this purpose, a fixed concentration of SL-176 or GSK2830371, chosen as the IC25 of the respective drug in IMR32 cells, or DMSO, was combined with five different concentrations of each of the 528 screening compounds, or DMSO. Both drugs were added to

IMR32 or SK-N-AS cells and incubated for 72 h, after which cell viability was assessed using an ATP-based luminescent viability assay. A drug sensitivity score (DSS) was calculated for each dose-response curve, and the ΔDSS was calculated as the difference between DSS of the single screening drug and DSS of the combination.

Results

SL-176 had cytotoxic effect with similar IC50 values on all tested neuroblastoma cell lines, while GSK2830371 only seemed effective in the WIP1-overexpressing cells. SL-176 treatment *in vivo* decreased xenograft growth by about 50% without significant side effects.
In the drug combination experiment, the two WIP1 inhibitors SL-176 and GSK2830371 showed different cytotoxic profiles measured by ΔDSS, suggesting different mechanisms of WIP1 inhibition. IMR32 cells were sensitive to a greater number of single drugs and drug combinations, as compared to SK-N-AS cells. For SL-176, the best ΔDSS in both SK-N-AS and IMR32 cells was found for the combination with GSK-J4, an inhibitor of H3K27 demethylation which has previously been identified as potential target in neuroblastoma. Regarding GSK2830371, combination with several MDM2 inhibitors yielded high ΔDSS in IMR32 cells. The combination of both WIP1 inhibitors with each other also seems beneficial in this cell line.

Summary/Conclusions

The WIP1 inhibitor SL-176 is cytotoxic/cytostatic to neuroblastoma cell lines, but has insufficient effect *in vivo*. Combination with GSK-J4 has shown promising results, and experiments to further investigate synergy *in vitro*, and subsequently *in vivo*, are ongoing. The mechanism of WIP1 inhibition by SL-176 also needs to be studied further.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 315

A Nationwide Phase II Study of Extremely Delayed Local Control Strategy in Children with High Risk Neuroblastoma : Japan Children's Cancer Group - Neuroblastoma Committee Trial (JN-H-11)

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

The survival rates of high risk neuroblastoma (HR-NB) patients are still unacceptable. The estimated 3-year progression-free and overall survival rates for 50 patients of JNBSG phase II clinical trial (JN-H-07) were 36.5±7.0% and 69.5±6.6% respectively. Increasing the time intensity and dose intensity of chemotherapy are both key factors for the treatment for HR-NB. In Japan the restart of chemotherapy after surgery tended to be significantly delayed, sometimes up to more than one month, due to surgical complications. Therefore, increasing the time intensity of

chemotherapy had been an important issue to be addressed.

Aims

JNBSG conducted a nationwide prospective single-arm clinical trial of time-intensive multimodal treatment with extremely delayed local control strategy (EDLC) .

Methods/Materials

Between May 2011 and September 2015, seventy-five patients with newly diagnosed HR-NB patients were enrolled. EDLC consisted of induction chemotherapy (IC) , myeloabrative chemotherapy(MAC), and local control treatment after MAC. IC consisted with cisplatin (100mg/m2), pirarubicin (40mg/m2), vincristine (1.5mg/m2), and cyclophosphamide (1,200mg/m2 in the 1st course and 2,400mg/m2 in the 2nd-5th course). After 5 courses of IC, all patients were immediately followed by MAC with melphalan (200mg/m2), etoposide (800mg/m2) and carboplatin (1600mg/m2)(MEC). After MAC local tumor eradication with surgery and irradiation (21Gy) were performed.

Results

A total of 75 patients were enrolled, 64 were evaluable: 40 male, 2.6 y/o average age, 56 stage 4, 51 Neuroblastoma, 21 MYCN amplified, 51 Unfavorable histology. 46 patients completed the protocol treatment, while the treatment was discontinued in 18 patients. The estimated 3-year event-free survival rates and overall survival rates (95%CI) of 64 patients were 44.4% [31.8% - 56.3%] and 80.7% [68.5% - 88.5%], respectively. The surgeries were performed safely in 56 patients. The response rates of INRC after MAC were 20.3%(13/64; 1 CR,12 VGPR) and 59.3%(38/64; 1 CR,12 VGPR,25 PR), respectively. The response rates of INRC after completion of protocol treatment were 51.6%(33/64; 23 CR, 10 VGPR) and 62.5% (33/64; 23 CR,10 VGPR, 9PR), respectively. Pathological response rates (Ef2+Ef3) were 74.4% in 43 patients evaluated. No patient died during the protocol treatment nor within 30 days after completion of the protocol treatment. Grade 4 adverse effects, excluding hematological adverse effects, occurred in 31 cases. EDLC was performed without affecting early relapse or progression in many cases.

Summary/Conclusions

Local control treatment could be postponed until after MAC in a notable number of cases. We considered that EDLC strategy is feasible. In the currently ongoing JNBSG HR-NB study (JN-H-15), EDLC is performed after BU/MEL treatment.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 316

Have fever and infections during induction chemotherapy in HR-neuroblastoma an impact on outcome?

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Topic Other

Background/Introduction

Fever and infections during induction chemotherapy indicate an activation of the endogenous defense system against the tumor, however, the necessary delay of the subsequent chemotherapy may be associated with some tumor revival.

Aims

The impact of inflammatory reactions and prolonged recovery time on outcome in neuroblastoma was investigated.

Methods/Materials

A retrospective data analysis of high-risk patients included in the GPOH trials NB2004-HR and NB97 (high-dose chemotherapy arm) was performed.
Eligibility criteria were neuroblastoma diagnosis according to the INSS criteria, stage 4 and age ≥18 months or non-stage 4 with MYCN amplification, and at least 6 courses of chemotherapy before their first progress/residual tumor/death.
Standard induction chemotherapy (SIC) consisted of six alternating courses N5 (cisplatin, etoposide, vindesine) and N6 (vincristine, dacarbazin, ifosfamide, doxorubicine). Patients of the NB2004-HR experimental arm received two additional courses consisting of topotecan,

cyclophosphamide and etoposide before SIC.
Event free (EFS) and overall survival (OS) were measured from day one of the sixth chemotherapy course. The adverse effects were graded according the CT-CAE system. The number of febrile episodes and infections, respectively, was limited to a maximum of one per course.

Results

484 patients were eligible and received 3318 courses of chemotherapy between March 1997 and December 2016. Fifteen percent had 0 infections throughout all courses, 9% 1,12% 2, 14% 3, 14% 4, 15% 5, 13% 6. No febrile episode was seen in 5%, 8% had 1 febrile episode, 11% 2, 15% 3, 16% 4, 14% 5, 19% 6, 6% 7, 6% 8. In total, 1657 infections (50%; grade 1-2: 41%, 3-4: 8%, unknown: 1%) and 2001 febrile episodes (60%; grade 1-2: 56%, 3-4: 2%, unknown: 2%) were registered. No differences were detected with regard to sex, age < vs. ≥18 months) and MYCN-status. The N5 courses 3, 5, and 7 had lower frequencies of infections and/or fever. The median time between two successive courses (day 1 to day 1) was 1 day longer with infections (26 days, IQR 23-30) compared to those without (25 days, IQR 22-28, P <0.001). Febrile episodes were also associated with a longer interval (26 days IQR 23-30 vs. 24 days, IQR 22-28, P <0.001).
Patients who did not experience any infection (grades 1-4) had a better EFS and OS (10y-EFS: 44%, 95%-CI: 32-56 vs. 30%, 95%-CI: 25-35, log-rank P = 0.013; 10y-OS: 52%, 95%-CI: 40-64 vs. 41%, 95%-CI: 36-46, log-rank P = 0.073). The cumulative number of infections (grades 1-4) was not relevant. More severe infections (grades 3-4) had no impact on EFS and OS compared to no and less severe infections (grades 0-2).
An association between febrile episodes (grades 1-4 vs. none; grades 3-4 vs. 0-2) and EFS or OS was not detected.

Summary/Conclusions

Only a minority of high-risk neuroblastoma patients did not experience infections and/or febrile episodes. Patients without infections during induction chemotherapy had a better EFS and OS. Febrile episodes were not associated with outcome differences.

Type Basic

Presentation Preference Traditional poster

Abstract nr. 317

Histone deacetylase inhibition suppresses telomerase activity in neuroblastoma cells harboring TERT rearrangements

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

Telomerase activation by genomic rearrangements inducing transcriptional upregulation of *TERT* defines a subgroup of high-risk neuroblastomas with poor outcome. Accordingly, telomerase activity presents a high-priority drug target with no currently available clinical candidates.

Aims

We aimed to assess the significance of histone deacetylase (HDAC) activity for *TERT* mRNA expression and telomerase activity in *TERT*-rearranged neuroblastoma cells, and estimate the effects of its inhibition by clinically approved drugs.

Methods/Materials

Transcriptional changes were analyzed via gene expression profiling. Telomerase activity was assessed with the TeloTAGGG™ Telomerase PCR ELISA^{PLUS} assay. Chromatin immunoprecipitation (ChIP) sequencing was performed using the Illumina NEBNext® ChIP-Seq

Library Prep Reagent Set and HiSeq 4000 in 50 single-read mode. The Illumina Infinium Human MethylationEPIC Beadchip was used for methylation studies. De novo RNA synthesis was blocked through addition of actinomycin D to the cell culture medium. Functional studies were performed in 2D and 3D cell cultures and subcutaneous neuroblastoma xenografts in mice.

Results

Changes in *TERT* transcript levels monitored in time-course in CLB-GA and GI-ME-N neuroblastoma cells following treatment with low nanomolar concentrations of panobinostat. *TERT* transcription was repressed after 2 hours of treatment, and remained strongly down-regulated throughout the 120-hour time-course. Treatment with vorinostat, mocetinostat or entinostat, which are either clinically approved or being evaluated in phase II or III clinical trials, similarly suppressed *TERT* transcript levels by 2- to 4.5-fold, supporting *TERT* repression as a common event of HDAC-inhibition in *TERT*-rearranged neuroblastoma cells. We assessed telomerase activity in CLB-GA and GI-ME-N whole-cell lysates to investigate whether TERT repression caused by HDAC inhibitor treatment is translated to the effector level, and detected an approximately 2-fold decrease. Transient plasmid-mediated enforced TERT expression partly rescued the anti-tumor effects induced by HDAC inhibitor treatment *in vitro*. Treating NMRI-Foxn1$^{nu/nu}$ mice carrying subcutaneous CLB-GA- or GI-ME-N-derived neuroblastoma xenografts with panobinostat in either preventive or therapeutic settings considerably attenuated tumor growth. We assessed *TERT* transcript levels and telomerase activity in these xenograft tumors, and detected a reduction of up to 5-fold. Altogether, our data indicate that HDAC inhibitors, including those in or being tested for clinical use, suppress TERT in TERT-driven neuroblastoma cells *in vitro* and *in vivo*. To test for underlying epigenetic changes, we performed ChIP sequencing in GI-ME-N cells treated with panobinostat or solvent control using antibodies against histone modifications known to mark active or repressive sites as well as transcriptional elongation, and looked for changes in the chromatin context of the *TERT* locus. We have detected no major differences between HDAC inhibitor-treated and control cells in our on-going experiments. Likewise, HDAC inhibition did not substantially alter the methylation profile in the region of interest. Blocking *de novo* RNA synthesis with actinomycin D, however, reduced *TERT* transcript levels by 2-fold in HDAC inhibitor-treated cells compared to controls, suggesting reduced *TERT* transcript stability as the underlying molecular mechanism.

Summary/Conclusions

Our results show high-level telomerase activity caused by genomic rearrangements is suppressed in neuroblastoma cells by treatment with clinically approved HDAC inhibitors, suggesting indirect druggability and a potential novel molecular rationale for therapeutic intervention.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 318

Relapsed Refractory Neuroblastoma – Retrospective Review in a Single Centre

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

Neuroblastoma is an aggressive paediatric tumour which accounts for approximately 15% of cancer-related mortalities in children. While children diagnosed with low- or intermediate-risk neuroblastomas have excellent long-term survival, more than 50% of high-risk neuroblastomas have tumour relapse or regrowth. About 50% of the patients present with stage 4 (metastatic) disease and while the survival rates of these patients have improved, treatment for these patients remains a difficult task with the risk of relapse still prevalent.

Aims

To study the epidemiological factors and clinical profile of patients in a single centre with relapse of neuroblastomas.

Methods/Materials

A total of 33 children with relapsed and refractory neuroblastoma in KKH, Singapore from 1997 – 2017 were studied. Data for patient’s initial disease characteristics - demographics (age of diagnosis, gender, race); biochemical data (urine HVA/VMA, serum ferritin and LDH); tumour characteristics (primary site, metastases); biological marker (NMYC); and final risk group according to the Children’s Oncology Group neuroblastoma risk strata – were collected. Data was primary studied with comparisons between the high risk and non-high risk group. Initial treatment details whether the patient underwent surgery, chemotherapy, radiotherapy and retinoic acid were also traced. Analysis of pattern of relapse was also done by collecting the time from first diagnosis and sites of relapse.

Results

A brief analysis of the initial characteristics shows a large percentage (97.0%) of them were of age more than 18 months during the diagnosis of neuroblastoma with the mean age of 4.6 years. According to the INSS stage, almost all the patients presented initially with Stage 3 or 4 (n=31 (93.9%)) but there was one stage 2B and one stage 1. Out of 33 patients, the NMYC status of 21 were traced and 1/3 showed amplification (n=7 (33.3%), total known=21). It was found that none of the non-high risk patients had NMYC amplification compared to the high risk group with had 26.9% of known NMYC amplified (n=7; total known high risk=18). The time of diagnosis to relapse between the high risk and non-high risk groups showed no significant difference with mean time in months 17.05 (±10.66 SD) for high risk and 17.08 (± 5.51 SD) for non-high risk. In the high risk group, 23.1% (n=6; total = 26; unknown = 2) relapsed to primary while the remaining 69.2% (n=18) to metastatic. It was found that 80.8% (n=21) of high risk and 71.4% (n=5) of non-high risk patients current disease status was dead. Only 1 (3.8%) out of 26 high risk patients and 2 (28.6%) non-high risk patients are in complete remission.

Summary/Conclusions

Neuroblastomas continue to be a challenging tumour to treat especially in higher age group patients and high risk patients. Fortunately, progress have been made over the years with intensive research and clinical trials and hopefully the overall survival risk of high risk group patients will improve.

Type Clinical

Presentation Preference Traditional poster

Abstract nr. 319

Synergistic activity of HDAC inhibitor Panobinostat and ALK inhibitor Lorlatinib in neuroblastoma cell lines harboring mutated ALK

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Topic Gene regulatory networks and epigenetics: from basic science to therapy

Background/Introduction

Targeted treatment approaches for patients with high-risk neuroblastoma are required in case treatment failure occurs after first- or second-line therapies. These approaches should ideally be well tolerated as many patients have already experienced significant therapy-related morbidity at that stage.

Aims

Inhibition of histone deacetylase (HDAC) activity by the clinically approved HDAC inhibitor Panobinostat has significant anti-tumor effects in preclinical models of *MYCN*-amplified and *TERT*-rearranged neuroblastoma, however, effects as stand-alone therapy are not curative in most cases. The purpose of this study was the identification of drugs that act synergistically with Panobinostat in neuroblastoma cells.

Methods/Materials

We screened four chemotherapeutic drugs implemented in first-line therapies and five targeted drugs, which are used in second-line therapies or are currently being tested in phase II or III clinical trials, for synergistic effects with Panobinostat in two neuroblastoma cell lines using the

Cell Titer Glo Luminescent *Cell* Viability *assay*. Each drug was applied in six different concentrations as stand-alone or combination therapy, and results were tested for synergy using the *SynergyFinder* software. Cell death assays were conducted with the top six drug combinations. A two-factorial mixed linear model with interaction and random effect for each experimental run was used to assess results for potential synergistic effects of the two-drug combinations using SAS PROC MIXED.

Results

Altogether, two chemotherapeutic agents (doxorubicine, temozolomide) and four targeted drugs inhibiting tyrosine kinases, mTOR, the proteasome or ALK attenuated neuroblastoma cell proliferation synergistically when combined with Panobinostat. Strongest synergistic killing effects were observed in combination treatment with the ALK inhibitor Lorlatinib. Findings were confirmed in six neuroblastoma cell lines harboring mutated *ALK*. Effects of the two-drug combination on cell cycle, DNA damage and replication stress response as well as on phosphorylation levels of selected proteins is investigated at the single-cell level using a *CyTOF* mass cytometer in ongoing experiments.

Summary/Conclusions

Panobinostat is effective against preclinical high-risk neuroblastoma models, and its effects can be strongly enhanced through simultaneous targeting of mutated *ALK*. This promising combination could be an important step towards improved second-line treatments for patients with high-risk neuroblastoma in case of treatment failure and warrants further testing in the framework of a clinical trial.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 320

Defects in 8-oxo-Guanine repair pathway cause high frequency of C>A substitutions in neuroblastoma

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

In neuroblastoma tumors C>A substitutions are the most common substitution type. C>A substitutions might be a result of defects in the 8-oxo-Guanine (8-OxoG) repair pathway. 8-oxoG is one of the most abundant DNA lesions that is generated by reactive oxygen species. During replication, DNA polymerases can insert an incorrect Adenine opposite of 8-oxoG. In the next round of replication, a Thymine will be inserted opposite the Adenine, finally resulting in a C>A substitution. 8-oxoG repair is initiated by excision of 8-oxoG by OGG1 or excision of the mis-inserted A, opposite 8-oxo-Guanine by MUTYH. *OGG1* and *MUTYH* are located on chromosome 3p and 1p, respectively, which are recurrently lost in neuroblastoma. This suggests that defects in OGG1 or MUTYH might contribute to the high levels of C>A substitutions in neuroblastoma.

Aims

Investigate whether defects in 8-OxoG repair cause high levels of C>A substitutions in neuroblastoma

Methods/Materials

We used whole genome sequencing (WGS) data to investigate the frequency of C>A substitutions in a cohort of neuroblastoma tumors and correlated these C>A frequencies to INSS stage and survival. We also determined *MUTYH* or *OGG1* copy number loss in this cohort and correlated this to C>A substitution frequencies. In addition, we created CRISPR CAS9 knockout clones of *MUTYH* or *OGG1* in a neuroblastoma cell line (CHP134) and performed WGS to investigate mutational patterns. We performed mutational signature analysis for the *MUTYH* or *OGG1* knockout clones and the neuroblastoma tumors. Finally, we performed compound screens on the knockout clones and evaluated a previously published dataset of primary and relapse neuroblastoma tumors for C>A substitutions in the RAS MAPK pathway.

Results

We identified high levels of somatic C>A substitutions in our cohort of neuroblastoma tumors, which was associated with a poor survival prognosis. In addition, we identified a strong association between increased levels of C>A substitutions and copy number loss of *OGG1* or *MUTYH*. In the *OGG1* or *MUTYH* single cell knockout clones we identified an increased accumulation of C>A substitutions. Mutational signature analysis of *OGG1* or *MUTYH* knockout clones revealed an enrichment for signatures 18 and 36, respectively. These clones cluster together with neuroblastoma tumors with *OGG1* or *MUTYH* copy number loss, which also show a strong contribution of signature 18 or signature 36. In a compound screen we did not identify differential sensitivities between the *OGG1* or *MUTYH* knockout clones compared to the mother line. Evaluation of a previously published dataset of primary and relapse neuroblastoma tumors indicates that in this small dataset 47% of the tumor evolution events in the RAS MAPK pathway are C>A substitutions.

Summary/Conclusions

Defects in the 8-oxoG repair pathway result in high frequencies of C>A substitutions in neuroblastoma cell line models and tumors. These high levels of C>A substitutions correlate with a poor survival prognosis, which might be related to an increased adaptive capacity of these tumors.

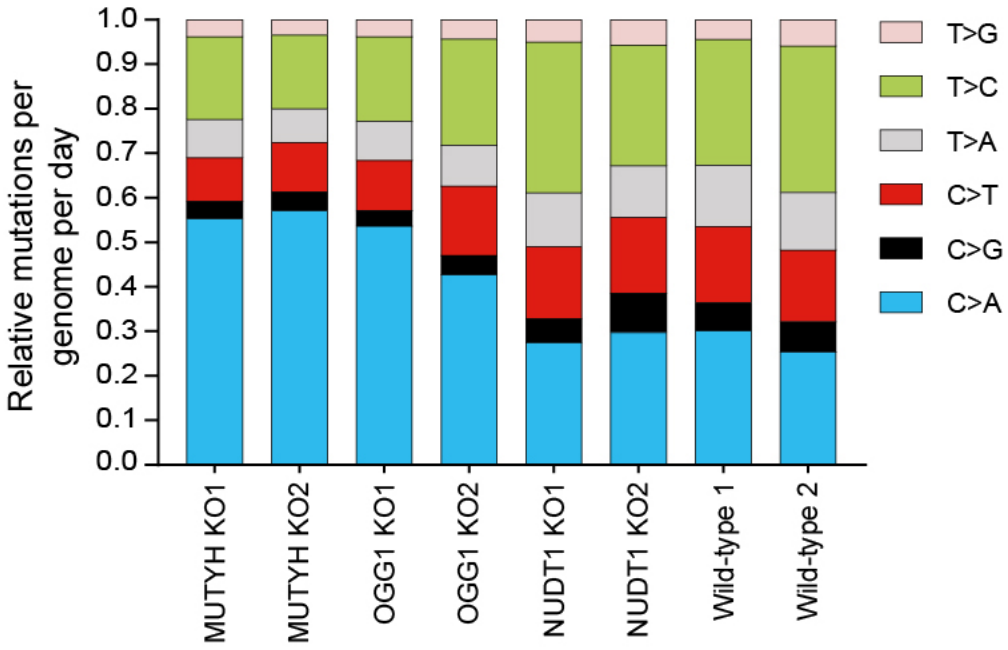

Figure 1. *MUTYH* or *OGG1* knockout results in accumulation of C>A substitutions in neuroblastoma cell line. Relative substitutions per genome per day that accumulated in *MUTYH* knockout, *OGG1* knockout, *NUDT1* knockout and wild-type CHP134 clones. Substitution types are indicated with colors.

MUTYH or OGG1 knockout results in accumulation of C>A substitutions in neuroblastoma cell line.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 321

3D in vitro scaffold-based cell model of neuroblastoma for evaluating genotoxic and miRNA-targeted therapeutics.

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

Neuroblastoma is an aggressive paediatric cancer of the sympathetic nervous system. Nearly ten new cases are reported annually in Ireland, 100 in the UK and 800 in the US. Current therapies are not effective in the long term for almost 80% of patients with clinically aggressive high-risk disease due to the development of metastasic loci resistant to multiple chemotherapeutic drugs. The accurate representation of the tumour architecture and patient diversity are two primary challenges in the identification of new agents for paediatric drug development particularly with the limited number of patients eligible for clinical trials.

Aims

We aimed to bridge the gap between conventional 2D culture and *in vivo* tumours for neuroblastoma by developing a 3D neuroblastoma tissue-engineered platform and exploring its therapeutic relevance to genotoxic and targeted drugs.

Methods/Materials

Chemotherapeutic sensitive Kelly and resistant KellyCis83 neuroblastoma cell lines were cultured in a 3D *in vitro* model on collagen-based scaffolds containing either glycosaminoglycan (Coll-GAG) or nanohydroxyapatite (Coll-nHA) and compared to 2D cell culture and an orthotopic murine model. The model was characterised by cell proliferation (DNA content) and viability, secretion of Chromogranin A (CgA) and fluorescent microscopy (DAPI). Chemosensitivity to cisplatin treatment was assessed in all tested models.

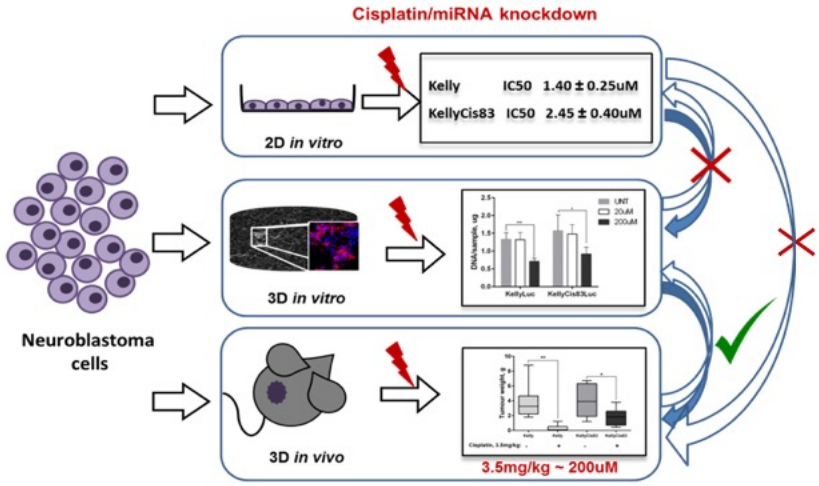
Results

Neuroblastoma cell lines actively infiltrated and proliferated over the 21-day timeframe and exhibited physiological activity by secreting CgA demonstrating the correlation between cell

numbers and concentration of CgA. Both cell lines responded to cisplatin, a cytotoxic drug commonly used in neuroblastoma treatment, displaying > 100-fold increased resistance to cisplatin treatment when compared to 2D cultures, exhibiting chemosensitivity similar to orthotopic xenograft *in vivo* models. The data is in agreement with previous studies reporting a significant increase in cancer cell resistance to chemotherapy when grown in 3D when compared to their 2D monolayer counterparts in various cancer types. The efficacy of cellular uptake and gene knockdown by liposomes bearing miR-324-5p was similar in both 2D and 3D *in vitro* culturing models highlighting the proof-of-principle for the applicability of this model for validation of miRNA function.

Summary/Conclusions

We successfully established and characterised a physiologically relevant, scaffold-based 3D neuroblastoma model, strongly supporting its potential value in the evaluation of chemotherapeutic and miRNA-based drugs and investigation of neuroblastoma biology.



Translational relevance of 3D in vitro neuroblastoma models

Type Translational

Presentation Preference Oral presentation

Abstract nr. 322

CDK12 AS A TARGET FOR SYNTHETIC LETHAL INTERACTION WITH THE HOMOLOGOUS DNA REPAIR MACHINERY IN NEUROBLASTOMA

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

Neuroblastoma (NB) is a deadly pediatric tumor arising from sympathetic neuronal progenitors. Current options for precision medicine are limited and overall survival of high-risk NB cases remains disappointingly low. In order to identify novel drug targets, we studied dynamic regulation of gene expression during MYCN driven NB formation in mice. We identified upregulation of key components of the homologous DNA repair machinery, including BRCA1 and RAD51. In addition, both genes were identified as hits of a genome wide siRNA screen in NB cells. Of further interest, we also noted elevated CDK12 expression levels over time. In view of the impact of CDK12 on *BRCA1* and *RAD51* expression levels through global suppression of intronic polyadenylation, we hypothesized that targeted pharmacological inhibition of CDK12 using THZ531, could mediate a BRCAness phenotype rendering these cells vulnerable to drugging of synthetic lethal targets.

Aims

Our aim was to evaluate drug synergism of the CDK12 inhibitor THZ531 in combination with compounds affecting DNA repair and replication fork stability.

Methods/Materials

We evaluated the half-maximum inhibitory concentration (IC50) of THZ531 in a panel of 10 NB cell lines and determined the impact on the NB transcriptome by RNA-seq 6h post-treatment of four cell lines. In addition, we scrutinized the effects of pharmacological CDK12 inhibition on DNA damage levels. Combination treatment was tested for a small in-house panel of compounds

including 3-AP (RRM2i), gemcitabine (RRM2i), talazoparib (PARPi), AZD1775 (WEE1i), LY2606368 (CHK1i), BAY1895344 (ATRi), nutlin-3 and idasanutlin (MDM2i). In addition, THZ531 was tested against a drug library of > 190 FDA approved compounds. Synergism was evaluated using the Bliss independence score.

Results

IC50 values for THZ531 after 48h exposure range between 40nM and 3000nM. RNA-sequencing upon treatment with THZ531 confirmed significant downregulation of amongst others *RAD51D, RAD50, BRCA1, EME1, MUS81* and *XRCC2* expression levels in at least 3 out of 4 cell lines. These findings were validated at RNA and protein level by RT-qPCR and immunoblotting, respectively. Compounds with known effect on replicative stress and DNA repair (3-AP, gemcitabine, talazoparib, and checkpoint inhibitors) yielded no synergistic effects. Unexpectedly, HSP90 and BCL2 inhibitors were found to generate synergistic effects on cell viability and are currently under further study. We also observed synergism with nutlin-3 (not idasanutlin) but only in TP53 mutated cell lines, most probably through the previously reported inhibitory effect on drug efflux pumps. In view of the recently reported broad synthetic lethal interaction between homologous repair proteins and DNA polymerase theta (POLQ), we will also evaluate the effects of combination treatment of THZ531 with a novel on-target POLQ inhibitor.

Summary/Conclusions

We confirm downregulation of multiple DNA repair proteins upon CDK12 inhibition in NB. We did not find evidence for synergistic interaction with drugs affecting DNA repair and cell cycle checkpoints. Synergistic effects were noted for combinations with BCL2 and HSP90 inhibitors which is currently under further study.

Princess Máxima Center

Department of Biomolecular Medicine

Princess Máxima Center

CDK12 AS A TARGET FOR SYNTHETIC LETHAL INTERACTION WITH THE HOMOLOGOUS DNA REPAIR MACHINERY IN NEUROBLASTOMA

Type Translational

Presentation Preference Oral presentation

Abstract nr. 323

Adrenergic-lineage genes are epigenetically repressed in immature MES-type neuroblastoma cells

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Topic Gene regulatory networks and epigenetics: from basic science to therapy

Background/Introduction

As reported recently, neuroblastoma tumors include two types of tumor cells with diverging characteristics. The majority of the tumor typically consists of adrenergic (ADRN) cells, which are chemosensitive and characterized by lineage markers like PHOX2A, PHOX2B and DBH. A minor proportion of the tumor cells are mesenchymal (MES) and have immature features and lack lineage differentiation markers. Interestingly, MES and ADRN cells can spontaneously interconvert into one another, which may have far reaching consequences on the resilience towards clinical intervention of neuroblastoma tumors. During reprogramming MES and ADRN cells undergo a major shift in epigenetic modification status.

Aims

To understand the role of epigenetic modification on establishing the MES- and ADRN-phenotypes and their transdifferentiation potential.

Methods/Materials

Four isogenic pairs of MES and ADRN cell lines were analyzed by ChIP-sequencing with anti-

H3K27me3 and anti-H3K4me3. The HiSeq sequencing result was analyzed and visualized on the R2 genomics analysis and visualization platform (http://r2.amc.nl/).

Results

Polycomb complexes mediate large-scale gene repression by H3K27me3 modification of chromatin. We therefore analyzed differential H3K27me3 modifications of MES- and ADRN-type neuroblastoma cell lines. ADRN-specific lineage commitment genes like ASCL1 and DBH are silent in MES-type cells. We observed that the transcription start site (TSS) of these genes have a H3K27me3 silencing mark in MES cells. Also ADRN-specific neuritogenesis genes, like NEFL, NEFM and NRCAM, carry an H3K27me3 modifications at their TSS sites in MES cells. The reverse pattern was not apparent: MES-specific genes that are silenced in ADRN-type cells have no H3K27me3 marks at their TSS site in ADRN cells.
It has been observed that in early embryogenesis, regulatory genes that are silent but have to be expressed later in lineage development may carry a bivalent modification of their TSS. This modification consists of a suppressive H3K27me3 mark and an activating H3K4me3 mark. We therefore further analyzed the TSS of the ADRN-specific genes that are silent in MES cells. Many of the silent regulatory ADRN genes have this bivalent modification in MES cells. However, MES-specific genes that are silenced in ADRN cells lack a bivalent mark. This suggests that MES and ADRN cells have maintained some of the normal epigenetic processes that control lineage development.

Summary/Conclusions

ADRN-specific genes are suppressed in MES-type cells by H3K27me3 modification of the TSS. However, these TSS sites can also carry a H3K4me3 modification, thus creating a bivalent mark typical for genes that are to be expressed in subsequent developmental stages. This suggests that basic regulatory principles of lineage development are preserved in MES- and ADRN-type neuroblastoma cells.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 324

Activation of downstream signalling pathways is a mechanism of ALK inhibitor resistance in neuroblastoma

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

About 8% of neuroblastomas harbour activating mutations in the kinase domain of the anaplastic lymphoma kinase receptor (ALK). The most frequent mutations are F1174L and R1275Q, causing constitutive kinase activation. While several patients responded initially well to treatment with ceritinib, a second-generation ALK inhibitor (ALKi), for their ALK-driven neuroblastoma, most eventually developed treatment resistance and relapsed or progressed.

Aims

Our aim was to identify and analyse potential mechanisms of ALK inhibitor resistance in neuroblastoma and develop strategies to overcome these resistance mechanisms.

Methods/Materials

A genome-wide CRSIPR/Cas9 screen activating or inhibiting genes was performed as an unsupervised approach to identify genes involved in ALKi resistance in neuroblastoma cell lines. Serial biopsies of ALKi-resistant neuroblastomas were analysed using whole genome or exome sequencing. Cell models harbouring resistance mutations observed in patients and *in vitro* screens were generated using CRISPR/Cas9 technology. Cell models were validated using Sanger sequencing, T7 assays and western blotting, and were characterized using cell-based assays and transcriptome sequencing. ALK downstream signalling in these models was analysed by mass spectrometry-based quantitative proteome and phosphoproteome profiling as well as immunoassay-based experiments and live-cell imaging during perturbation experiments. Sensitivity of resistant clones to several compounds was assessed.

Results

An unbiased, genome-wide CRISPR/Cas9 screen to identify ALKi resistance genes in neuroblastoma cell lines harbouring ALK mutations revealed several genes, including *SIK3* and *CDCP1*, regulating the canonical ALK downstream signalling pathways, RAS/MAPK and PI3K/mTOR. We also identified *ABCE1,* an ATP-binding cassette transporter that has previously been described in human cancers and associated with drug resistance. In line with these results, we observed *de novo* mutations or deletions in *NF1*, a negative RAS/MAPK pathway regulator, in relapsed tumours following ALKi therapy among the serial neuroblastoma biopsies analysed by whole genome or exome sequencing. Concordantly, inactivating NF1 in neuroblastoma cells harbouring *ALK* mutations and sensitive to ALKi using CRIPR/Cas9 technology conferred resistance against ALKi treatment. Western blotting confirmed ALK-independent activation of the RAS/MAPK pathway. However, (phospho-)proteome profiling and perturbation experiments with targeted phospho-protein analysis revealed complex rewiring of signalling pathways downstream of ALK, which is currently being deciphered in an ongoing computational modelling approach. Gene expression profiling revealed increased *RET* expression in *NF1* knockout cells in comparison to the parental control cells, pointing to a potential downstream mechanism of resistance. In line, *NF1* knockout cells displayed stable *ETV5* expression after 6 hours of ceritinib treatment in comparison to the parental control cells in which *ETV5* expression was reduced. To identify vulnerabilities in resistant clones, *NF1* knockout cells were treated with different inhibitors. While the parental control cells harbouring ALK mutations retained intermediate sensitivity, ALKi-resistant NF1-deficient clones were ultra-sensitive to MEK inhibitors.

Summary/Conclusions

(Re-)activation of ALK downstream signalling pathways is a mechanism of ALKi resistance observed in preclinical models as well as patients. Mutations inducing ALKi resistance confer new vulnerabilities, thereby pointing to potential combinatorial and/or sequential therapeutic strategies to overcome resistance.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 325

T-SNE and DeMixT applied to identify core biological processes and subsets of cells from which the signals originate.

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Topic Gene regulatory networks and epigenetics: from basic science to therapy

Background/Introduction

Many mRNA profiling studies have highlighted the strong expression differences between low- and high-risk neuroblastoma. Nonetheless, both disease entities are histopathologically very similar and are both thought to arise from the sympatho-adrenergic lineage.

Aims

We therefore analyzed neuroblastoma mRNA profiles not only for differences, but also for commonalities between both low- and high-risk tumours to identify key transcriptional networks underlying different molecular subtypes of neuroblastoma.

Methods/Materials

To achieve this, we performed gene-based T-SNE dimension reduction analysis on mRNA profiling datasets of NB followed by cellular deconvolution using the algorithm DeMixT.

Results

We identify 3 central dimensions of the disease, consisting of gene-networks regulating epithelial to mesenchymal transition (EMT), immune cell/inflammatory response and finally cell cycle/MYCN. These core networks were validated and remained stable across 3 independent NB datasets consisting of over 900 tumours and were identifiable independent of patient stage, risk-status or MYCN-amplification. Application of DeMixT suggests that signals from these networks originated from different cellular subsets within the tumour, including adrenergic, mesenchymal and stromal cellular components. Furthermore, clustering of patients based on these 3 core networks identified sub-division of patients within the each of the clinically relevant NB subgroups (low-risk, high-risk and MYCN-amplified).

Summary/Conclusions

This suggests underlying biological subtypes of NB that differ based on root biological networks independent of the aggressiveness of the disease. In addition, these NB subtypes are likely reflective of different cellular subtype composition within the tumour. These findings may provide insight into identifying key therapeutic networks and targets within both high-risk and MYCN-amplified patients that may have been previously overlooked.

Type Basic

Presentation Preference Traditional poster

Abstract nr. 326

The antibody-drug conjugate D3-GPC2-PBD is potently efficacious against diverse preclinical models of neuroblastoma and other cancers via engagement of a tumor-specific conformational GPC2 epitope

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

We identified glypican 2 (GPC2) as a MYCN-driven neuroblastoma oncoprotein that is robustly differentially expressed between tumor and normal tissues, and developed an antibody-drug conjugate (ADC) specific to mouse and human GPC2 (D3-GPC2-PBD; *Cancer Cell*, *2017*).

Aims

Here we sought to comprehensively characterize the anti-tumor efficacy of D3-GPC2-PBD in diverse preclinical models as well as its mechanisms of cell death. We also aimed to solve the crystal structure of the D3 antibody bound to GPC2 to aid in clinical development.

Methods/Materials

We performed GPC2-directed immunohistochemistry (IHC) on 61 high-risk neuroblastomas and

flow cytometry on 8 neuroblastoma patient-derived xenografts (PDXs). We then performed preclinical trials of the D3-GPC2-PBD ADC in 4 neuroblastoma PDXs with varying GPC2 expression. We quantified ADC-induced DNA damage, apoptosis and bystander cell killing and performed X-ray crystallography and mutational and isoform studies on the D3 antibody-GPC2 binding interaction.

Results

GPC2 was expressed on 59/61 high-risk neuroblastomas by IHC and on 8/8 neuroblastoma PDXs by flow cytometry. GPC2 expression by flow was consistently bimodal (GPC2-Hi and GPC2-UltraHi cell populations), with the GPC2-UltraHi cells having 20-164-fold-higher GPC2 protein and co-expressing significantly higher levels of the stem cell markers CD133, CD338 and CD117. The GPC2-UltraHi cell population was further enriched when neuroblastoma cell lines were cultured in serum-free neurobasal media. Additionally, forced GPC2 overexpression in SY5Y cells (low native GPC2) was sufficient to induce a 3.4-fold increase in CD133 expression and markedly enriched tumor initiating cells by limiting dilution assay. D3-GPC2-PBD induced maintained complete responses (MCRs) in all 4 neuroblastoma PDXs tested with diverse oncogenic drivers, including locally advanced tumors with volumes >1 cm^3, while the D3 antibody alone had no effect on tumor growth and did not induce significant antibody-dependent cellular cytotoxicity. ADC dose finding studies showed MCRs of up to 100 days in 70% (26/37) of mice treated with 1 mg/kg ADC, 95% (35/37) of mice treated with 3 mg/kg ADC and 92% (33/36) of mice treated with 1 mg/kg ADC given twice weekly x 2 weeks. ADC treatment induced robust upregulation of γH2AX, cleaved PARP and cleaved caspase-3 throughout the tumor. Furthermore, ADC treatment of co-incubated GPC2-high/low expressing cells induced 10-62% more cytotoxicity than expected, consistent with potent bystander cell killing. X-ray crystallography showed that the D3 antibody binds to a conformational and tumor specific core GPC2 epitope, findings validated by binding kinetics experiments with GPC2 mutants, and flow cytometry and ADC susceptibility studies with GPC2 isoforms. Similar studies in other GPC2-expressing tumors (small-cell lung cancer, uterine carcinosarcomas, medulloblastomas and high-grade gliomas) are ongoing and early observations of similar potent ADC activity will be reported.

Summary/Conclusions

Neuroblastomas express high-levels of GPC2 in the plasma membrane, including significant enrichment in the stem cell compartment. The D3-GPC2-PBD ADC targets a conformational and tumor specific GPC2 epitope and is potently efficacious against a diverse panel of neuroblastomas and other GPC2-expressing cancers, supporting the clinical development of GPC2-directed ADCs and chimeric antigen receptor engineered T cells.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 327

Outcomes and toxicities associated with ch14.18 (dinutuximab), GM-CSF, IL-2 and isotretinoin in the non-randomized cohort of patients treated on ANBL0032: A Children's Oncology Group (COG) Study

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

COG ANBL0032 demonstrated that immunotherapy with the anti-GD2 antibody dinutuximab in combination with sargramostim (GM-CSF), aldesleukin (IL-2) and isotretinoin in the post-consolidation setting improved event-free survival (EFS) and overall survival (OS) for patients with high-risk neuroblastoma (HRNBL). Randomization was halted in 2009, and the immunotherapy arm remained open to enrollment to ensure dinutuximab availability and to obtain additional outcome and toxicity data in a larger cohort of patients.

Aims

Our aims are to: 1) Determine the EFS and OS of patients treated with immunotherapy in the

ANBL0032 non-randomized cohort, and 2) Identify the CTCAEv4.0 Grade 3 or higher toxicities associated with immunotherapy.

Methods/Materials

Patients <31 years of age with HRNBL and a pre-autologous stem cell transplant (ASCT) response of partial response (PR) or better by the 1993 International Neuroblastoma Response Criteria (INRC) were eligible. Patient demographics, International Neuroblastoma Staging System (INSS) stage, tumor biology, pre-ASCT response by INRC and toxicities were summarized using descriptive statistics. Five-year EFS and OS estimates from the time of study enrollment post-consolidation were generated.

Results

From 2009-2015, 1,183 patients were non-randomly assigned to receive immunotherapy. Among these, 96.7% (n=1,144) were >=18 months of age at enrollment and 83.1% (n=765/921) had INSS stage 4 disease. Among those tumors with known biology, 45.1% (n=363/805) harbored *MYCN* amplification, 94.5% (n=749/793) had unfavorable histology, and 54.9% (n=397/723) had diploid tumors. Pre-ASCT response included complete response (CR) (n=352; 29.8%), very good partial response (VGPR) (n=418; 35.3%), or PR (n=413; 34.9%). Patients underwent a single (n=1,042; 88.1%) or tandem (n=141; 11.9%) ASCT prior to immunotherapy. For the entire cohort, 5-year EFS from the time of enrollment was 61.1±1.9% and 5-year OS was 71.9±1.7%. Among patients >=18 months of age with INSS stage 4 disease (n=746), 5-year EFS and OS were 58.4±2.3% and 71.0±2.1%, respectively. Five-year EFS and OS were 82.3±4.8% and 86.7±4.2% among patients with INSS stage 3 disease (n=110). EFS but not OS was superior for those with a CR/VGPR pre-ASCT compared to those with a PR pre-ASCT (5-year EFS: 64.2±2.2% vs. 55.4±3.2%, p=0.0133; OS: 72.7±2.1% vs. 70.5±2.9%, p=0.3811). There was a trend for improved OS for those treated with tandem transplant versus single transplant, although the difference did not reach statistical significance (5-year EFS: 65.9±4.3% vs. 60.4±2.1%, p=0.1282; OS: 76.5±3.8% vs. 71.2±1.9%, p=0.0704). Six cycles of therapy were completed in 83.9% of patients, with IL-2 replaced by GM-CSF in 1.8% of patients. Grade 3 or higher toxicities occurring in >10% of patients during GM-CSF and IL-2-containing cycles, respectively, included pain (15.6/11.4%), fever (15.1/32.7%), anemia (18.9/21.7%), thrombocytopenia (13.9/17.4%), lymphopenia (12.3/16.0%), and hypokalemia (13.3/25.2%). During GM-CSF-containing cycles, hypoxia occurred in 10.1% of patients. During IL-2-containing cycles, additional toxicities included anaphylaxis (12.0%), neutropenia (16.1%), hyponatremia (16.5%), and hypotension (13.8%).

Summary/Conclusions

Aligned with prior reports, we observed a 5-year EFS of 61.1% among a large cohort of HRNBL patients treated with dinutuximab immunotherapy. EFS was higher for patients with INSS stage 3 disease and pre-ASCT disease response of CR/VGPR. The number of Grade 3 or higher toxicities occurring in >10% patients was greater during IL-2-containing cycles.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 328

Genomic characterization of high-risk neuroblastoma by cancer gene panel tests

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Neuroblastoma (NB) is known to exhibit a variety of clinical features and its tumor genome signature has been considered as one of the useful risk classification markers. Cancer gene panel testing can examine mutations of several hundreds of cancer-related genes in tumor specimens, and is one of the promising ways for current tumor diagnosis and therapeutic drugs selection. In Japan, two types of cancer gene panels have been approved for national health insurance coverage from June 2019 and their clinical application have been rapidly spreading mainly for adult cancer, however, such system has not yet been well established for pediatric cancers including NB.

Aims

To construct a system for better cancer genome medicine for NB by utilizing cancer gene panel, we made a gene panel with pediatric cancer-related genes and evaluated it retrospectively with high-risk NBs.

Methods/Materials

Specimens were obtained from the patients registered in the clinical studies for high-risk NBs (JN-H-07 and JN-H-11) conducted by the Japan Children's Cancer Group (JCCG) Neuroblastoma Committee (JNBSG). Tumor and paired lymphocyte (if available) DNAs were analyzed by using Ion Torrent Comprehensive Cancer Panel (409 genes) or a custom-made cancer gene panel (211 genes) with Ion Proton (n=45) or Illumina NextSeq sequencers (n=28), respectively (read depth>600).

Results

Among the 45 NB tumors (JN-H-07 study) analyzed by the Ion Torrent Comprehensive Cancer Panel, 41 tumors had at least one mutation (*ATRX*:3, *ALK*:5, *ARID1A*:1). Ultra-high-risk NBs (died within 2y) had *MYCN* mutation (n=1), *CDK4* amplification (n=1) or tyrosine kinase mutations (*ALK*:1, *EGFR*:1, *FGFR1*:1). As a next step, we modified the "NCC Oncopanel" which is clinically available and approved for national health insurance coverage in Japan to make the customized "NCC Oncopanel Ped". It can survey mutations in 211 genes as well as 9 known gene fusions frequently observed in pediatric cancer. We applied it for sequencing of 28 tumors from the JN-H-11 study. Copy number alterations were clearly detected by both gene panels, and calculated *MYCN* copy numbers showed good correlation with those by quantitative real-time PCR. At least one mutation was detected in 27 tumors, and we found three non-*MYCN*-amplified tumors had actionable gene alterations involved in cell cycle regulations: *CDK4* and *MDM2* amplifications, *CCND1* amplification, and *CDKN2A*/*2B* homozygous deletion. For these tumors, CDK4/6 inhibitors might be possible candidate drugs. *HRAS* and *NRAS* representative mutations were also observed in other tumors.

Summary/Conclusions

Cancer gene panel analysis will provide beneficial information for considering subsequent therapeutic strategies for high risk NBs by showing targetable gene mutations, mutation frequencies and copy number alterations. We have added analysis of other 24 tumors from JN-H-11 patients (including 3 recurrent tumor samples), so results of 49 cases will be discussed.

Type Basic

Presentation Preference Traditional poster

Abstract nr. 329

Circulating adrenergic neuroblastoma mRNAs predict outcome in stage M children during treatment: a SIOPEN study.

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Topic Other

Background/Introduction

Twenty percent of children with high-risk neuroblastoma present with tumours that are resistant to current induction treatment and 40% of those who attain complete or partial remission will relapse and die from disease within 5 years of diagnosis. Early identification of these children could mean modification of their treatment at diagnosis or during early treatment, with the goal of improving outcomes. High levels of adrenergic neuroblastoma mRNAs in bone marrow aspirates and blood in stage M children receiving frontline treatment for high-risk neuroblastoma predict poor outcome [1], making them attractive candidates as circulating biomarkers of risk.

Aims

To prospectively evaluate the predictive power of the adrenergic neuroblastoma mRNAs paired-like homeobox 2B *(*PHOX2B*)* and tyrosine hydroxylase (TH) in peripheral blood from children with stage M neuroblastoma at sequential time-points of the HR-NBL-1/SIOPEN protocol (www.SIOPEN-R-NET.org).

Methods/Materials

Peripheral blood samples from 271 children were analysed by reverse transcriptase polymerase chain reaction (RTqPCR) for PHOX2B and TH mRNAs as previously described[1]. The predictive power of PHOX2B and TH mRNAs was evaluated using Kaplan-Meier survival curves and Cox regression analyses. Event-free survival (EFS) was calculated from the date that the blood sample was taken to the date of an event (relapse, death) or censored alive at the last clinic evaluation.

Results

Consistent with previous results[1], high levels of TH and PHOX2B mRNA in blood **at diagnosis** identified 1 in 5 children with rapidly progressing disease. **At the end of induction treatment** (COJEC) detection of PHOX2B and TH mRNA in blood identified 11% (30/271, chi^2= 4.76, HR=1.57, p=0.029) of children with an increased risk of an event. In blood taken **pre-** and **post-treatment for minimal disease** PHOX2B and TH mRNA remained associated with an increased risk of an event. This was most striking when evaluated at the **end of treatment**, the detection of TH and PHOX2B mRNAs identifying 8% (10/121) of children with a more than 6-fold increased risk of an event (chi^2= 32.0, HR=6.6, p=0.000). Five year event-free survival (EFS) for children with PCR positive blood was 10% compared to 61% for those with PCR negative blood. In a multivariate analysis the detection of high levels of TH and PHOX2B mRNAs in blood at diagnosis dominated and predicted those children that had PCR positive blood at the end of induction treatment. In the minimal disease setting, children with both TH and PHOX2B mRNAs in blood were also at greater risk of death from disease than children with PCR negative blood samples; 11% (15/131) children had an 8-fold increased risk of death (chi^2= 56.8, HR=8.1, p=0.000). This was independent of MYC-N status, age, type of myeloablative treatment and whether immunotherapy was given.

Summary/Conclusions

TH and PHOX2B mRNAs in blood identify children with stage M disease at greatest risk of relapse. This simple blood test could be used to save specific children from ineffective toxic treatment and fast-track them for alternative possibly more effective investigational treatments.

[1]Viprey et al, 2014, JCO,32;1074-83.

	Number of children	Number PCR negative	Number PCR positive	% positive	Log rank Chi^2	P value	Hazard Ratio	95% CI	5 Year EFS PCR negative (Std. Error)	5 Year EFS PCR positive (Std. Error)
End of induction	271	241	30	11%	4.76	0.029	1.57	1.04-2.36	34% (0.03)	17% (0.07)
Pre-MRD treatment	173	156	17	10%	6.77	0.009	2.20	1.20-4.04	49% (0.04)	29% (0.11)
Post-MRD treatment	121	111	10	8%	31.89	0.000	6.56	3.10-13.88	61% (0.05)	10% (0.1)

The detection of TH and PHOX2B mRNAs in blood predicts EFS
Type Translational

Presentation Preference Oral presentation

Abstract nr. 330

Differentiation of Residual Neuroblastoma Manifestations in Patients with Neuroblastoma Stage 4S

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

Neuroblastoma stage 4S is known for its excellent prognosis. Spontaneous regression is regularly observed, but differentiation of neuroblastoma manifestations has also been reported.

Aims

We here analysed data of a large cohort of patients with neuroblastoma stage 4S with special attention to differentiation of residual neuroblastoma manifestations and outcome.

Methods/Materials

Patients with stage 4S metastatic pattern were included if registered in the national trials NB97 and NB2004 between 1997 and 2016; diagnosed in the first year of life and presenting with MYCN-non-amplified tumors. For event-free-survival, progression to stage 4 metastatic pattern, any progression after the first year of life, secondary malignancy and death of any cause were considered, but not progression of primary or liver, skin, or soft tissue metastases during the first year of life typically known for patients with stage 4S.

Results

A total of 253 patients met the inclusion criteria and were included in the analysis. Median follow up was 9.1 years (range: 0.13-22 years).
The patients presented at a median of 3.0 months of age (range 0-11.7 months) with metastases

in the bone marrow (68%), liver (75%) and/or skin (11%). Chemotherapy was only given to 118 out of 253 patients (47%) either at initial presentation or for typical stage 4S progression of primary or metastases (with and without symptoms) in the first year of life. In median two cycles (range 1-10) were applied. Ninety-seven patients underwent resection of the primary. Of interest, the primary regressed completely in 45 out of 57 patients (79%) never treated with chemotherapy or resected.
For the whole cohort, 5-year-EFS was 0.82 ± 0.03 and 5-year-OS 0.93 ± 0.02. Eleven patients (4.4%) died from early fatal progression of liver metastases. One patient each developed a second malignancy, died from chemotherapy or died from other causes.
Progression to stage 4 was observed in 22 patients (8.7%) in median after 11.2 months (range 1.5–83 months). All 22 patients received chemotherapy for progression. Nine patients died, but only one patient diagnosed with progression within the first year of life.
After the first year of life, 11 patients (4.4%) presented either with progression of residual primary (n=6), relapse at the primary site (n=2) or progression of metastases (n=3, skin or testes). Progression occurred in median after 14.1 months (range 8.3-51.6 months). Only two out of 11 patients underwent chemotherapy for progression/relapse. Histologic assessment at relapse/progression revealed differentiation in 4 out of 11 patients (ganglioneuroma, n=3; ganglioneuroblastoma intermixed, n=1).
Similarly, in seven out of 15 patients where a residual primary was resected beyond the first year of live, differentiation was observed (ganglioneuroma, n=6; ganglioneuroblastoma intermixed, n=1).
At last follow, 231 patients were alive either in complete remission (n=177; 77%) or presenting with minor or major residuals (n=54; 23%).

Summary/Conclusions

This analysis confirms the excellent prognosis of neuroblastoma stage 4S and the potential for regression. Besides progression, differentiation was observed as well in residual tumors as in tumors progressing after the first year of life.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 331

Combination of Carboplatin and Etoposide in Patients with Relapsed or Progressing Neuroblastoma

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

Despite therapeutic advances, outcome following relapse/progression of patients with neuroblastoma remains poor. We analyzed patients with relapsed/progressing neuroblastoma treated with a combination of carboplatin and etoposide (CE).

Aims

Methods/Materials

In this analysis we included patients registered in the prospective national neuroblastoma trials between 1997 and 2019 who were treated with CE (standard dose carboplatin 300 mg/m²/d and etoposide 300 mg/m²/d for two to three days) for relapse/progression. Patients receiving additional other cytotoxic agents were excluded. We correlated first and best response evaluations with clinical features, MYCN, and calculated time-to-progression and post-relapse overall-survival.

Results

A total of 38 patients with 43 relapses have been identified. Twenty-seven patients (71.1%) were diagnosed with INSS stage 4 neuroblastoma at >18 months of age or were *MYCN* amplified at first diagnosis and therefore stratified as INRG high-risk (HR)-patients. The remaining eleven patients (28.9%) either presented with metastastatic relapse/progression after first-line treatment (n=4, 10.5%) or localized relapse/progression after previous intermediate risk therapy (n=7, 18.4%).

Twelve of 43 (27.9%) relapses were locoregional, n=14 (32.6%) were metastatic only, n=17 (39.5%) were combined. 37/38 patients had previously been treated with etoposide/platinum containing regimens.
Among 43 relapses, CE was given for the patient's first relapse in 19 (44.2%), for the second in 10 (23.3%), and for the third or more in 14 (32.6%). A median of 2 CE cycles was applied (range 1-10 cycles), 1 cycle in 8 (18.6%), 2 cycles in 17 (39.5%) and ≥3 cycles in 18 treatment episodes (41.9%). Dose reduction for toxicity of CE was necessary in 7/43 (16.3%), delayed bone marrow recovery (>28 days between cycles) was observed in 12/43 (27.9%).
First response evaluation was done after 1 or 2 CE cycles in 29 patients and demonstrated very good partial response (VGPR) in 1 (3.4%), partial response (PR) in 6 (20.7%), stable disease (SD) in 7 (24.1%) and progressive disease (PD) in 14 (48.3%). One patient was not evaluable. Best response evaluation was performed in 36 patients after a median of 2 CE cycles (range 1-5 cycles) and revealed complete remission in 1 (2.8%), VGPR in 3 (8.3%), SD in 5 (13.9%), PR in 11 (30.6%) and PD in 16 (44.4%). In patients with PD, median time-to-progression was 2.6 months (range 0.2 – 9.2 months).
Neither for early nor for best response evaluation, response (SD or better vs. PD) correlated with number of previous events, HR at first presentation or age at relapse. Patients without *MYCN* amplification were by trend more likely to respond (SD or better in 12/16 non-amplified patients vs. PD in 7/17 patients with *MYCN* amplification, p=0.08).
Twelve of 20 patients received other treatments after objective response to CE, five patients were without response evaluation. Of those, six patients are alive at last follow-up, five of these presented with an early response. For the whole cohort 3-year-post-relapse overall-survival was 0.19 ± 0.06.

Summary/Conclusions

Despite previous treatment with etoposide and platinum containing regimens, treatment with CE was effective leading to stabilization or response in a substantial part of patients with relapsed/progressing neuroblastoma. This combination therapy warrants further evaluation.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 332

Functional connection between neuroblastoma hotspot locus 6p22.3 lncRNAs and oncogenic/tumor suppressor pathways: Exploring molecular mechanisms to therapeutic approaches

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Topic Gene regulatory networks and epigenetics: from basic science to therapy

Background/Introduction

Long noncoding RNAs (lncRNAs) have emerged as important players in cancer development and progression but mechanistically how they contribute to the disease remain less understood. Genome wide association studies (GWAS) have identified several loci which are associated with the aggressive neuroblastoma (NB) and till date the most significant locus associated with aggressive NB is 6p22.3, which harbors three lncRNAs *CASC15-003*, *CASC15-004* and *NBAT1* (6p22 lncRNAs). 6p22 lncRNAs show decreased expression in high-risk NB and their lower expression correlates with poor disease outcome. Previously, we demonstrated that 6p22 lncRNAs regulate differentiation of the NB cells by regulating REST/NRSF and

SOX9/CHD7/USP36 pathways, and that their interplay determines undifferentiated state which is a major hall mark of NB.

Aims

6p22 lncRNAs in the regulation of oncogenic/tumor pathways that drive NB tumor aggressiveness

Methods/Materials

Tissues from NB patients and NB cell lines were used in the study.
Methods used in this study: Chromatin immunoprecipitation (ChIP), Luciferase assay, Immunocytochemistry (ICC), NB cell line derived xenografts, immunoblot, RNA extraction, sequencing and transcriptomic analysis, Proximity ligation assay (PLA), Immunoprecipitation and Ubiquitination assays.

Results

In the current study we have explored how the lower expression of 6p22.3 locus encoded lncRNAs influences p53 and MYCN dependent pathways that may contribute to tumor aggressiveness.
NB has low mutation rate for the p53 gene. Alternative ways of p53 inactivation have been proposed in NB via an abnormal cytoplasmic accumulation of wild-type p53. However, mechanisms leading to p53 inactivation via cytoplasmic accumulation are not well investigated. We show that NB risk-associated 6p22.3 locus derived tumor suppressor lncRNA *NBAT1* is a p53 responsive lncRNA and regulates p53 sub-cellular localization. Low *NBAT1* expression provides resistance to genotoxic drugs by promoting cytoplasmic p53 accumulation. Altered CRM1 function in the *NBAT1* depleted cells contributes to p53 cytoplasmic accumulation and the loss of p53 dependent nuclear gene expression during genotoxic drug treatment. CRM1 inhibition rescues p53 dependent nuclear functions in the *NBAT1* depleted cells and sensitize the cells to genotoxic drugs. Combined inhibition of CRM1 and MDM2 was even more effective in sensitizing aggressive NB cells with p53 cytoplasmic localization.
Interestingly, we found that the levels of 6p22lncRNAs also correlate with MYCN protein levels. Overexpression of 6p22 lncRNAs in MYCN amplified cell lines and non-amplified cell lines leads to decreased MYCN protein levels whereas their KD resulted in stabilization of MYCN. We show that 6p22lncRNA and its common interacting protein USP36, a ubiquitin-specific protease, determine MYCN levels. ICC and Co-IP studies reveal MYCN interaction with USP36 and their colocalization at the nucleolus, respectively. Importantly, we have characterized 6p22 lncRNAs/USP36/MYCN pathway regulated genes that can contribute to MYCN dependent NB disease progression.

Summary/Conclusions

This study, using NB as a disease model system, has uncovered how lncRNA mediated oncogenic and tumor suppressor pathways converge to regulate cancer progression. Of note, our mechanistic studies of 6p22 lncRNA/p53/CRM1 propose a novel combination therapy for high-risk NB.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 333

Clinical response from entrectinib and ALK-TRK-ROS1 inhibition in treatment-refractory metastatic neuroblastoma with tumor ALK activation and germline ALKAL2 mutation

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

Activation of the receptor tyrosine kinases ALK or TRKA/B/C is indicted as an oncogenic event in a fraction of neuroblastomas. Their inhibitors are not routinely used in children with neuroblastoma, but have proven very beneficial in some.

Aims

We report clinical response in a patient treated with the ALK-TRK-ROS1 inhibitor entrectinib after failing five lines of conventional clinical chemotherapy.

Methods/Materials

A six-month-old previously healthy boy presented with vomiting, weight loss, abdominal mass and high arterial blood pressure. He was found to have a poorly differentiated neuroblastoma without MYCN amplification but with genetic aberrations including 17q-gain, 1p-deletion, and 2q-gain (involving MYCN, ALK and ALKAL2). There were extensive hepatic, pulmonary, and bone

metastases. Bone marrow showed 0.1–0.3% involvement. Urine catecholamines were extremely elevated and hypertension necessitated triple pharmacological therapy.
The patient failed to respond initial intermediate-risk treatment (LINES, four courses etoposide/carboplatin followed by four courses cyclophosphamide/doxorubicin/vincristine). COJEC (HR-NBL-1/SIOPEN) did not induce remission but increasing bone marrow involvement. After primary tumor extirpation 9.5 months from diagnosis, metastases remained viable. Celecoxib (220mg/m2 x2 p.o.) with four courses topotecan/vincristine/doxorubicin followed by two courses temozolamid/topotecan induced only limited incomplete response, remaining extensive metastatic disease and elevated catecholamine markers.
Tumor tissue stained positive for ALK and phosphorylated ALK demonstrating ALK activation, whereas sequencing showed wild-type ALK. However, a constitutional mutation in the ligand ALKAL2 was detected. Moreover, pronounced Trk-activation was detected. To target both RTK-activations, experimental treatment was started with the ALK-TRK-ROS1 inhibitor entrectinib at initial oral dose 200mg/day, increasing to 300mg and 400mg after 10 and 29 months, respectively.

Results

The child is alive and well after 39 months of continuous still ongoing oral therapy with entrectinib and celecoxib. General condition and tumor symptoms improved rapidly while extremely elevated urine catecholamines have gradually decreased to normal or near-normal levels. Despite excellent clinical condition, core-needle biopsy of liver metastases still showed viable poorly differentiated tumor two months after treatment start. However, metastatic extent (number and size) in liver and lungs has continued decreasing.
Entrectinib is well tolerated and pathologic tibiae and fibulae fractures are attributed to previous chemotherapy. Growth, development and social life are completely normal 4.5 years from diagnosis.

Summary/Conclusions

We report a patient with refractory metastatic neuroblastoma who, in the setting of exhausted therapeutic options, has responded favorably reaching a stable clinical situation from ALK-TRK-ROS1 inhibitor, entrectinib, treatment. To our knowledge, this is the first successful report of ALK inhibitor response in ALK activation possibly mediated by ALK-ligand, ALKAL2, mutation.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 334

ADRA1B as a novel pharmacological target for neuroblastoma

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Topic Gene regulatory networks and epigenetics: from basic science to therapy

Background/Introduction

High-risk neuroblastoma (NB) is an aggressive tumour with a survival rate below 50%. A drug combination strategy can provide new answers to this unmet therapeutic need. 13-cis retinoic acid (13-cisRA) is an anti-proliferative and pro-differentiative agent used in the post-consolidation phase of NB therapy, while naturally occurring polyphenols have been shown to exert anti-proliferative effects in several cancer cell lines. To identify molecules able to potentiate the activity of 13-cisRA, we screened a library of 169 natural compounds for their effects on the viability of the NB cells CHP134 challenged with a sub-lethal dose of 13-cisRA. The i*n vitro* screening identified *isorhamnetin* as a synergistic molecule leading to 80% reduction in cell viability.

Aims

The project aims to identify the molecular basis of the synergism 13cis-RA and isorhamnetin, in order to explore its possible role as a novel therapeutic target for neuroblastoma treatment.

Methods/Materials

We generated NB cell lines knocked out for ADRA1B. We used two NB cell lines: CHP134, sensitive to 13cis-RA, and SK-N-AS, which are known to be unresponsive the retinoid. Cells were treated with 13-cisRA alone or in combination with isorhamnetin. The cell response was evaluated via metabolic assay, apoptosis assay, differentiation assay, and gene expression analysis.

Results

We found that the synergistic effect of 13cis-RA and isorhamnetin is accompanied by a marked increase in the expression of the catecholamine receptor adrenergic receptor alpha-1B (ADRA1B). The exposure of CHP134 ADRA1B KO cells to 13-cisRA provokes a reduction of cell viability, accompanied by an activation of Caspase3/7, comparable with what occurs in WT cells treated

with both 13cis-RA and isorhamnetin. Furthermore, CHP134 KO cells challenged with 13-cisRA show an increase in the percentage of neuritis protrusion, hallmark of neural differentiation, confirmed at the molecular level by the rise of the expression of neural differentiation markers β3-Tubulin and tyrosine hydroxylase. Nonetheless, we detected a dramatic overexpression of the transcription factor SOX9 both at mRNA and protein level, followed by a coherent promotion of HES1 mRNA transcription. None of these markers resulted to be induced in SK-N-AS NB cells exposed to 13-cisRA, neither if we KO ADRA1B. Consistently, SK-N-AS cells do not show neurites protrusion. Testing the effect of combining the alpha-adrenergic antagonist, L765,314, with 13-cisRA, we observed a decrease of cells viability in CHP134 WT cells in line with that of KO cells exposed to the retinoid.

Summary/Conclusions

We can conclude that the deletion of ADRA1B sensitizes NB cells to 13-CisRA in terms of induction of apoptosis and neural differentiation. Since NB is a catecholamine-rich tumor, we propose that the interruption by KO of ADRA1B of the pro-proliferative circuit generated by catecholamines in NB restores in cells the ability to undergo the pro-differentiative and pro-apoptotic programs promoted by 13-CisRA and mediated by activation of SOX9, which has already been reported to be necessary to 13-cisRA to exert its effects in human breast cancer cell lines. Considering the druggable nature of the ADRA1B receptors, we indicate ADRA1B as a novel pharmacological target for treating neuroblastoma.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 335

Developmental and oncogenic programs in neuroblastomas dissected by single-cell analysis

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Topic Neuroblastoma as developmental disease

Background/Introduction

Neuroblastoma arises in the adrenal medulla and paraspinal sympathetic ganglia, suggesting that tumors develop from precursors of adrenal chromaffin cells and sympathetic neurons. However, the definitive cells of origin remain unknown. Two types of neuroblastoma tumor cells with different gene expression profiles, committed adrenergic cells and mesenchymal cells, have been described. The two subtypes are regulated by distinct transcription factor networks and are thought to resemble cells from different lineages probably arising from divergent cells of origin. Since this current view on neuroblastoma cellular heterogeneity is exclusively based on bulk sequencing studies, single-cell techniques are needed for an in-depth analysis of intratumoral heterogeneity.

Aims

The aim of this project is to understand the cellular composition of neuroblastoma tumors and identify putative neuroblastoma cells of origin. In addition, we investigate influences of oncogenic pathway activation on lineage identity and differentiation.

Methods/Materials

To approach these aims, we profiled neuroblastoma tumors and normal human adrenal glands from five time points during embryonic and fetal development by single-nucleus RNA-seq using the 10x Genomics Chromium Single Cell 3' and Smart-seq2 protocols. In total, about 35.000 cells from healthy pre-natal human tissues and 60.000 cells from neuroblastoma tumors, including four low risk tumors, five tumors with amplification of the oncogene *MYCN*, four tumors with overexpression of the oncogene *TERT* and one tumor with alternative lengthening of telomeres were analyzed. We further studied neuroblastoma cell lines by MARS-seq and assessed transcriptional changes upon deregulation of oncogenic signaling pathways like the RAS pathway.

Results

Using a shared nearest-neighbor algorithm, we segregated transcriptomes from healthy human adrenal gland cells into distinct clusters. Comparison of cluster-defining marker genes to canonical cell type markers allowed the assignment of cell identities to each cluster. Adrenal medullary cells included sympathetic neuroblasts, chromaffin cells and Schwann cells during different stages of maturation. Pseudotime analysis captured the underlying dynamics of cellular differentiation. In addition, we identified adrenal cortical cells, vascular endothelial cells, immune cells and stromal cells.

To classify single cells from neuroblastoma tumors into malignant and non-malignant, we determined copy number variations (CNVs) on the basis of the scRNA-seq data and additionally clustered cells from all tumor samples based on their transcriptomes. In contrast to malignant cells, which showed copy number alterations and clustered primarily by patient, non-malignant cells from multiple patients clustered together, showed no CNVs and/or expressed markers of immune cells, vascular endothelial cells, stromal cells or Schwann cells.

To relate neuroblastoma single-cell profiles to normal cell types, we projected single malignant cells onto healthy cells of the adrenal medulla. The majority of tumor cells showed the highest similarity to normal neuroblasts. However, a subset of malignant cells within the same tumor mapped to chromaffin or Schwann cells, suggesting that tumors contain a mixed population of cells with divergent differentiation.

Summary/Conclusions

In conclusion, we identify the cell types that constitute the healthy adrenal medulla and their differentiation dynamics during embryonic development. Comparison of the transcriptome of single neuroblastoma tumor cells to these normal cell types identifies similarities of neuroblastoma tumors to normal developmental hierarchies.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 336

Linking telomere structures and processing to neuroblastoma tumor cell differentiation and malignancy

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Topic Neuroblastoma as developmental disease

Background/Introduction

Neuroblastoma (NB) tumor cells were previously shown to manifest three distinct neural crest differentiation states: neuroblastic (N-type), substrate-adherent non-neuronal (S-type), and intermediate (I-type). These lineage-specific cell types can inter-convert and the resulting phenotypic diversity appears to contribute to tumor progression and relapse. Recent studies also showed that telomere maintenance mechanisms (TMMs) are critical for high-risk NB and positive TMMs are strong prognostic indicators for poor outcomes. However, the impacts of telomeres and TMMs on the development/differentiation of neural crest and neuroblastoma cells are poorly understood.

Aims

Profile telomere structures in NB tumors/cell lines and normal neural tissues to identify telomere features in NB that are related to its developmental program.
Analyze telomere structures, TMMs, and telomere protein compositions in matched N, S and I clones to explore the roles of telomeres in NB cell differentiation.

Methods/Materials

Previous studies of NB telomeres were mostly limited to the determination of average telomere lengths and telomerase activity. We conducted a detailed survey of telomeres in 5 cell lines and 30 surgical samples from high-risk NB patients using a combination of assays that assess different aspects of telomere structure and processing (Clinicaltrials.gov, NCT00588068) with IRB aproval. Most importantly, the levels of telomere trimming activity (a telomere shortening pathway) was examined using in-gel hybridization and C-circle assays. We also used STELA, a PCR-based assay that captures individual telomeres, to gain a high-resolution view of telomere length distributions. The results from NB samples were compared to those from normal neural tissues. The same telomere assays were applied to a series of longitudinal tumor samples from individual

patients to identify aspects of telomere structures or processing that are related to tumor progression.
The telomere protein compositions and telomerase activity in matched N, S and I cells were compared by Western and TRAP assays.

Results

We discovered robust “telomere trimming” activity (evidenced by high levels of C-strand single-stranded DNA and low levels of C-circles) in NB samples and normal neural tissues, but not in other cancers. This suggests that telomere trimming is active in normal neural development and is retained in the majority of NB tumors. Furthermore, we found that the trimming activity tends to diminish during tumor relapse, suggesting that tumors with low trimming activity may be more resistant to therapy. Hence, TMMs may be especially critical for in high-risk NB tumors growth by countering a developmental program of telomere shortening. We also observed strong connections between telomeres and the morphologically distinct, lineage-specific NB cells. Specifically, in comparison to highly malignant N-type cells, S-type cells consistently manifested low levels of telomerase activity and telomere proteins. Conversion of I-type cells to S-type cells through BrdU treatment also triggered reductions in telomerase activity and telomere proteins. Our data indicate that telomere remodeling is tightly linked to the developmental and differentiation pathways in NB tumor cells.

Summary/Conclusions

Our observations reinforce crucial connections between telomeres, NB tumor cell differentiation, and tumor malignancy. Better understanding of the mechanistic basis of these connections may lead to new biomarkers and therapies for NB.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 337

The therapeutic potential of ERK inhibition in neuroblastoma

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Topic Other

Background/Introduction

Neuroblastoma (NB) is the most common extracranial malignant solid tumor in children and drug resistance is a major reason for poor outcome of neuroblastoma. Dysregulation of MAPK pathway has been implicated in the pathogenesis of relapsed and refractory NB patients, which underscores the possibility of targeting MAPK signaling cascade as a novel therapeutic strategy. Ulixertinib is a small-molecule ERK1/2 inhibitor and assessment of Ulixertinib in clinical trials is underway in many malignancies.

Aims

To evaluate the effect of ERK inhibition using Ulixertinib on neuroblastoma growth.

Methods/Materials

We first examined the effect of Ulixertinib on NB cell proliferation using a panel of NB cell lines including three MYCN-amplified cell lines (NGP, IMR32, and SK-N-BE2) and four non-MYCN-amplified cell lines (CHLA255, SH-SY-5Y, SK-N-AS, and LAN-6). 2.0×10^4 cells were seeded in each well of a 96-well plate and treated with serial dilutions of Ulixertinib for 48 hours, and cell viability was determined using Cell Counting Kit-8 (CCK-8).Then the soft agar assay was performed to test the inhibitory effect of Ulixertinib on NB cell anchorage-independence growth. A CHLA255 xenograft mouse model was used to determine the effect of Ulixertnib on tumor growth. CHLA255-Fluc cells at the concentration of 5×10^6 were injected into NSG mice by tail vein. A week post-injection, the mice were given Ulixertnib 50 mg/kg by intraperitoneal injection every two days. Tumor growth was monitored weekly by bioluminescent imaging.

Results

Ulixertinib inhibited ERK1/2-mediated signaling and suppressed cell proliferation by inducing cell apoptosis. Furthermore, Ulixertinib significantly inhibited NB tumor growth *in vivo*.

Summary/Conclusions

Ulixertinib may be a potential therapeutic agent for NB treatment.

Type Translational

Presentation Preference Traditional poster

Abstract nr. 338

Antibody-drug conjugate efficacy in neuroblastoma

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

The efficacy of antibody-drug conjugates (ADCs) in neuroblastoma relies on several target independent (payload sensitivity, drug transporter expression, drug resistance mutations) and target dependent (cell surface target density, antibody/target internalization kinetics) factors.

Aims

Here we aim to define the complex interplay of the multiple elements driving the efficacy of ADCs in neuroblastoma using the GPC2-directed D3-GPC2-PBD ADC (*Cancer Cell*, *2017*) as an example.

Methods/Materials

Drug transporter expression, drug resistance mutations, GPC2 cell-surface density, D3-GPC2-IgG1 internalization kinetics and ADC payload sensitivity were quantified in a panel of 11 neuroblastoma cell lines utilizing RNA/DNA sequencing, flow cytometry, a fluorescent D3-GPC2-IgG1 antibody and ADC payload IC_{50} assays. Payloads tested included tubulin (MMAE, DM1, DM4, dolastatin, HTI-286) and DNA (PNU-159682, PBD, duocarmycin, and N-acetyl-calicheamicin γ1) interacting drugs. Cell lines were also treated with the D3-GPC2-PBD ADC to define the role of target molecule expression and antibody internalization kinetics in ADC efficacy.

Results

Neuroblastoma cell lines were significantly more sensitive to DNA vs. tubulin interacting ADC payloads (n=4 vs. 5; mean IC_{50} = 48 pM, range = 14 - 79 pM vs. mean IC_{50} = 1,453 pM, range =

137 - 2,784 pM, $p = 0.04$). PNU-159682 was the most potent payload overall (mean IC_{50} = 14 pM, range 3.2 - 44 pM), followed by N-acetyl-calicheamicin γ1 (mean IC_{50} = 37 pM, range 5-116 pM) and duocarmycin (mean IC_{50} = 61 pM, range 23 - 170 pM). The most potent tubulin interacting payload was dolastatin (mean IC_{50} = 137 pM, range 44 - 435 pM). DNA interacting payload treatment induced upregulation of γH2AX, cleaved PARP and cleaved caspase-3 consistent with on-target DNA damage and apoptosis. MDR1 overexpression and *TP53* mutations explained some of the ADC payload resistance patterns observed across this panel which will be reported. D3-GPC2-IgG1 antibody internalization was significantly correlated with GPC2 cell-surface density (r = 0.7, $p = 0.02$) but not with D3-GPC2-PBD IC_{50} (r = - 0.1, $p = 0.55$). In fact, cell lines that were sensitive to PBD (n = 7 of 11; PBD IC_{50} <40 pM), with over a 6-fold-range in D3-GPC2-IgG1 internalization, all responded robustly to the GPC2 ADC (IC_{50} range 1 - 6 pM), except the very low D3-GPC2-IgG1 antibody internalizing NBLS cell line (mean IC_{50} >5,000 pM). However, the CHP134 cell line similarly internalized a low amount of antibody, yet was remarkably susceptible to PBD (mean IC_{50} = 6 vs. 27 pM for NBLS), and thus was also sensitive to the ADC (mean IC_{50} = 1.6 pM). Conversely, the NB-1643 cell line internalized the greatest amount of the D3-GPC2-IgG1 antibody and was only modestly susceptible to PBD (mean IC_{50} = 49 pM), yet was also very sensitive to the ADC (mean IC_{50} = 3 pM). Taken together, these data reveal the critical interplay between antibody internalization and payload sensitivity in ADC efficacy.

Summary/Conclusions

DNA interacting ADC payloads are significantly more cytotoxic to neuroblastoma cells than payloads that bind tubulin. Both target dependent and independent elements contribute to the efficacy of ADCs in neuroblastoma.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 339

Generation of patient-specific iPSC from patients with familial neuroblastoma.

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Topic Other

Background/Introduction

Neuroblastoma (NB) is a developmental disorder whose biology remains poorly understood. Clinical models are essential in gaining a full understanding of NB biology and creating better therapies. One potential approach is using patient-specific induced pluripotent stem cells (iPSCs) derived from patients with familial NB for *in vitro* modelling of the disease by directed differentiation. Leveraging a precision medicine pipeline and the resources of the Paediatric Translational Medicine Program at Memorial Sloan Kettering Cancer Center (MSKCC), and in collaboration with the Developmental Biology Program and Stem Cell Research Core Facility at MSKCC, we prospectively identified four families with germline mutations responsible for familial NB and generated four patient-specific iPSC lines.

Aims

Using the existing infrastructure at MSKCC, we aim to develop a pipeline to generate iPSCs from paediatric cancer patients, including NB patients, harbouring mutations of interest. This will lay the foundations for a biobank consisting of rigorously validated IPSC lines with prospectively shown, relevant mutations for further paediatric cancer research.

Methods/Materials

After obtaining Institutional Review Board approval, we reprogrammed peripheral white blood cells from four different familial NB patients with the following mutations: TP53 R248Q, PHOX2B p.G197D-BDNFp.R209L, PHOX2B c.234_240delCGCCGCA and ALK R1275Q. The generated iPSC lines were validated using short tandem repeat testing, shallow Whole Genome Sequencing and targeted sequencing of a panel of 468 cancer-related genes (MSK IMPACT) for lineage tracing and analysis of chromosomal aberrations and mutations, respectively. The pluripotency of the iPSC lines was confirmed using FACS analysis for stem cell markers (i.e., OCT3/4 and SOX2) and tri-lineage differentiation. After validation, the developed cell lines will be submitted to the cell repository in the Antibody and Bioresource core facility at MSKCC.

Results

To date, we have successfully generated and validated four different iPSC lines. STR analysis showed correct lineage tracing. sWGS and IMPACT testing verified the presence of the mutation of interest as well as lack of co-mutations or copy number variations. Pluripotency testing using FACS analysis for OCT3/4 and SOX2 confirmed reprogramming to the stem cell state. Further proof of pluripotency is ongoing by performing tri-lineage differentiations. The developed iPSC lines will be used for *in vitro* differentiation experiments to elucidate the biology of NB development. The two PHOX2B mutations reported here represent novel mutations not previously described in familial NB and illustrate the potential of this resource to explore normal differentiation and cancer development.

Summary/Conclusions

We have developed a pipeline to create a biobank of iPSC lines carrying prospectively validated mutations of interest. These resources will be made available to interested researchers and will allow researchers to use these generated iPSC lines to further investigate the impact of a mutation-of-interest on development and thus identify essential signalling pathways and/or test promising new therapies. Prospective identification of patients with pathogenic germline mutations is on-going, and additional iPSC lines will be generated and made available to the research community as a resource for the study of NB and other paediatric cancers.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 340

Response to Post Induction Therapy prior to Autologous Stem Cell Transplant is Prognostic of Outcome in Patients with Refractory High Risk Neuroblastoma

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

Outcome for high-risk neuroblastoma (HRNBL) patients with refractory disease at end of induction (EOI) is poor. It is unknown if additional therapies such as I-131-Metaiodobenzylguanidine (MIBG) or combination therapy with irinotecan, temozolomide, and dinutuximab (I/T/DIN) prior to autologous stem cell transplant (ASCT) will improve outcome.

Aims

The primary study aim is to determine the 3-year event-free survival (EFS) and overall survival (OS) of patients with HRNBL who had refractory disease at EOI treated with versus without post-induction therapy prior to ASCT.

Methods/Materials

A retrospective chart review of HRNBL patients diagnosed between 2008 and 2018 and found to have refractory disease at EOI was conducted at seven centers. Demographics, tumor biology, treatment response, and outcomes were abstracted. Response was assessed using the 2017 International Neuroblastoma Response Criteria (INRC). The Kaplan-Meier method was used to assess 3-year EFS and OS from the time of diagnosis.

Results

Three-year EFS and OS were 54% and 79% for the cohort (n=136). Ninety-one patients received no additional therapy prior to ASCT (Cohort 1); 32 patients received post-induction therapy prior to ASCT (Cohort 2); and 13 patients did not undergo ASCT (Cohort 3). Three-year EFS and OS were not statistically significantly different between Cohort 1 (3-year EFS and OS; 62% and 81%) and Cohort 2 [3-year EFS and OS; 49% (p=0.48) and 82% (p=0.19)]. Outcome for Cohort 3 patients was significantly worse than Cohort 1 [3-year EFS: 15% vs. 62% (p<.001) and 3-year OS: 48% vs. 81% (p=0.003)] and Cohort 2 [3-year EFS: 15% vs. 49% (p<.001) and 3-year OS 48% vs. 82% (p=0.035)]. For Cohort 2 patients, post-induction therapy for the 31 patients with metastatic disease at EOI included I/T/DIN (n=12), MIBG (n=16), MIBG plus I/T/DIN (n=1), and other (n=2). Improved response in metastatic sites was observed in 10 of the 12 (83%) patients who received I/T/DIN and 9 of the 16 (56%) who received MIBG. Two additional patients responded to MIBG plus I/T/DIN (n=1) or MIBG with chemotherapy (n=1). Among the 21 patients with metastatic disease response to post-induction therapy, 3-year EFS and OS were 69% and 94%; significantly better than Cohort 2 patients who did not respond to post induction therapy [3-year EFS and OS: 11% (p=0.016) and 66% (p=0.2)]. Six patients achieved a complete response (CR) in metastatic sites following I/T/DIN (n=5) or MIBG (n=1) prior to ASCT, and all are alive without relapse with median follow-up of 3.4 years.

Summary/Conclusions

No difference in EFS or OS was detected in the overall cohort of HRNBL patients with refractory disease at EOI treated with versus without post-induction therapy prior to ASCT. However, outcomes were significantly better for patients whose metastatic disease responded to post-induction therapy compared to those whose disease did not respond. Excellent survival was observed in the small cohort of patients who achieved a CR in metastatic sites prior to ASCT. Additional prospective studies testing the efficacy to I/T/DIN prior to ASCT in patients with refractory metastatic disease at EOI are warranted.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 341

Tackling MHC class I downregulation to develop effective immunotherapy for neuroblastoma

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

Neuroblastoma is the most common pediatric solid tumor and responsible for about 15% of all pediatric cancer deaths. The majority of high-risk patients suffers from relapse after intense therapy regimens, resulting in a 5-year survival rate of only 40%. Even though the potential of immune interference in neuroblastoma is shown by the additive effect of anti-GD2 monoclonal antibody therapy to the treatment protocol, long-term follow-up studies reveal that the beneficial effect of immunotherapy diminishes over time. We hypothesize that this is a result of inadequate adaptive immune engagement caused by the extensive immunomodulatory capacity of neuroblastoma and its microenvironment. One of the most remarkable immunomodulatory strategies of neuroblastoma tumors is the downregulation of MHC-I surface expression, thereby preventing cytotoxic T cell recognition and killing. MHC-I lacking cells are known to be subjected to NK-cell mediated cytotoxicity, however, we show that neuroblastoma is able to evade this by temporary upregulating surface expression of MHC-I, thereby becoming more prone to T-cell mediated cytotoxicity. Addition of MHC-I upregulatory cytokines to neuroblastoma cells results in improved MHC-I expression and T-cell cytotoxicity, however, the drawback of toxicity diminishes the potential of these cytokines in therapeutic strategies. Therefore, we are aiming to identify FDA-approved compounds with similar effects on MHC-I expression. Pharmaceutical upregulation of MHC-I expression could foster adaptive anti-tumor immunity in neuroblastoma patients.

Aims

To screen FDA-approved drug libraries to identify MHC-I upregulatory compounds to increase neuroblastoma-specific T-cell recognition and killing.

Methods/Materials

We have developed a high-throughput flow cytometry antibody staining protocol for adherent cells which we have applied to screen FDA-approved drug libraries to identify compounds affecting

MHC-I surface expression in neuroblastoma cell lines. Libraries have been screened at a concentration range between 0.1 nM and 20 µM. Observed effects were validated, after which neuroblastoma cell lines have been co-cultured with PRAME reactive tumor-specific T-cells in presence/absence of identified compounds to determine the effect of the observed MHC-I upregulation on T-cell cytotoxicity *in vitro*.

Results

~1200 FDA-approved compounds have been screened on their effect on MHC-I surface expression in neuroblastoma cell lines, which resulted in the identification of 21 potential MHC-I upregulatory compounds. The upregulatory effect of two classes of drugs, topoisomerase inhibitors and DNA demethylating agents, have been studied and confirmed in more detail. We show that neuroblastoma cells treated to increase MHC-I cell surface expression show increased susceptibility to PRAME-reactive tumor-specific T-cell mediated cytotoxicity as compared to untreated controls.

Summary/Conclusions

We show that pharmacological upregulation of MHC-I can be achieved with cellular treatment of topoisomerase inhibitors and/or DNA demethylating agents. MHC-I upregulation in neuroblastoma leads to improved T-cell mediated cytotoxicity *in vitro* and is a potential strategy to improve adaptive immune engagement and therewith immunogenicity of neuroblastoma.

Type Translational

Presentation Preference Oral presentation

Advances in Neuroblastoma Research (ANR) Meeting
ANR 2020
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Abstract nr. 342

PARP and CHK1 inhibitors synergize in killing MYCN amplified neuroblastoma via an ATM-dependent pathway

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

MYCN amplification (MNA) is strongly correlated with poor prognosis and treatment failure in patients with neuroblastoma. Despite intense multimodal therapies, about 50% of these patients succumb to their disease, making the search for effective and less toxic therapies an absolute priority.

Activation of oncogenes such as MYCN is associated with high replication stress (RS) and DNA damage, making MYCN-driven tumors addicted to the activity of molecules involved in RS and DNA damage responses. Prompted by the relevant role of PARPs in controlling RS and DNA repair, we previously demonstrated that olaparib (a pharmacological inhibitor of PARP) enhanced MYCN-induced RS and caused cell death via mitotic catastrophe, in high-risk MNA and overexpressing neuroblastoma cells. Inhibition of the CHK1-dependent S-phase checkpoint raised by olaparib anticipated and increased the occurrence of mitotic catastrophe.

Aims

Together with the observation that both PARP and CHK1 expression is significantly increased in MNA compared to MYCN single copy (MNSC) neuroblastomas, our data suggested that MYCN-induced RS could make MNA neuroblastoma strongly addicted to PARP and CHK1 activity, providing a rationale to test their combined inhibition for therapeutic purposes.

Methods/Materials

We used a large panel of neuroblastoma cell lines to address the effectiveness of multiple PARP and CHK1 inhibitors to induce DNA damage, cell growth inhibition and cell death, as measured by comet assay, MTS, trypan blue exclusion test, immunoblot assay and mRNA quantification. For *in vivo* experiments, mice were subcutaneously or orthotopically injected with IMR32 cells and treated with vehicle, olaparib, the CHK1 inhibitor MK-8776 or their combination. Tumor growth was measured by caliper or bio-luminescent imaging. Genetic correction of mutant ATM in IMR32 cells was obtained by CRISPR/Cas9 technology.

Results

PARP inhibitors sharply increased the sensitivity of neuroblastoma cells to CHK1 inhibitors. The efficacy of this combination was far higher in MNA cell lines than in MNSC cells, *in vitro*. Indeed, the MK-8776+olaparib combination led to accumulation of DNA damage and cell death in a synergistic and MYCN-dependent manner, allowing the use of suboptimal doses of MK-8776. Consistently, suboptimal doses of MK-8776 plus olaparib significantly reduced the growth of MNA neuroblastoma subcutaneous and orthotopic xenografts, and the growth of a SHH-MYCN medulloblastoma model, with no major toxicities.
IMR32, the most responsive cell line, bears a putatively pathogenic ATM mutation. Since it has been reported that genetic loss of homologous recombination repair genes, including ATM, may predict sensitivity to PARPi, in the 11q-deleted neuroblastoma, we asked whether ATM was involved in the responses to PARPi+CHK1i combination in this MNA neuroblastoma cell line. CRISPR/Cas9-based genetic correction of mutant ATM showed that this mutation did not fully abolish ATM kinase function and was irrelevant for the biological responses to PARPi+CHK1i combination. Rather, ATM pharmacological inhibition revealed that its kinase activity was required for the proapoptotic effect of PARPi+CHK1i combination, in MNA neuroblastoma cells.

Summary/Conclusions

Our data highlight a new potential chemo-free strategy to treat MNA neuroblastoma and shed light on the involvement of ATM in shaping cell responses to PARPi+CHK1i in the MNA background.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 343

Long-term evaluation of a combined polychemotherapy, MIBGI131 in vivo purging, autologous bone marrow transplantation (ABMT) and dendritic cell vaccine (DCV) program for the treatment of high-risk (HR) neuroblastomas (NB)

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

HR NB represent an enormous problem for Pediatric Oncology. The impact of combinations including platinum derivatives (PD), the value of stem-cell (SC) transplants and more recently of anti-GD2, are the most significant steps for their treatment. Not all resources are easily available for institutions throughout the world. To stablish a program that can truly add potential curative

objectives, considering all these difficulties, is a true challenge.

Aims

To provide a feasible and useful program for Brazilian institutions, considering the disparity of resources existent within the country.

Methods/Materials

From 2000 to 2017, 439 children with NB were referred for treatment to the Institute for the Treatment of Pediatric Cancer (ITACI). 150/439 (34%) had been previously treated in other institutions. Only 357/439 (81.30%) had enough data allowing adequate analysis. This study enclosed 252/357 (70.6%) children over 18 months of age (median: 34.5 months) at diagnosis, either stage 4 or 3 with regional node involvement and/or amplified *MYCN*. 27 months was their median follow-up. Treatment: i = induction with cycles of: A) Topotecan/Cyclophosphamide; B) Vincristine/Cyclophosphamide/Adriamycin & Carboplatin/Etoposide (up to 2010: A-A-B-B-B- B; after 2010: A-A-B-B-A-A-B-B); ii = delayed surgery (primary site); iii = collection of peripheral blood SC (starting in 2002, after previous exposure to therapeutic MIBGI^{131} [10 mci/kg, as previously defined in a phase I study, allowing enough peripheral stem-cell collection {PBSCC}, capable of recovering marrow function]); iv = ABMT, conditioning regimen until 2012: Carboplatin/Etoposide/Melphalan; Busulfan/Melphalan afterwards); v = individualized radiotherapy; vi = cis-retinoic acid and; vii = DCV (tumor cells from the patient's tumor and allogeneic dendritic cells). Response criteria: at the time of PBSCC the children should have: absence of any sign of progressive disease (PD), absence of bone marrow (BM) involvement (9 BM aspirations & 2 biopsies & negative immunohistochemical study for NB84 and synaptophysin), normal lactate dehydrogenase levels and total absence of progression in images. Exclusion criteria: any sign of PD at the time of PBSCC.

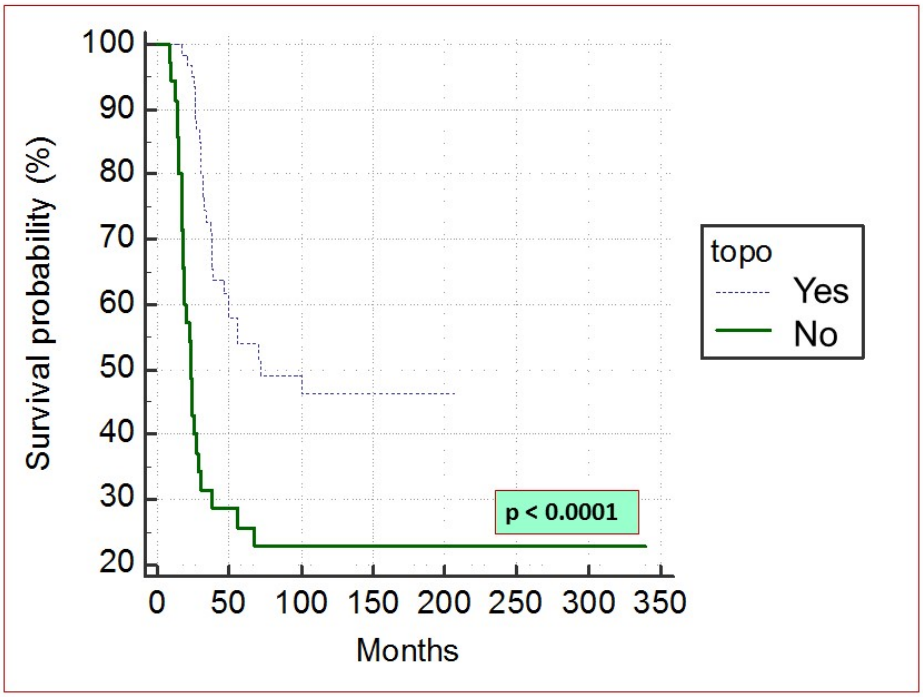
Results

Overall results revealed (all patients had PD): 35.9±5.08%, without significant interferences with the use of ABMT or not and, for the transplanted patients, the use of MIBG purging or DCV. The only significant difference found was regarding the favorable use of CYCLOPHOSPHAMIDE/ETOPOSIDE (figure 1). When the analysis was restricted to patients entirely followed at ITACI, the overall survival was of 55.8±7.65% for the patients who did not experience either recurrences or progression during their management, without any change of the original chemotherapy program, compared to the ones who had modifications in their original programs due to progression or recurrences, before the point where ABMT was to be done (figure 2).

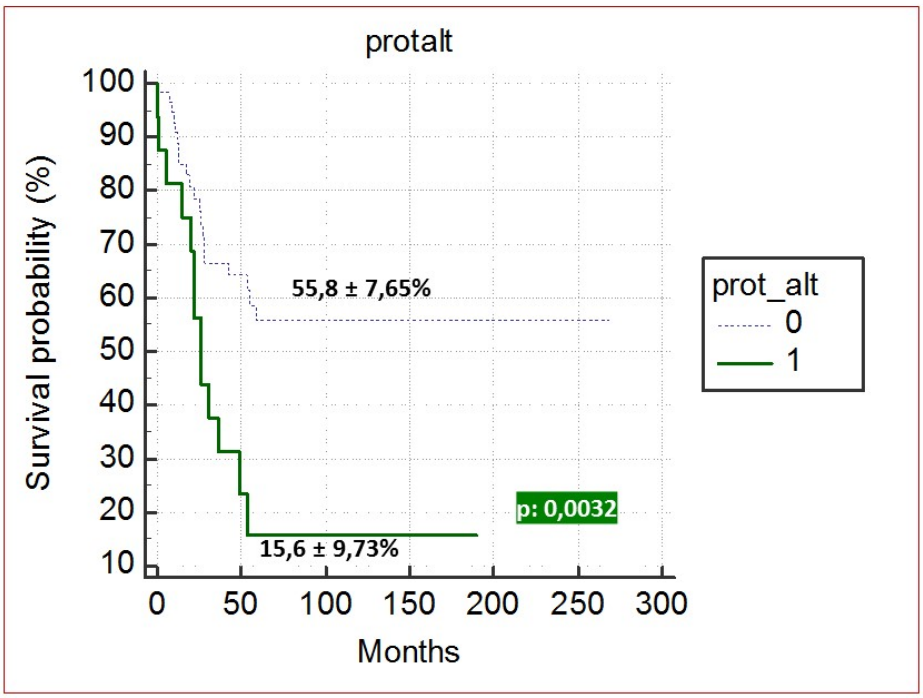
Summary/Conclusions

The above described program may be able to confer results parallel to the best ones in the literature. However, the objective of a general application in Brazil revealed impossible, being essentially restricted to one or very few institutions. Although the definite role of each component of the program cannot be established, DCV are to be maintained for future propositions. The regular addition of anti-GD2 is the key point of these new possibilities.

OS, PATIENTS AFTER ABMT, CYCLO + TOPO X NO



OS, ABMT, up-front treatment X alternative chemo, single institution treatment



Type Clinical

Presentation Preference Oral presentation

Abstract nr. 344

Delayed local treatment and Busulfan/Melphalan as a myeloablative chemotherapy for high-risk neuroblastoma (JN-H-15): a report from Neuroblastoma Committee of Japan Children's Cancer Group (JCCG) Trial

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

Neuroblastoma Committee (JNBSG) of Japan Children's Cancer Group (JCCG) conducted a phase II nation-wide clinical trial, JN-H-15, for high-risk neuroblastoma. This protocol was characterized by "delayed local treatment" in which tumor resection was performed after completing all chemotherapeutic courses including myeloablative high-dose chemotherapy (HDC), and by using busulfan/ melphalan (180 mg/m^2) as HDC.

Aims

To reveal the feasibility and effectiveness of JN-H-15 protocol.

Methods/Materials

Between February 2015 and March 2018, 66 patients with high-risk neuroblastoma enrolled. One patient was excluded because of misdiagnosis of staging. All were diagnosed pathologically according to the central review as ganglioneuroblastoma (7), neuroblastoma poorly differentiated (57) and neuroblastoma undifferentiated (2). The construction of JN-H-15 protocol was similar to previous JN-H-11 protocol except for the following courses; two courses of 05A3 regimen in the induction therapy were substituted to two courses of ICE regimen, and MEC regimen for myeloablative chemotherapy was substituted to busulfan/melphalan (BuMel) regimen (busulfan on days −7 to −4 (0.8 to 1.2 mg/kg according to body weight) and melphalan (90 mg/m^2) on day -3, -4).

Results

Sixty-five patients evaluated were INRG stage M metastatic neuroblastoma (n=59) or *MYCN* amplified INRG stage L2 neuroblastoma (n=6). *MYCN* amplification was observed in 29 patients in total. Median age at diagnosis was 3.1 years old (range 0-10). Forty-seven patients (72.3%) completed the protocol and 18 patients discontinued because of progressive disease (1), parents' desire (8) or physicians' decision (6) due to poor response of induction chemotherapies.
The grade 4 adverse effects except for hematological adverse effects were seen in 5 cases which was fewer than JN-H-11; liver dysfunction (2), anaphylaxis (1), acute respiratory distress (1) and coagulopathy (1). VOD after BuMel was experienced in 5 patients but all resolved including grade 3 hyperbilirubinemia in 2 patients. No patients died of treatment-related complication.
At the end of September 2019, thirty-eight out of 47 therapy-completed patients survived without disease. The response rates before HDC by INRC was 49.2% (32/65; CR(2), VGPR(7), PR(23), MR(8), NR(7)) and that at 30 days after HDC was 53.9% (35/65; CR(2), VGPR(6), PR(27), MR(5), NR(7)).The response rate after completion of protocol treatment were 46.2%(CR+VGPR) and 64.6%(CR+VGPR+PR) (30/65 and 42/65 respectively; CR(27), VGPR(3), PR(12), MR(1), NR(1), PD(1)), which was comparable to that in JN-H-11 protocol. Delayed resections of primary tumors were performed in 41 patients, and pathological response rate (Ef2+Ef3) was 73.7% in 38 patients evaluated, which was similar to the result of JN-H-11.

Summary/Conclusions

JN-H-15 protocol, which included "delayed local treatment", ICE and BuMel regimen to improve the outcome, appears to be feasible and effective without anti-GD2 for high-risk neuroblastoma. Response rate was similar to JN-H-11 protocol, but the severe adverse events caused in JN-H-15 were fewer than that in JN-H-11, which were due to mostly using BuMel as HDC. Long-term effectiveness of JN-H-15 protocol should be evaluated.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 345

Spinal canal invasion in peripheral neuroblastic tumors: a change in the front-line treatment approach according to the interim results of a prospective study

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Topic Other

Background/Introduction

Patients with Peripheral Neuroblastic Tumors (PNTs) and spinal canal invasion (SCI) have an excellent survival probability but are prone to develop late neurologic and orthopedic sequelae. The therapeutic approach may include neurosurgical decompression, chemotherapy, and radiation

therapy. During the last 2 decades, studies have focused more on primary chemotherapy management of patients with spinal cord compression although there is a bias of treatment for patients with severe symptoms favoring surgical approach. The optimal treatment for these patients has not been determined yet.

Aims

The aim of the study was to describe the natural history of PNTs presenting with SCI and the applied treatments, to evaluate the combined effects of risk factors and to develop treatment guidelines.

Methods/Materials

We set up a prospective registry collecting clinical, pathological, biological, therapeutic and follow-up data on symptomatic and asymptomatic patients with SCI.

Results

135 patients have been registered from 18 countries. 60% had localized disease. The intraspinal extension occupied more than two third of the spinal canal in 66% of the patients. 64% were symptomatic, with motor deficit (41%) and pain (26%) being the most common symptoms. Patients were treated by SIOPEN/institutional protocols. Chemotherapy at diagnosis was performed in 78% of cases; early neurosurgery in 16% and correlated with the presence of symptoms; other approaches at diagnosis were extraspinal tumor resection (3%) and wait and see (3%). The treatment decision was significantly influenced by the presence of symptoms, but not by the degree of symptoms or spinal canal invasion. Improvement of symptoms (motor deficit), if any, was possible within the first 6-12 months since diagnosis. Neurosurgery is more likely to be used in symptomatic patients, although the medium-term outcome is comparable to that of chemotherapy only.
.

Summary/Conclusions

This is the first prospective study collecting data on patient characteristics, treatment approaches and outcome of PNT's presenting with SCI. The study allows the correlation between clinical features at diagnosis and type of treatment with clinical response and late effects. Chemotherapy is the most frequent front-line approach with comparable outcome to neurosurgery. Logistic regression analysis is planned when more data will be available.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 346

Early detection and molecular characterization of therapy-related leukemia in children with neuroblastoma reveals patterns of leukemia transformation with implications for future surveillance protocols

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Therapy-related myelodysplastic syndrome (t-MDS) and acute leukemias (AL) represent a major cause of non-relapse morbidity and mortality in childhood cancer survivors. Neuroblastoma (NB) patients receive multimodality therapy and have had high rates of t-MDS/AL. While specific therapeutic modalities have been associated with distinct abnormalities, our understanding of the relationships between the timing of mutation acquisition, dynamics of clonal selection in relation to specific therapeutic modalities, and how these result in overt leukemia, remains limited.

Aims

Methods/Materials

We studied 199 samples from 53 NB patients treated at MSKCC (enrolled on NCT00588068),

including 17 patients with t-MDS/AL, 14 with transient cytogenetic abnormalities, and 21 matched controls. Comprehensive genomic profiling of bone marrow (BM) was performed with targeted gene sequencing (MSK Hemepact) and fusion detection (Archer FusionPlex Pan-Heme panel) to identify somatic mutations, chromosome level copy number alterations (CNA), and gene fusions at the time of t-MDS/AL diagnosis. Backtracking studies were performed in longitudinal samples with complete molecular characterization. For patients in the transient cohort, samples were sequenced throughout therapy, with a focus on times surrounding cytogenetic abnormalities and treatment windows. In the control cohort, samples were chosen at 1-2 time points at the end of treatment windows.

Results

We detected at least one disease-defining alteration in all cases with t-MDS/AL at time of diagnosis, most commonly MLL fusions (n=6 patients) or TP53 mutations (n=9 variants, n=5 patients). These events were generally mutually exclusive, with the exception of one patient with an apparent subclonal mutation in *TP53* and clonal *MLL* fusion. The remaining cases acquired gene mutations in *NPM1, IDH1, PTPN11, NRAS, CUX1, STAG2, WT1, PPM1D* amongst other genes, at a median variant allele frequency (VAF) 21% (range 2.6-74), with or without chromosomal aneuploidies. High-confidence fusions detected by Archer FusionPlex were limited to MLL-rearrangements, though clinical cytogenetic analysis demonstrated other translocations. All MLL-rearrangements involved the canonical MLL breakpoint region. Backtracking studies identified at least one of these mutations or fusions in the majority (12/17) of patients at a median of 18 months prior to diagnosis (range 1.3-36) using a 0.02 VAF detection threshold.
In contrast, only three patients from the transient cytogenetic cohort, had putative oncogenic somatic mutations identified at any time point sequenced, with a median maximum VAF of 3% (range: 2.5-8.5), with resolution of the molecular alterations in subsequent samples. One control case had an identified presumed oncogenic mutation in BCOR, though this patient died of NB with limited hematologic follow-up after the last sample, >7 years after initial NB diagnosis.

Summary/Conclusions

These findings demonstrate that molecular abnormalities are a hallmark of t-MDS/AL in NB patients and that these are often identifiable months to years prior to leukemia transformation. This presents an opportunity for the development of early detection studies for patients with neuroblastoma undergoing intensive therapy and should inform studies into mechanisms of leukemic transformation.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 347

Targeting polyamine addiction in neuroblastoma: optimizing combined inhibition of polyamine biosynthesis and transport as a novel therapeutic approach

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

Polyamines are essential cations frequently upregulated in tumours for survival, proliferation, and immune evasion. *MYCN* directly regulates the rate-limiting enzyme in polyamine synthesis, ornithine decarboxylase 1 (ODC1). Treatment of neuroblastoma cells with the ODC1 inhibitor difluoromethylornithine (DFMO), although a promising therapeutic strategy, is only partially effective at impeding cell growth due to activation of compensatory mechanisms resulting in increased polyamine uptake from the surrounding microenvironment. We have recently identified the solute carrier SLC3A2 as a key transporter responsible for the compensatory polyamine uptake induced by DFMO. We have further shown that the polyamine transport inhibitor, AMXT 1501, which blocks polyamine uptake, can substantially enhance DFMO efficacy both *in vitro* and in neuroblastoma mouse models (Sci. Trans. Med. 2019), opening the way for clinical trials of combined inhibition of polyamine synthesis and uptake. SLC3A2 is known to frequently dimerise with one of a number of light chains to form a transporter complex, but it is unclear if any of these cotransporters are involved in polyamine uptake, and as such, whether further benefit could be obtained by relevant co-transporter inhibition.

Aims

To further elucidate the mechanisms underlying polyamine uptake in neuroblastoma by identifying SLC3A2 binding partners that contribute to polyamine uptake.

Methods/Materials

Colony and cytotoxic assays were used to determine tumour cell growth. CRISPR-Cas9 technology was used to create SLC3A2 knockout neuroblastoma cell lines. Polyamine uptake was measured by using radiolabelled and fluorescent polyamines.

Results

Among the six light-chain proteins that are known to form binding partnerships with SLC3A2, we have shown that high expression of three of these binding partners (SLC7A5, A6 and A10), like SLC3A2, is strongly associated with poor prognosis in primary neuroblastoma ($P < 0.001$). Of these, the L-type amino acid transporter SLC7A5, which is the most extensively studied SLC3A2 cotransporter, is highly expressed in a broad range of cancers. Expression of SLC7A5 is strongly correlated with SLC3A2 in both primary human neuroblastoma tumors ($R2 = 0.645$, $P < 0.0001$) and PDX samples ($R2 = 0.538$, $P = 0.0005$). Like AMXT 1501, inhibition of SLC7A5 with the selective inhibitor, JPH-203, synergises with DFMO to reduce colony formation in neuroblastoma cell lines ($P = 0.0249$), with this effect being more pronounced in SLC3A2 knockout cells. We have further developed a rapid, simple FACS-based polyamine uptake assay using fluorescently labelled polyamine species, producing comparable results to those obtained through the traditional radiolabelled polyamine uptake assay, which is potentially valuable for biomarker studies in the clinical context.

Summary/Conclusions

Our results suggest that inhibiting the SLC3A2 co-transporter SLC7A5 may synergise with DFMO to reduce neuroblastoma growth. We are currently conducting *in vivo* studies to determine the optimal combination of polyamine transport and synthesis inhibitors, in preparation for clinical trial.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 348

A large-scale in vivo mutagenesis screen identifies Runx1t1 as a critical gene in the development of MYCN-driven neuroblastoma

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

MYCN has a clearly established role in driving the pathogenesis of neuroblastoma, however, the critical molecular interactions involving MYCN have not been sufficiently well defined to allow a clear understanding of how neuroblastoma develops. *N*-ethyl-*N*-nitrosurea (ENU) is a powerful mutagen that causes heritable mutations in male mice by creating DNA adducts in sperm cells.

Aims

To use ENU mutagenesis in *Th-MYCN* transgenic mice, in order to identify new genes/pathways associated with the development of neuroblastoma.

Methods/Materials

Male homozygous *Th-MYCN* mice were injected with ENU, following treatment with cyclophosphamide to prolong survival sufficiently for mating with female homozygous mice. A heritable point mutation associated with greatly delayed tumour development, was mapped and sequenced using exome sequencing and backcrossing to Balb/c and C57BL/6 mice, and shown to be in the *Runx1t1* gene. NMR spectroscopy was used to characterise the effect of the mutation on the RUNX1T1 structure. *Runx1t1* knock-out mice (Calabi, F., et al. (2001). Molecular and cellular

biology *21*, 5658-5666) were imported and crossed with *Th-MYCN* mice.

Results

Of 1716 viable offspring screened, 50 mice displayed a delay in tumour onset of >7.5 weeks. In one line, the founder mouse developed a tumour (a teratoma) at 49 weeks of age, an unprecedented delay compared to untreated homozygous mice. In this founder line, the tumour abrogation phenotype was inherited as a dominant Mendelian trait, with 50% of 22 offspring of this founder developing neuroblastoma by 6 weeks and the remainder displaying suppression of tumour development - either failing to develop tumours at all (>52 weeks), or in two cases developing avascular tumours (18-25 weeks). Mapping and sequencing pinpointed the mutated region to the proximal end of chromosome 4, and more specifically in the gene *Runt Related Transcription Factor 1; Translocated to, 1* (*Runx1t1*, also known as *ETO*), a gene involved in the AML1:ETO translocation of acute myeloid leukaemia. The *Runx1t1* thymine to cytosine point mutation occurred within a highly conserved zinc finger domain (Nervy Homology Region 4), and resulted in an amino acid change of a tyrosine to a histidine. Analysis by NMR spectroscopy showed complete disruption of the normally well-folded zinc finger domain within the mutant peptide. To confirm that the point mutation leads to a loss of function, a knock-out model of *Runx1t1*, with targeted disruption in exon 2, was crossed with the *Th-MYCN* mice. Mice homozygous for *MYCN* and wild-type for *Runx1t1* had almost complete tumour penetrance, with 92.08% of the mice developing tumours. However, loss of only one copy of *Runx1t1* decreased tumour incidence to 6.54%, thus phenocopying the results observed with ENU mutagenesis.

Summary/Conclusions

Using a forward genetic screen, we have identified *Runx1t1* as a critical gene involved in MYCN-driven neuroblastoma. Although *Runx1t1* has not previously been implicated in this disease, loss of only one functional allele, either through mutation or deletion, almost completely inhibits tumour formation in *Th-MYCN* mice, making this gene a potentially attractive target for neuroblastoma therapy.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 349

Combined MDM2 and Survivin inhibition as a new therapeutic strategy in neuroblastoma

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

Inactivation of TP53 is frequently caused by amplification and overexpression of TP53 negative regulators, of which the best studied is MDM2. Targeted activation of the TP53 pathway via inhibition of MDM2-TP53 interaction has been extensively studied in neuroblastoma. The prioritized MDM2 inhibitor Idasanutlin (RG7388) has demonstrated high efficacy in neuroblastoma and entered a pediatric clinical trial. However, single agent treatment with MDM2 inhibitors has been reported to result in therapy resistances through acquisition of *de novo* mutations. Survivin is encoded by the baculoviral inhibitor of apoptosis repeat-containing (*BIRC5*) gene that is mapped to chromosome 17q25, a region with a frequent copy number gain in high-risk neuroblastoma. Survivin overexpression is correlated with poor patient prognosis, metastatic spread and resistance to chemotherapy, making it an attractive drug target. YM155 is a small molecule drug that targets Survivin on a transcriptional level and demonstrates activity against a broad range of cancer entities.

Aims

It is of high interest to preclinically investigate effective combination therapies, to achieve more durable therapy responses while reducing toxicity. Therefore, we evaluated the effect of the MDM2 inhibitor Idasanutlin in combination with the Survivin inhibitor YM155 and further characterized the synergistic effects of the combination therapy in order to create a basis for further clinical application.

Methods/Materials

The effects of the YM155/ Idasanutlin combination treatment on cell viability, migration and apoptosis was investigated *in vitro* using established neuroblastoma cell lines with varying genetic backgrounds. The effects of the combination treatment on neuroblastoma cell viability was investigated using XTT. Cell viability values were converted to relative compound effect values and CI values were calculated, using CompuSyn software according to Chou & Talalay (1984). Scratch Assays were performed to investigate the effects of the combination treatment on migration. Apoptosis induction was detected by flow cytometry using Annexin/PI and Caspase-3 antibody and by immunoblot based detection of PARP cleavage. TP53 dependency was tested using neuroblastoma cell lines overexpressing a dominant negative TP53 mutant. Mode-of-action analysis was performed using western blotting experiments.

Results

We evaluated the effect of the MDM2 inhibitor Idasanutlin in combination with the Survivin inhibitor YM155 and observed high degrees of synergism in a preclinical neuroblastoma model. We demonstrated that the combination of MDM2 and Survivin inhibition consistently reduces viability and resulted in highly synergistic CI values in neuroblastoma cell lines with varying genetic backgrounds. Migration capability of neuroblastoma cells was reduced when cells were treated with the combination therapy. Flow cytometric detection of annexin V/propidium iodide (PI) staining and caspase 3 activity, as well as immunoblotting based detection of cleaved PARP showed that the combination treatment enhances the apoptosis induction in neuroblastoma cell lines in a dose-dependent manner. Stable, ectopic dn-TP53 expression attenuated the Idasanutlin single agent treatment effect but did not affect the YM155 or combination treatment mediated apoptosis-induction.

Summary/Conclusions

In conclusion, our preclinical data provide a rational framework for a combination therapy consisting of Idasanutlin and YM155 for high risk neuroblastoma treatment.

Type Basic

Presentation Preference Traditional poster

Abstract nr. 350

Innovative armed oncolytic adenovirus immunotherapeutic approach for the treatment of refractory or metastatic neuroblastoma patients.

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

Neuroblastoma (NB) is the most common extracranial solid tumour of childhood. Despite advances in multimodal therapy, children with high-risk (HR-NB) have a poor prognosis and new therapeutic approaches are urgently needed. Immunotherapy based on oncolytic viruses (OV) might represent an important and effective immunotherapeutic strategy but so far even though results in animal experiments are promising, the efficacy in first clinical trials has been suboptimal.

Aims

Improvement of OV immunotherapy by investigating the mechanisms limiting their efficacy in vivo.

Investigating OV immunotherapy as a platform for combinatorial treatment approaches.

Methods/Materials

We first investigated the efficacy of viral production after infection with OV in different types of NB cell lines by virus titration focusing first on oncolytic adenovirus (OA). Thereby, we identified that the difference depended on the ability to metastasize and we focused our attention on proteins involved in this process. We silenced these proteins and investigated the effects both at the molecular level (gene and proteomic expression), virus production (titration) and functional efficiency in vitro and in vivo (anti-tumour effect, activation of autophagy and production of exosomes).

Results

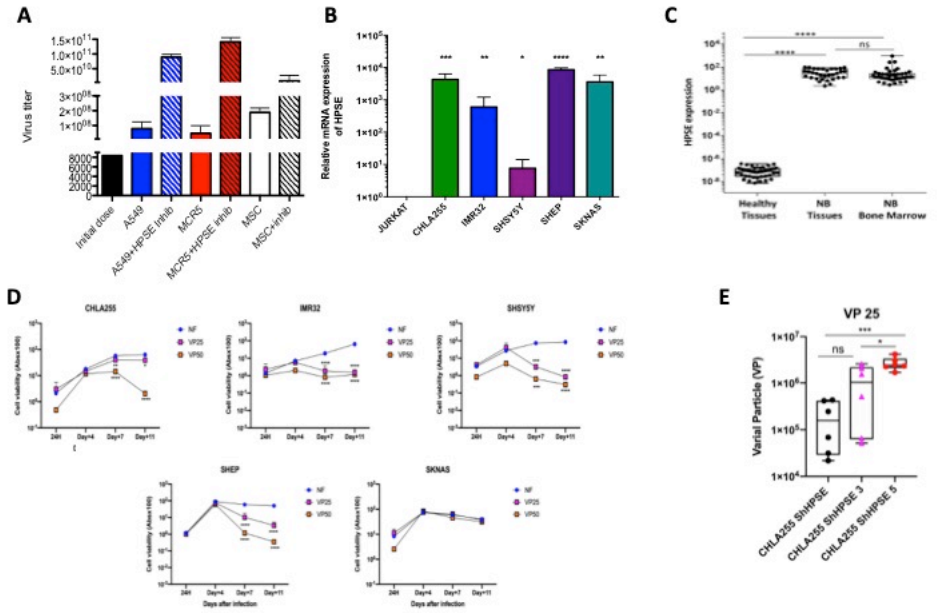
Viral amplification studies revealed a correlation between the reduced number of new viral particles (VP) and the expression of Heparanase (HPSE), an enzyme responsible for modeling the extracellular matrix (ECM) by degradation of heparin-sulfate (HS), a constituent of viral capsids and cellular membranes.

We demonstrated, by immunohistochemistry and qPCR, a significant expression of HPSE in 32 HR-NB ($p<0.001$) both at diagnosis and relapse. This data was confirmed in a public database (https://hgserver1.amc.nl/cgi-bin/r2/main.cgi), showing a negative correlation between HPSE expression and outcome of these patients.

CHLA-255 and SKNAS NB cell lines show high levels of HPSE, metastatic behaviour in an orthotopic xenograft mouse model and a significant resistance to OA ($p<0.05$). Lentiviral viruses encoding different sh-HPSE have been used to silence the two cell lines. Silencing induced a strong inhibition of HPSE expression and impaired proliferation both in vitro and in *vivo.* Notably, reduced expression of HPSE leads to a decreased tumour progression, a significant reduction of metabolic activity and exosome production. Following OA infection, the silenced cells produce a greater amount of VP ($p=0.001$), show a block of autophagy and a down-regulation of pro-tumoural genes. This phenomenon translates in a different protein expression profile compared to the controls. Furthermore, silenced and infected tumour cells show a strong cytopathic effect of OA as well as a more efficient and robust immune presentation increasing the response against adenovirus antigens and tumour.

Summary/Conclusions

In conclusion, HPSE modulation is a promising strategy to implement viral oncolytic immunotherapies in patients with oncological disease. This strategy might be a useful component for the development of a combined treatment with gene modified cellular products such as Chimeric Antigen Receptor T cells.



Panel A shows the impact of HPSE on virus production after OA infection. Panel B and C show the expression of HPSE in NB cell lines and primary NB samples. Panel D shows the susceptibility of NB cell lines to OA infection. Panel E reveals the improvement of VP production upon silencing of HPSE in CHLA255 NB cell line.

Influence of HPSE expression on new VP production.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 351

Neuroblastoma between 1990 and 2014 in the Netherlands: Increased incidence and improved survival of high-risk neuroblastoma

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

Trends of longitudinal incidence of neuroblastoma on a population based scale are not well understood. Similarly, long-term trends in neuroblastoma incidence and survival in unscreened populations are unknown.

Aims

In this study we explored trends in incidence, stage and age at diagnosis, treatment and survival of neuroblastoma in the Netherlands from 1990-2014.

Methods/Materials

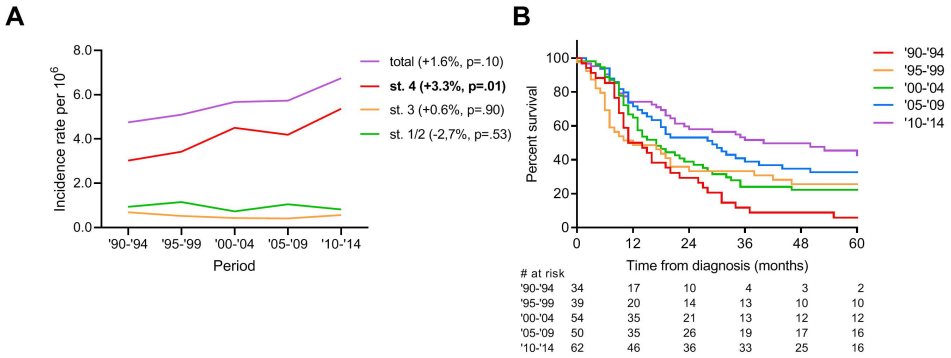
Data on all patients aged <18 years diagnosed with a neuroblastoma between 1990 and 2014 were provided by the Netherlands Cancer Registry (NCR). Trends in incidence by age group and stage at diagnosis were evaluated by calculating the average annual percentage change (AAPC). Univariate and multivariable survival analyses were performed for stage 4 disease to test whether changes in treatment are associated with survival.

Results

The cohort consisted of 593 newly diagnosed neuroblastoma cases. Forty-five percent was <18 months of age at diagnosis and 52% had stage 4 disease. The age-standardized incidence rate of stage 4 disease increased from 3.2 to 5.3 per million children per year (AAPC + 2.9%, p<.01). This increase in incidence of stage 4 disease was exclusively caused by patients aged ≥18 months (3.0 to 5.4; AAPC +3.3%, p=.01, Figure 1A). Five-year OS of all patients improved from 44±5% to 61±4% from 1990 to 2014 (p<.01) and from 19±6% to 44±6% (p<.01) for patients with stage 4 disease. For patients ≥18 months old with stage 4 disease the 5-yr OS improved from 6±4% to 43±7% from 1990 to 2014 (p<0.01, Figure 1B). Multivariable analysis revealed that high-dose chemotherapy followed by autologous stem cell rescue and anti-GD2 based immunotherapy were associated with the improved survival of stage 4 neuroblastoma patients (HR 0.46, p<.01 and HR 0.37, p<.01 respectively).

Summary/Conclusions

Incidence of stage 4 neuroblastoma has increased exclusively in patients aged ≥18 months since 1990, while the incidence of other stages has remained stable. The 5-year OS of stage 4 patients improved, mostly due to the introduction of high-dose chemotherapy followed by stem cell rescue and immunotherapy.



A) Time trends of incidence rates per million children ≥18 months B) Five year OS by period for patients with stage 4 neuroblastoma ≥18 months.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 352

Establishing zebrafish xenografts for pediatric High Risk Neuroblastoma

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

High Risk neuroblastoma (HR-NB) accounts for approximately 50 % of neuroblastoma cases. These patients show aggressive metastatic tumors with a very poor response to therapy. More than 90 % of HR-NB patients present with disseminated tumor cells in the bone marrow and other parts of the body already at the time of diagnosis. To date the survival rate for HR-NB patients is below 40 % despite intensive multimodal treatment. HR-NB patients are treated according to the European HR protocol regardless of their response to induction chemotherapy and genetic makeup. This is in part due to the lack of detailed knowledge of the driver genes as well as the lack of reliable disease models for pre-clinical drug testing. In recent years, zebrafish has developed into a novel powerful tool to study cancer via xenotransplantation of human cell lines. However, xenotransplantation of human neuroblastoma derived from primary cells into the zebrafish larvae has been challenging and automated screening methods were not available.

Aims

The aim of this study is to establish zebrafish xenografts as a reliable, fast and personalized model for studying HR-NB. This will allow us to perform high-throughput image-based drug screens for addressing patients that are not responsive to current therapies.

Methods/Materials

We characterized three neuroblastoma cell lines, derived from HR-NB patients with different genetic makeup by whole genome sequencing, tagged with GFP, sorted and injected them into the perivitelline space of 2-days-post-fertilisation zebrafish larvae. Xenografts were treated with a combination of drugs that have been approved in clinical trials for HR-NB patients.

Results

In this study we demonstrate the successful engraftment of human neuroblastoma cells into the perivitelline space of 2-days-post-fertilisation zebrafish larvae. Upon transplantation, larvae were kept at 35 °C, a compromised temperature between the optimal temperature for human cell growth (37 °C) and zebrafish physiological development (28.5 °C). The injected larvae did not develop any health defects or malformations. Similarly, we also show that there was no significant difference in viability, apoptosis and migration between human NB cells that grew in culture at 35 °C and 37 °C. We were able to visualize and quantify the tumor cell growth *in vivo* for up to 8 days post injection, using the Operetta high-content live imaging system (PerkinElmer). As a proof-of-principle we estimated the IC50 of two drugs that have been reported to reduce growth of neuroblastoma cell lines *in vitro*. Based on the determined IC50 values we are now testing the drug effects on the xenotransplated cell lines growth *in vivo.*

Summary/Conclusions

The successful establishment of a preclinical model for pediatric HR-NB will enable us to address the unmet need of increasing the survival rate of the aggressive disease. Our ultimate aim is to exploit patient-derived neuroblastoma xenografts for quick identification of novel drug targets and combination therapies for precision medicine.

Type Basic

Presentation Preference Traditional poster

Abstract nr. 353

Resolving neuroblastoma heterogeneity via CD surface antigen codes

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction
Differential expression patterns of cluster-of-differentiation (CD) antigens have long been exploited to define cellular heterogeneity in immunology, hematopoiesis and hematological malignancies.

Aims
Having initiated the comprehensive characterization of CD surface molecules in neural cell types, our research is aimed at resolving neuroblastoma heterogeneity via combinatorial CD surface antigen codes.

Methods/Materials
Using a range of established neuroblastoma cell lines we determined the expression profiles of >240 CD antigens and applied SPADE (Spanning-tree Progression Analysis of Density-normalized Events) to automatically extract subpopulation clusters from multidimensional flow cytometry data (see Hindley *et al.*, 2016; Ferlemann *et al.*, 2017). Fluorescence-activated cell sorting (FACS), immunoblot and immunohistological expression analysis and *in vitro* functional assays were used for validation of distinct cellular subsets.

Results
Our work provides a comprehensive analysis of CD antigen expression patterns in neuroblastoma cell lines. Based upon this, we identified surface marker profiles specifically associated with NMYC-amplified tumor entities as well with YAP-TAZ expressing migratory neuroblastoma subtypes. Moreover, we demonstrated the utility of complex multicolor flow cytometric readout for small molecule screens towards identifying differential responsiveness of neuroblastoma

subpopulations to established and novel pharmacological candidates.

Summary/Conclusions

Our work may contribute to expanding the neuro-histopathological arsenal of neuroblastoma subpopulation markers. Complementing transcriptomics and epigenetic analyses of neuroblastoma heterogeneity, CD surface antigen expression dynamics and cellular profiles may aid in refining more targeted therapeutic approaches including immunotherapeutic paradigms.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 354

ASCL1 activation by dephosphorylation can direct a genome-wide re-engagement of a latent differentiation programme in neuroblastoma

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Topic Neuroblastoma as developmental disease

Background/Introduction

Neuroblastoma is thought to arise from sympathetic neuroblast precursors that fail to engage the neuronal differentiation programme, but instead are locked in a pro-proliferative state, resembling a developmental intermediate. Achaete-scute homolog 1 (ASCL1) is a master transcriptional regulator, which modulates both proliferation and differentiation of sympathetic neuroblast precursor cells. ASCL1 transcriptional activity is controlled by multi-site phosphorylation on serine/proline sites. The phosphorylated form of ASCL1 transcribing genes involved in proliferation and neural progenitor cell maintenance, the dephosphorylated form driving differentiation. During development, ASCL1 is transiently expressed and is dephosphorylated and downregulated during differentiation. In high risk neuroblastomas, the levels of ASCL1 remain high and the protein is highly phosphorylated, maintaining the cells in a proliferative mode. Here we show that CDK-dependent phosphorylation of the transcriptional master regulator ASCL1 plays a critical role in controlling the balance between cell proliferation and differentiation, and can be modulated to re-engage a programme of differentiation in neuroblastoma.

Aims

Here we aim to build on our understanding of the phospho-regulation of ASCL1. We look at alterations to cellular proliferation and morphology, as well as genome-wide changes to ASCL1 binding and ASCL1-driven transcription in response to expression of different phospho-forms of ASCL1. We also want to understand if CDK inhibitor-mediated dephosphorylation of ASCL1 is sufficient to re-engage a programme of differentiation in neuroblastoma cells.

Methods/Materials

We are using chemical inhibitors and western blotting to characterise the phospho-regulation of ASCL1 in neuroblastoma cells and its effect on neuroblastoma cell differentiation. We are also using inducible expression of wild-type and phospho-mutant ASCL1 to characterize phosphorylation status-related changes in neuroblastoma cell behaviour. For example, we are investigating DNA binding alterations (by ChIP) and genome-wide transcriptome changes (by RNA-seq) when phosphorylated and dephosphorylated ASCL1 is expressed.

Results

We show that ASCL1 is phosphorylated by Cyclin-Dependent Kinases and that phospho-ASCL1 supports the pro-proliferative programme in neuroblastoma cells. Preventing CDK-dependent phosphorylation of ASCL1 results in changes in the genome-wide transcriptional programme of neuroblastoma cells, leading to suppression of pro-proliferative targets and simultaneous activation of genes that drive cell cycle exit and differentiation.
Mechanistically, ASCL1 ChIP-Seq reveals enhanced binding of un(der)phosphorylated ASCL1 at sites associated with pro-differentiation targets. Moreover, PHOX protein binding at key downstream regulatory elements is also modulated by ASCL1 phosphorylation. Finally, we also show that chemical CDK inhibition is sufficient to drive differentiation of neuroblastoma cells in a manner dependent on endogenous ASCL1.

Summary/Conclusions

We conclude that CDK-dependent phosphorylation of ASCL1 acts as a critical fulcrum controlling the balance between proliferation and differentiation of neuroblastoma cells. By characterising and manipulating the mechanisms restraining the progenitor differentiation programme in neuroblastoma, we are aiming to develop pro-differentiation therapies to inhibit tumorigenesis. Indeed, an understanding of the how to modulate the differentiation-proliferation axis provides a powerful opportunity to exploit cellular fate decision processes for therapeutic benefit.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 355

6p22.3 Locus lncRNAs regulate MYCN stability to determine the neuroblastoma susceptibility

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Topic Gene regulatory networks and epigenetics: from basic science to therapy

Background/Introduction

Neuroblastoma (NB) is a childhood cancer and the most common extra cranial solid tumor, arises from neural crest cells of the developing sympathetic nervous system. Non-random chromosomal aberrations, including MYCN amplification, are commonly seen NBs. In NB, MYCN amplification correlates with tumor aggressiveness, and as a transcriptional driver, it is thought to determine cancer initiation and progression. In our previous findings we demonstrated lncRNAs CASC15-003 and NBAT1 (6p22 lncRNA) encoded by NB risk associated locus 6p22.3 as NBs-specific independent progonostic biomarkers (Pandey et al., 2014; Tanmoy et al., 2018). Here we attempt to understand the functional connection between 6p22 lncRNAs and MYCN dependent oncogenic pathways.

Aims

6p22.3 lncRNA levels negatively correlate with MYCN protein levels. This intrigued us to find the functional nexus between 6p22 lncRNAs and MYCN dependent oncogenic program.

Methods/Materials

Methods: Immunocytochemistry (ICC), Xenografts, Western blot analysis, Co-immunoprecipitation (Co-IP) assay, ChIP, RNA sequencing and transcriptomic analysis, Ubiquitination assays.
Materials: NB tissues from patients and NB cell lines (MYCN amplified and non-MYCN amplified cell lines)

Results

Ectopic expression of 6p22 lncRNAs in MYCN amplified and non-amplified cell lines decreased the MYCN protein levels, whereas their depletion in the NB cell lines resulted in increased levels of MYCN protein. We show that 6p22 lncRNAs and their common interacting ribonucleoprotein USP36, ubiquitin-specific protease, regulate MYCN protein levels. Consistent with functional connection between USP36 and MYCN, Co-IP experiments demonstrate the interaction between MYCN and USP36 and also, they co-localize at the nucleolus, as judged by ICC. Knockdown of USP36 in MYCN amplified NB cells resulted in reduced levels of MYCN protein and decreased cell proliferation. Ectopically expressed USP36 reduced MYCN ubiquitination, suggesting that USP36 deubiquitinates MYCN. Transcriptomic analysis revealed a set of common genes regulated by 6p22.3 lncRNAs/USP36/MYCN which are involved in cell proliferation and cell adhesion, there by contributing to NB disease progression. Knock down of 6p22 lncRNAs/USP36/MYCN regulated genes, such as Col18A1, in the 6p22 lncRNA depleted NB cells rescues molecular and phenotypic effects, which occur due to the loss of 6p22 lncRNAs.

Summary/Conclusions

In summary, our data demonstrate the functional link between 6p22 lncRNAs and MYCN. Identification of 6p22 lncRNAs/USP36/MYCN regulated genes uncovers novel MYCN dependent oncogenic program, leading to new MYCN dependent therapeutic options for high-risk NBs.

Type Basic

Presentation Preference Rapid Fire Poster Presentation

Abstract nr. 356

In vitro modelling of neuroblastoma initiation using human pluipotent stem cells

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Topic Neuroblastoma as developmental disease

Background/Introduction

Neuroblastoma (NB)is considered as a “developmental tumour” due to its embryonic origins: NB tumours are primarily found in the adrenal gland/sympathetic ganglia and therefore are thought to arise during early embryonic development as a result of oncogenic transformation of multipotent trunk neural crest (NC) cells or their derivatives such as sympathoadrenal progenitors (SAP). Consequently, transformed trunk NC/SAP cells fail to differentiate properly, a phenomenon that is likely to contribute to the wide spectrum of cellular phenotypes defining the inter- and intra-tumoural heterogeneity observed in NBs. Since NB heterogeneity has been linked to non-uniform response to treatment and recurrence, it is essential to define precisely how NB tumourigenesis correlates with the distorted differentiation trajectories adopted by transformed trunk NC cells. Current models for studying NB biology involve the use of transgenic animals/primary explants, patient-derived cell lines and xenograft models. Although such approaches have provided valuable insights, they do not fully recapitulate the pathology of human NB due to species differences and only provide a snapshot of late disease stages. Thus these models do not allow the temporal dissection of the events associated with tumour initiation during human trunk NC development, while the limited availability of early-stage human foetal material precludes the direct examination of non-transformed trunk NC/SAP cells in comparison to transformed cells. An attractive solution involves the use of human pluripotent stem cells (hPSCs), which can generate every cell type in the body and therefore represent an attractive in vitro model of early human development. However, conventional hPSC differentiation approaches induce only a modest yield of trunk NC and this limitation is a major obstacle to the hPSC-based modelling of NB tumourigenesis.

Aims

Here, we aimed to define an efficient protocol for the in vitro generation of trunk NC cells and their derivatives from hPSCs.

Methods/Materials

Results

We show that cultures of hPSC-derived, Brachyury-positive neuromesodermal progenitors, the posteriorly-located drivers of embryonic axis elongation, contain trunk NC precursors. These can be directed to efficiently generate functional trunk NC cells as well as sympathoadrenal progenitors and sympathetic neurons.

Summary/Conclusions

This strategy represents a considerable improvement over current differentiation approaches and is an ideal platform for the mechanistic dissection of NB initiation at the cellular and molecular level. To this end, we are currently investigating the behaviour of trunk NC/SAP cells derived from hPSCs carrying two principal NB-associated genetic aberrations: gain of chromosome arm 17q and inducible MYCN overexpression to mimic MYCN gene amplification.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 357

The m6A RNA modification sustains neuroblastoma tumour aggressiveness and is a promising new pharmacological target

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Topic Other

Background/Introduction

Neuroblastoma (NB) is a neuroendocrine tumour of the developing sympathoadrenal lineage that arises from the transformation of neural crest cells (NCCs). NB presents few somatic mutations, and it is mostly characterized by dramatic genomic copy number abnormalities. Therefore, a quantitative rather than a qualitative transcriptome imbalance should be at the heart of this disease. Moreover, the ability of some NBs which are metastatic at diagnosis to spontaneous regress suggests the involvement of reversible alterations. M^6A is the most pervasive post-transcriptional modification in the transcriptome, controlling almost every aspect of mRNA biology. Strong evidence shows now consistent links between alteration in the m^6A content and cancer progression, and the m^6A modification has been also demonstrated to influence embryonic development. From the analysis of public datasets, we discovered that some m^6A key regulators (METTL14, ALKBH5 and YTHDF1) are aberrantly expressed in high-risk neuroblastoma patients.

Aims

We aim to propose an innovative target for NB treatment, the m^6A mRNA methylation, by proving the association between increase disease aggressiveness in patients and the causality for increased aggressiveness *in vitro* and *in vivo*.

Methods/Materials

NB cell lines were genetically engineered to overexpress METTL14, ALKBH5 or YTHDF1 and cellular and in vivo experiments were performed to demonstrate a role of the m6A in neuroblastoma aggressiveness and progression. We also analysed the methylome, the translatome and the transcriptome of NB cells and integrated all the data to find those target that are most affected by m6A methylation, and that therefore could play a role in the neuroblastoma tumor phenotype.

Results

We find that overexpression of the methyltransferase METTL14 and the RNA-binding protein YTHDF1 increase neuroblastoma cell clonal potential and invasion both in adherent and 3D growing cells. Tumour growth *in vivo* is also enhanced. The restoration of the demethylase ALKBH5 acts in the opposite fashion, by strongly decreasing neuroblastoma progression. Molecularly, METTL14 positively controls neuroblastoma cell translation, while ALKBH5 play a role in the regulation of transcript stability. We finally provide proof-of-principle of the feasibility of pharmacological intervention on NB by interfering with the transduction of the m6A signal after a screening-based selection of a small molecule.

Summary/Conclusions

NB is currently an urgent medical need in paediatric oncology. We discovered a new potential drug target for NB, the m6A RNA modification. We show that NB aggressiveness is positively correlated with the m6A-induced signal transduction in NB cells, leading to the effect of increased tumour fitness evident both in cultured in vitro conditions and in nude mice, and due to distinct actions of writers and erasers on mRNA. In the last 40 years, only few therapeutics solution have been added to the clinician toolkit to high-risk NB apart from the classic, heavy and highly toxic therapeutic approach of myeloablative chemotherapy followed by autologous stem cell transplantation. We propose that the suppression of m6A transduction is a new, promising drug target for high-risk NB.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 358

Hypermethylated RASSF1A and tumor specific DNA breakpoints as circulating tumor markers for detection of minimal residual disease

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Circulating tumor DNA (ctDNA) has been used for disease monitoring in several types of cancer. However, most current methods to detect ctDNA in neuroblastoma lack the sensitivity, needed for minimal residual disease (MRD) detection during and after treatment. Neuroblastoma tumors lack recurrent mutations, but chromosomal breakpoints and methylation changes are common in high-risk tumors. Tumor-specific breakpoints can be identified by Targeted locus amplification (TLA) technology (de Vree, 2014). TLA is a technique that selectively amplifies and sequences entire genes on the basis of crosslinking of physically proximal DNA loci. These breakpoints could potentially be used for personalized MRD detection. Previously, our group also demonstrated that the *RASSF1A* gene is inactivated by hypermethylation in all stage M and MS and in 86% of localized neuroblastoma tumors. With the absence of recurrent mutations in neuroblastoma, detection of hypermethylated RASSF1A ($RASSF1A_M$) is a potentially attractive biomarker for diagnosis as well as follow-up.

Aims

To show feasibility of sensitive ctDNA detection for MRD testing, with both tumor specific breakpoint markers and hypermethylated RASSF1A as a more generic marker.

Methods/Materials

Plasma was collected from patients with neuroblastoma at diagnosis (n=47) and during treatment

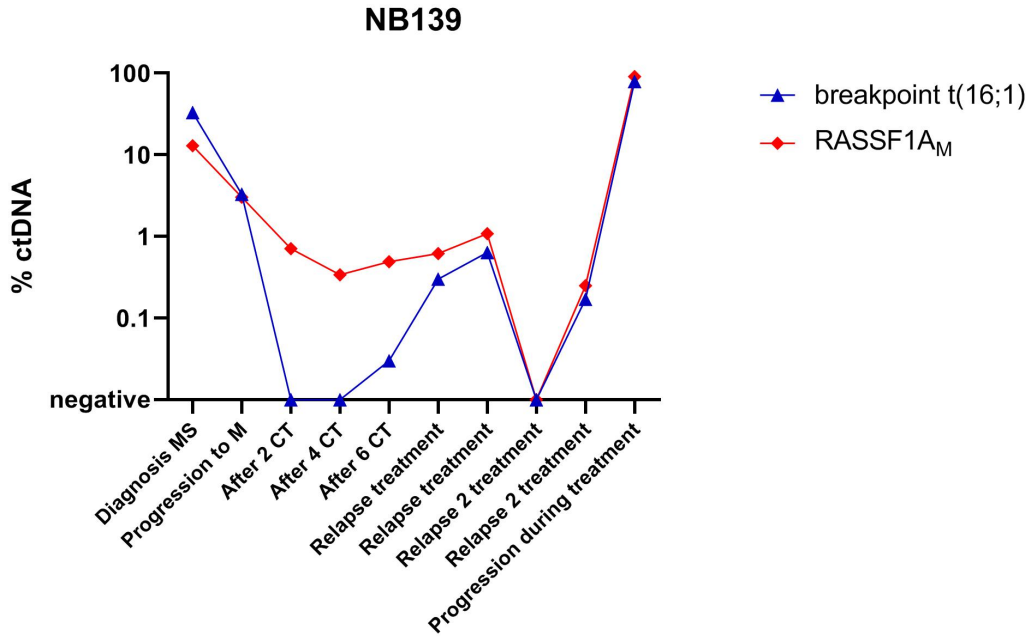
and follow up (n=180). Initially, cell free DNA (cfDNA) was isolated, bisulfite treated and tested by qPCR for actin beta (ACTB), unmethylated and methylated RASSF1A (35 diagnostic and 121 follow-up neuroblastoma samples. Subsequently, a droplet digital PCR (ddPCR) method for $RASSF1A_M$ and ACTB, including a methylation-specific restriction enzymatic digestion, was applied on the remaining samples to ensure accurate quantification and reducing bisulfite induced cfDNA loss. A threshold for detection of $RASSF1A_M$ was established by testing plasma from adult and pediatric controls (73 by qPCR, 25 by ddPCR). For 4 patients, DNA from tumor-derived organoids was used to identify tumor specific breakpoints by TLA. These breakpoints were used to design tumor- and patient-specific ddPCR assays, which were tested on sequential plasma samples.

Results

The total cfDNA level (ng/mL; median) was significantly higher in patients with metastatic neuroblastoma (84.0) and localized neuroblastoma (34.4) compared to healthy adult controls (2.2), but not significantly different from pediatric controls (6.9). CtDNA was present in all patients with metastatic neuroblastoma, with a median percentage of 80% of total cfDNA. $RASSF1A_M$ was detected in 10/21 localized neuroblastoma (median of 8.4% ctDNA). TLA-based breakpoint sequences could be translated into functional ddPCR assays for all 4 patients. CtDNA levels ($RASSF1A_M$ or breakpoint assay) decreased during therapy and re-appeared at relapse. Interestingly, in 4 patients we were able to detect elevated ctDNA ($RASSF1A_M$ or breakpoint assay) prior to relapse or disease progression.

Summary/Conclusions

Our findings demonstrate the value of $RASSF1A_M$ and DNA breakpoints as molecular circulating tumor markers in neuroblastoma. We developed a sensitive and quantitative ddPCR-based assay for $RASSF1A_M$ detection. Furthermore, we were able to develop patient-specific breakpoint assays for ddPCR after TLA-based breakpoint identification. Based on the data presented, RASSF1A hypermethylation and DNA breakpoints are interesting biomarkers for MRD detection in neuroblastoma.



Example of a patient with sequential samples tested for ctDNA by both breakpoint assay (t16;1) and hypermethylated RASSF1A.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 359

GD2-directed bispecific trifunctional antibody demonstrates therapeutic activity in a metastasized murine neuroblastoma model

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

Treatment with the ch14.18 monoclonal antibody (Dinutuximab beta) has improved survival in high-risk patients, and this antibody therapy was recently introduced into standard therapy. Bispecific trifunctional antibodies (trAbs) are IgG-like molecules that can redirect T cells and accessory immune cells towards tumor cells. Thus, trAbs increase the amount of effector cells fighting against the tumor, compared to monoclonal antibodies. TrAbs, directed against the GD2 disialoganglioside, have demonstrated promising preclinical activity in a mouse model of melanoma, but have not yet been tested in preclinical neuroblastoma models.

Aims

We aimed to preclinically evaluate anti-tumor activity of trAbs, directed against GD2, in models of neuroblastoma to assess whether it might be used to treat patients with high-risk neuroblastoma.

Methods/Materials

Two different trAb constructs were used in experiments. EKTOMUN, which is directed against GD2 and human CD3, and SUREK, which is directed against GD2 and murine Cd3. A tumor-unspecific trAb and the ch14.18 monoclonal antibody served as negative and positive controls,

respectively. We established a co-culture model with both human peripheral blood mononuclear cells (PBMCs) and neuroblastoma cell lines to evaluate trAb efficacy *in vitro* using a luciferase-based cytotoxicity assay, the ELISA-based release of proinflammatory cytokines by the PBMCs and the flow cytometric assessment of PBMC activation profiles. SUREK activity was also assessed in a syngeneic immunocompetent neuroblastoma mouse model for experimental metastases. A/J mice intravenously received 5 x 10^5 NXS2 cells, a GD2 expressing hybrid murine neuroblastoma cell line, which is derived from C1300, a neuroblastoma tumor that spontaneously arose in A/J mice. SUREK or control antibodies were intraperitoneally administered on days +1, +5, and +10, and the number of liver metastases was assessed on day +21.

Results

The EKTOMUN bispecific trifunctional antibody mediated a GD2-dependent cytotoxic effect *in vitro* against human neuroblastoma cell lines harboring *MYCN* amplifications or with diploid *MYCN* status in the presence of PBMCs. EKTOMUN also induced the GD2-dependent release of proinflammatory cytokines from co-cultured PBMCs and activated T cells in co-culture with neuroblastoma cell lines. SUREK administration to mouse models *in vivo* activated $Cd4^+$ tumor-infiltrating T cells and expanded the population of $Cd8^+$ tumor-infiltrating T cells. Treatment with SUREK strongly reduced or completely prevented liver metastases in the mouse model for highly aggressive murine neuroblastoma. SUREK also outperformed the current state-of-the-art in antibody therapy (ch14.18 anti-GD2 antibody) when administered at equal doses in the syngeneic immunocompetent neuroblastoma mouse model.

Summary/Conclusions

Here we provide the first evidence that a GD2-directed bispecific trifunctional antibody is a promising new therapeutic agent against high-risk neuroblastoma.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 360

Evaluation of chromosomal aberrations and its influence on treatment results in patients with stage 3 neuroblastoma without MYCN amplification.

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

Treatment strategies in children with localized unresectable neuroblastoma (NBL) are heterogenous. Except of age and MYCN amplification there are no unequivocal risk factors predictive of outcome. Presence of segmental chromosomal aberration may also influence the prognosis. Better defining of risk factors at diagnosis is necessary to optimize the treatment strategies.

Aims

The aim of the study is evaluation of influence of genetic aberrations on treatment results in patient with stage 3 NBL without MYCN amplification and its correlation with clinical and laboratory risk factors of treatment failure.

Methods/Materials

Retrospective analysis of 40 stage 3 patients treated in 2000-2017 in 3 centers of Polish Pediatric Solid Tumors Group with complete follow-up data was performed. All children received standard chemotherapy, including 14 treated with radiotherapy as well. All potential known risk factor and laboratory markers and kind of treatment were evaluated. To evaluate genetic profile, in all children in which fresh or paraffin embedded (FFPE) tumor samples were available, comparative genomic hybrydyzation (CGH) and fluorescence in situ hybridization (FISH) for ALK mutations were done. The Agilent SurePrint G3 CGH ISCA v2 Microarray Kit 8x60K array platform was used for genome evaluation. The resolution for aCGH evaluation was established on 150 kb. Frozen

and FFPE aCGH profiles were matched for 7 patients to confirm and validate results. Two the most common mutations in the *ALK* gene (Phe1174Leu and Arg1275Gln) were performed.

Results

We analyzed 40 children aged 0.2-127.8 months (mean 23.7, median 14.1), including 16 infants, 9 children aged 12-18 months and 15 over 18 months. Overall survival (OS) and progression free survival (PFS) at 5 years were 0.95 and 0.9, respectively. CGH results were available in 29 pts, including 14 NCA and 11 SCA profiles. ALK mutation was found only in 1 infant with SCA profile, who responded to standard therapy and is disease free for over 5 years. Complete remission was obtained in 60%, partial response in 32,5%. Three relapses (1 distant and 2 local) and 1 progression were diagnosed, all 3 with known profile had SCA. Two children died, including 1 death of toxicity. PFS was lower is SCA group (5-y 0.78 vs 1.0, p=0.05. Relapses occurred 3, 4 and 6 years after primary diagnosis. Three of 4 patients with therapy failure had no previous radiotherapy. Multivariate analyses showed the age >18 months (p=0.0) and SCA profile (p=0.03) at diagnosis were the risk factors of relapse.

Summary/Conclusions

All observed treatment failures were found in patients over 18 months, and with SCA profile (3/4 known profiles), and mostly (3/4) without radiotherapy. Children with stage 3 NBL without MYCN amplification over 18 months and with SCA profile should also have radiotherapy considered. As relapse may occur late after therapy, patients with SCA >18 months should be strictly followed after treatment.

Type Clinical

Presentation Preference Traditional poster

Abstract nr. 361

Drug resistance and phenotype of MES- and ADRN-type neuroblastoma cells faithfully reflect consecutive stages of normal adrenergic lineage development.

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Topic Neuroblastoma as developmental disease

Background/Introduction

Neuroblastoma is thought to originate from stalled development of the peripheral sympathetic lineage. We have established that neuroblastoma is composed of two phenotypically divergent types of tumour cells, i.e. lineage-committed adrenergic (ADRN) cells and undifferentiated mesenchymal (MES) tumour cells (van Groningen *et al.*, 2017). MES and ADRN cells have highly divergent transcriptional states and super-enhancers but they can transdifferentiate into one another (van Groningen *et al.*, 2019). MES cells are more chemo-resistant *in vitro*, form a small minority in primary neuroblastoma, but are enriched post-therapy. It is unknown how drug resistance and MES- and ADRN-phenotypes relate to normal development of the peripheral sympathetic lineage.

Aims

To analyse whether MES- and ADRN phenotypes and their drug-resistance profiles relate to normal developmental stages of the sympathetic nervous system.

Methods/Materials

Single cell gene expression data (Furlan *et al*., 2017) were used to relate MES and ADRN cell types to embryonic stages of the peripheral adrenergic lineage. Drug resistance patterns in MES and ADRN cells were established, the underlying pathways were identified and compared to activity in the normal embryonal stages of the peripheral sympathetic lineage.

Results

We show that MES- and ADRN neuroblastoma cells correspond to different stages of sympathetic nervous system development. MES-type neuroblastoma cells resemble normal immature Schwann Cell Precursor (SCP) cells, while ADRN neuroblastoma cells resemble normal lineage-committed Suprarenal Ganglion (SRG) cells. The gene expression programs of normal SCP and SRG cells are conserved in MES and ADRN neuroblastoma cells, respectively.
We then analysed drug-resistance profiles of MES and ADRN cells. ADRN cells were highly sensitive to Retinoic Acid (RA) and the ALK inhibitor Lorlatinib, but MES cells were completely resistant to both drugs. Conversely, we found that TRAIL efficiently killed MES cells but not ADRN cells. For each of these three drugs, we clarified the molecular pathways underlying drug resistance and their key differences between MES and ADRN cells. E.g. MES cells were not only resistant to RA, but even synthesized this metabolite themselves. This was mediated by expression of ALDH genes that synthesize RA from retinol.
We analysed how the three pathways that control resistance or sensitivity to RA, ALK inhibition and TRAIL are expressed during normal development of the peripheral sympathetic lineage. The single cell analysis data showed that normal SCP cells, which correspond to MES tumour cells, have a gene expression program consistent with resistance to RA and ALK-inhibitors and susceptibility to TRAIL. The SRG cells, which correspond to ADRN cells, have a gene expression program consistent with sensitivity to RA and ALK-inhibitors and resistance to TRAIL.

Summary/Conclusions

Our results demonstrate that intratumor heterogeneity faithfully reflects consecutive stages of normal adrenergic lineage development. Resistance or sensitivity to drugs in tumour cell types is controlled by gene expression programs that belong to these normal developmental stages. Resistance to drugs is therefore determined by normal pathways active in subsets of naïve tumour cells, and not necessarily caused by gene mutations.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 362

Evaluation of V600E BRAF mutation in neuroblastic tumors – an immunohistochemical study

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Topic Other

Background/Introduction

BRAF is a serine/threonine kinase immediately downstream of KRAS in the MAPK signaling pathway. The mutation most commonly expressed in human neoplasia, V600E, is expressed in a variety of common malignancies, among them are colon cancer, melanoma, thyroid cancer, glioma and more. Previous studies demonstrated that in some tumors this mutation is a prognostic marker and can be utilized for targeted therapy. In general, few studies investigated the presence of this mutation in pediatric tumors, and in particular, the evaluation of neuroblastoma, has been minimal.

Aims

The aim of this study was to investigate the expression of the most common BRAF mutation - V600E, in a variety of neuroblastic tumors.

Methods/Materials

We used the antibody Mouse anti-human BRAF V600E monoclonal antibody (clone VE1). Demographic as well as clinical and pathologic data was collected for these patients including age, gender, stage and outcome. The immunohistochemical stains were evaluated using a 3-tired semiquantitative method.

Results

The study included tumor samples from sixty four patients: 55 Neuroblastomas, 7 Ganglioneuroblastomas and 2 Ganglioneuromas. The immune stains from all samples were negative for the BRAF. The controls were all positive.

Summary/Conclusions

These results cannot support the existence of BRAF mutation (V600E) in neuroblastic tumors. Therefore, this mutation cannot be considered as a target for treatment of neuroblastic tumors.

Type Clinical

Presentation Preference Rapid Fire Poster Presentation

Abstract nr. 363

A Cross-Sectional Study of the profile of Neuroblastoma at an Indian Tertiary Cancer Centre

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Topic Other

Background/Introduction

The tremendous progress in diagnostics and therapy for neuroblastoma worldwide has not ricocheted in most developing countries. Apart from these major lacunae, there is a constant strain to describe the incidence and distribution of neuroblastoma.

Aims

Since the information on disease burden in a community is an intricate part of the health care planning to guide quality improvement initiatives and policy formulation process we intended to review the profile and the treatment offered to patients with neuroblastoma presenting at a tertiary referral cancer which caters to essentially entire India.

Methods/Materials

All patients under 15 years with histologically confirmed neuroblastic tumours presenting at a tertiary cancer center from July 2006 to December 2018 were identified from the hospital database. Patients without a definite diagnosis of malignancy irrespective of the clinicoradiological features and prior therapy for suspected or proven disease elsewhere were excluded from the analysis. Staging evaluations included cross-sectional imaging for assessment of the primary, core biopsy for confirmation of the histology and assessment of MYCN status either by polymerase chain reaction and/or fluorescent in situ hybridization. Metastatic workup included a bone marrow aspirate and biopsy, I 131 meta-iodo-benzyl guanidine scan, bone scan and/or fluorodeoxyglucose positron emission tomogram scan. Patients were risk-stratified according to the COG risk stratification schema into low, intermediate and high-risk. The treatment included surgery alone or

with chemotherapy for patients with low and intermediate-risk tumors. For high-risk tumors induction chemotherapy followed by surgery and radiotherapy was utilized. Autologous stem cell transplant (ASCT) was not feasible in all patients due to several reasons and immunotherapy is not available in our country. Overall the treatment was categorized into four groups, prior treatment only, prior treatment and treatment at our institute, treatment only at our institute and no cancer-directed treatment.

Results

The median age of the entire cohort of eligible 791 patients was 3 years with the majority (67.3%) in the 0-4 years' age group. The male to female ratio was 1.7:1 with 503 (63.6%) males and 288 (36.4%) females. The majority of patients were categorized as high-risk (51.4%) while low and intermediate-risk was present in 21% and 15% respectively. 12% of the patients could not be assigned risk due to inadequate work-up either due to the fulminant course of the disease or treatment abandonment. Of the 791 patients, 41 had presented with recurrent disease after receiving curative treatment elsewhere. Of the 750 patients with a primary presentation, 55.5% had treatment only at TMH and an additional 14% who had prior incomplete treatment also received treatment in TMH. 12% had prior treatment only and 18.5% had no cancer-directed treatment.

Summary/Conclusions

Predictably, high-risk tumors form the bulk of our patients. A very high incidence of no cancer-directed treatment was observed in our population due to several patients (large burden of disease, suboptimal nutritional status, the toxicity of the treatment and poor family motivation) and administrative factors (non-availability of ASCT, immunotherapy, etc).

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 364

Autologous stem cell mobilization in paediatric oncology patients; characteristics of Poor Mobilisers and the use of Filgrastim, Pegfilgrastim and Plerixafor.

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

Mobilization of a sufficient amount of peripheral blood stem cells (PBSC) can be a challenge in paediatric patients with cancer. Recently, Pegfilgrastim and Plerixafor, a CXCR4 antagonist, are used for hematopoietic stem cell (HSC) mobilisation but data on their use in the paediatric population is limited.

Aims

To identify characteristics of Poor Mobilisers (PM) and compare success of mobilisation, apheresis yield, quality of stem cells and engraftment between different mobilisation regimens.

Methods/Materials

In this retrospective study we analysed medical records of 215 patients who underwent PBSC mobilization from August 2005 to December 2019 at the Princess Maxima Centre and Academic Medical Centre Amsterdam, The Netherlands. Mobilization regimens consisted of either daily injections Filgrastim 10mcg/kg or one single injection Pegfilgrastim (100, 150 or 200mcg/kg) after chemotherapy. Plerixafor was administered when peripheral blood CD34+ (PBCD34) counts failed

to reach 20cells/ul. Patients were classified as PM when harvest counts were below $2*10^6$ CD34+cells/kg (within maximum 3 consecutive days) or in case Plerixafor was used.

Results

Two hundred fifteen paediatric oncology patients (age 1-19) scheduled for PBSC collection were consecutively included. One hundred four children were diagnosed with Neuroblastoma (NBL); median age 3 (range 0-19); 61% male, 39% female. Seventy-five NBL-patients (72,1%) received Filgrastim, 8 patients (7,7%) received Pegfilgrastim, 4 patients (3,8%) received Pegfilgrastim + Filgrastim, 6 patients (5,7%) received on-demand Plerixafor during initial mobilization, 9 patients (8,7%) received Plerixafor after initial failed harvest with (Peg)filgrastim. Twenty-one NBL-patients (20%) were classified as PM; NBL-patients accounted for 70% of all PM. Bone marrow (BM) involvement at both diagnosis and start of mobilization was more common in PM (90,5% vs. 60,5%; p=0.04 and 57,1% vs. 26,5%; p=0.01). NBL patients had more BM involvement at diagnosis and mobilisation (72,1 vs. 4,5%; p<0.01 and 32,7 vs. 0%; p<0.01) and received more cycles of chemotherapy prior to mobilisation compared to other malignancies (4 vs. 3; p<0.01). After exclusion of PM, PBCD34+ counts before apheresis (40,46 vs. 99 cells/ul; p<0.05), harvest yield (2,69 vs. 6,35 $*10^6$cells/kg; p<0.05) and CFU-GM/CD34+ ratio (0,20 vs. 0,30; p<0.05) were lower in the NBL group. With the use of Plerixafor, 76% of all PM and 71% of PM with NBL were able to achieve a successful harvest. Successful engraftment was achieved in all PM and 94% of good mobilisers with NBL. No differences in neutrophil (>0,5 * 10^9/L) and platelet (>20 $*10^9$/L) engraftment were observed between PM and good mobilisers. Median time to platelet engraftment was 32,5 days in NBL-patients and 19 days for patients with other malignancies (p<0.05). Further analyses will be performed on harvest products of selected patients to compare phenotypes of CD34+ cells mobilised with Filgrastim, PEGfilgrastim and Plerixafor.

Summary/Conclusions

A significant proportion of NBL patients fails initial mobilisation with (Peg)filgrastim. The use of Plerixafor seems effective in most but not all poor mobilisers. NBL patients showed lower apheresis yield and longer time to platelet engraftment compared to patients with other malignancies. Further testing of harvest material will provide more information on CD34+ cell function and phenotype mobilised using different mobilization agents.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 365

Immunophenotypic characterization of bone marrow metastases and NK cells aimed at predicting sensitivity / resistance to immunotherapy in patients with high risk neuroblastoma

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

High-risk neuroblastomas (HR-NB) are particularly aggressive childhood tumors characterized by metastatic dissemination, typically affecting the bone marrow. The 5-year survival remains <40% despite the aggressiveness of the therapy which includes autologous stem cell transplantation and immunotherapy with specific antibodies for the GD_2 oncofetal antigen. Patients with similar clinical pathological parameters and receiving the same treatment may have markedly different clinical outcome.

Aims

Our study aims to analyze the variables that might reduce/abolish the effectiveness of treatments, in particular the efficacy of anti-GD2 immunotherapy. They could include: i) the presence of $GD2^{neg}$ or $GD2^{low}$ metastatic variants; ii) the presence of high levels of ligands involved in immune-checkpoints axis (such as B7-H3 or PD-Ls,); iii) a reduced post-transplant number of immune cells, in particular of CD16+ Natural Killer (NK) capable of being effectively engaged by the antibody; iv) surface expression of CD16 allotypes (158V or 158F) with different binding affinity for the Fc portion of the antibody; v) reduced expression of DNAM-1 and NKp30, highly involved in spontaneous NB cell killing; vi) percentage of long-term differentiated NK cells (expressing different markers including CD57 and KIRs).

Methods/Materials

We analyzed NB and/or NK cell compartment in more than 20 liquid biopsies (Peripheral blood and bone marrow aspirates) from HR-NB patients at the onset at relapse and post autologous stem cell transplantation by using a polychromatic cytofluorimetric method. Moreover, NK cell

degranulation capability against NB cells has been evaluated in combination with different NK cell phenotypes, using an in vitro CD107a assay

Results

Our study allowed us to set up a cytofluorimetric method showing an almost complete overlapping with results derived from histopathologic analysis in terms of presence/absence of NB infiltration. Rare unmatched results are under investigation. Thus, our method showed a great specificity and sensibility. Moreover, importantly, our analysis allowed quantification of CD56, GD2 and B7-H3 surface expression to be correlated to the efficacy of immunotherapy or other clinical parameters. PD-L1 surface expression has never been detected on the bone marrow metastases analyzed. About NK cell compartment, at time of immunotherapy a relevant percentage of HR-NB patients showed a significant proportion of CD16 negative cells almost in bone marrow and a heterogeneous surface expression of the other molecules analyzed. A correlation between different NK cell phenotypes and degranulation capability against NB in the presence of dinutuximab beta has been performed

Summary/Conclusions

In conclusion our study set up an unbiased cytofluorimetric method to unequivocally identify NB infiltration in liquid biopsies and to characterize NB in terms of surface expression of GD2, B7-H3 and other molecules possibly impacting on response to therapy and on patients' survival.
Our analysis also provided a wide still lacking characterization of NK cell compartment at onset at relapse and before immunotherapy in HR-NB patients.
Overall our study contributes to optimize diagnosis and to predict response to anti-GD2 immunotherapy in HR-NB patients. Moreover, our results highlight some aspects of the immune landscape in NB patients to be considered for current and future novel therapeutic approaches.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 366

Integration of High-Throughput Drug Screening on Patient-Derived Organoids into the Princess Máxima Center iTHER Precision Medicine Program: The Future is Now!

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

One of the most promising options to improve outcomes for pediatric cancer patients with relapsed or refractory disease is through molecular profiling guided treatment approaches. However, only 50% of these tumors harbor actionable events according to the first results of pediatric precision medicine programs. Compound screening on organoids grown from patient-derived tumor tissues might reveal additional treatment options. As a proof-of-concept study we have generated tumor organoid models of relapsed neuroblastoma patients in our iTHER program to test a 190-compound screen. Data was referred to a large cohort of neuroblastoma cell lines that was exposed to the same library and compared to genomic patterns and clinical

characteristics in the online R2 bio portal.

Aims

To establish a neuroblastoma specific organoid model for high-throughput drug screening within a pediatric precision medicine program to confirm or exclude drug sensitivity and to identify additional treatment options.

Methods/Materials

The Princess Máxima Center individualized THERapies program (PMC-iTHER) performs comprehensive molecular profiling (WES, lcWGS, RNAseq, Affymetrix microarray and methylation profiling - INFORM pipeline) on biopsy samples of primary very high risk or relapsed/refractory patients. Data are analyzed using the R2 bioinformatic platform and recommendations are made by a national Multidisciplinary Tumor Board. Simultaneously, tumor-derived organoid cultures are generated and exposed for 5 days to a 190-compound library. Effects on cell viability are assessed using CellTiter-Glo and organoid DNA/RNA is profiled through the same pipeline to compare molecular events. The same compound screen and profiling was performed on a neuroblastoma cell line panel (N=23) to generate a reference dataset.

Results

To date, 171 patients have been enrolled in the iTHER trial including 35 neuroblastoma. For 86% of the included neuroblastoma patients, one or more actionable events with priority score intermediate or higher could be identified, most frequently aberrations in the RAS-MAPK and cell-cycle-checkpoint pathways. In addition, samples showed high mutational burden, supporting immunotherapy treatment. In 23% of the cases, patients received matching targeted therapy based on molecular profiling results (crizotinib; ribociclib) with stable disease as best response. Isolation and *in vitro* growth of neuroblastoma organoids has been optimized by comparing culture conditions with varying growth factors based on the expression of growth factor receptors and matching ligands in neuroblastoma organoid cultures versus neuroblastoma biopsies and healthy tissues. Adding human plasma to the culture medium resulted in the formation of a 3D matrix, allowing the growth of single-cell derived neuroblastoma spheres. Neuroblastoma organoids retained molecular features of the parental tumor. High-throughput drug screening with 0.1 nmol/L-10 µmol/L concentration of all compounds of the first 10 neuroblastoma organoids (5 from relapsed and 5 from primary) was compared to reference data of 23 fully characterized neuroblastoma cell lines. Data analysis using modified AUC, IC50, LC50 and Z-scores over the cohort confirmed sensitivity to drugs targeting known driving molecular events such as ALK and NRAS. Additional treatment options in DNA damage repair with clinically significant concentrations could be identified for patients without relevant molecular targets.

Summary/Conclusions

Patient-specific drug testing on neuroblastoma tumor organoids is a feasible model to confirm as well as exclude drug sensitivity and explore new therapeutic options for children with neuroblastoma. Clinical benefit will be evaluated in our upcoming clinical trial iTHER 2.0.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 367

MYCN-amplified neuroblastoma with vasoactive intestinal peptide syndrome and BRAF V600E mutation

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

Neuroblastoma, the most common extracranial solid tumor in children, occasionally produces vasoactive intestinal peptide (VIP) resulting in secretory diarrhea (VIP-D). Patients with this paraneoplastic phenomenon usually have low-risk localized disease; resection is typically curative of both the neoplasm and the VIP-D. Rarely, patients present with metastatic high-risk neuroblastoma (HR-NB) and then develop VIP-D during induction. Chemotherapy is hypothesized to induce differentiation and VIP expression in these patients. Management is a major challenge because of the VIP-D in the setting of strongly myelosuppressive chemotherapy.

Aims

To report on two patients with HR-NB who developed VIP-D during induction chemotherapy.

Methods/Materials

With IRB approval, clinical and genomic information on two patients with VIP-secreting HR-NB treated at Memorial Sloan Kettering Cancer Center was retrospectively analyzed. Tumor sequencing was performed by CLIA-certified platforms: either MSK-IMPACT or Foundation One.

Results

Two females with *MYCN*-amplified HR-NB (abdominal primary with metastases to bones, bone marrow, and liver) acutely developed massive VIP-D after two cycles of induction chemotherapy with cyclophosphamide and topotecan as per ANBL0532. Both patients had a somatic BRAF V600E mutation. In patient #1 (age 19 months at diagnosis), severe VIP-D persisted despite chemotherapy, octreotide therapy, and tumor resection. However, secretory diarrhea promptly

resolved and serum VIP levels decreased from >3200pg/mL to 438pg/mL within 14 days after starting dabrafenib (BRAF inhibitor). Four weeks later, the patient relapsed in brain with BRAF wild-type neuroblastoma. Dabrafenib was stopped; diarrhea recurred and VIP levels rose to 879pg/mL. After management of the hemorrhagic brain metastases, trametinib (MEK inhibitor) was added and dabrafenib restarted. Although the diarrhea improved, the neuroblastoma rapidly progressed leading to death 13 months post-diagnosis. Patient #2 (age 11 months at diagnosis) had prompt resolution of diarrhea after starting dabrafenib and trametinib with induction cycle five. Serum VIP levels decreased from 1416pg/mL to 209pg/mL within six days of starting the tyrosine kinase inhibitors (TKIs). These agents were discontinued after six cycles of post-induction chemo-immunotherapy without return of diarrhea or elevation of VIP levels (remained <50pg/mL). Subsequent therapy included anti-GD2 antibody and anti-neuroblastoma vaccine. She remains progression-free 27 months post-diagnosis. At the time of primary tumor resection, metastatic sites (liver and lymph nodes) showed the BRAF mutation, but the adrenal neuroblastoma that was previously biopsied and BRAF-positive no longer had the BRAF mutation.

Summary/Conclusions

Patients with HR-NB who develop VIP-D during induction chemotherapy may have an association with somatic BRAF V600 mutations. Treatment with BRAF inhibitors leads to rapid improvement in symptoms and facilitates continuation of anti-neuroblastoma therapy. Both patients demonstrated tumor heterogeneity, suggesting that combination therapy with BRAF and MEK inhibitors is warranted. Concurrent therapy with TKIs and anti-neuroblastoma chemotherapy was found to be safe and feasible in patient #2. Tumor sequencing should be performed in all patients with HR-NB, especially those with VIP-D since timely treatment with appropriate TKIs can contribute to a favorable outcome in these rare patients.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 368

Phase I Trial of Lorlatinib in Children and Adults with ALK-driven Refractory or Relapsed Neuroblastoma: A New Approaches to Neuroblastoma Consortium (NANT) Study.

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

Neuroblastomas (NB) harboring *ALK* aberrations are differentially sensitive to crizotinib, with certain mutations conferring intrinsic resistance. Lorlatinib, a macrocyclic ATP-competitive potent ALK inhibitor, exerts unprecedented activity against NB-derived xenografts harboring the most common *ALK* mutations.

Aims

Determine the safety, pharmacokinetics (PK), recommended phase 2 dose (RP2D), and explore the anti-tumor activity of lorlatinib in patients with relapsed/refractory ALK-driven NB.

Methods/Materials

Relapsed/refractory patients >12 months, with *ALK* mutations or amplification were eligible; prior ALK inhibitor (ALKi) treatment was allowed. Lorlatinib was orally administered once daily in 28-day courses (C). For patients <18 years and <1.73m^2, 4 dose levels (DL) (45, 60, 75, 95 mg/m^2/day) were assessed. DL5 (115 mg/m^2/day) is currently enrolling (cohort A1). For patients >18 years or >1.73m^2, one DL (100 mg/day, adult RP2D) was assessed. DL 150mg/day is currently enrolling (cohort A2). The primary endpoint was dose-limiting toxicity (DLT) during C1 and neurocognitive

toxicity through C2. Lorlatinib safety, PK, and activity data for patients enrolled in cohorts A1and A2 are reported.

Results

Between 9/2017 and 12/2019, 32 eligible patients enrolled (13 with prior ALKi therapy), with median age (range) 5.5 years (2-17) on A1, 21.5 years (15-50) on A2. In cohort A1, 3 patients each enrolled onto DL1-3, none developed DLT during C1, or neurotoxicity during C2. Ten patients enrolled on DL4; 5 completed 2 courses, none developed DLT. In cohort A2, 5 patients enrolled at 100 mg/day with no DLT's; 5 enrolled at 150 mg/day and one experienced a DLT (grade 4 psychosis/hallucinations) that recurred after dose reduction in a patient with undisclosed pre-existing psychiatric history. Most common treatment-related adverse events were weight gain (90%, grade 1-3), hyperlipidemia (90%, grade 1-3), AST/ALT elevation (63%, grade 1-2), concentration/memory impairment (23%, grade 1-2), peripheral neuropathy (13%; grade 1-2, A2 only), and peripheral edema (10%; grade 1, A2 only). Lorlatinib steady state exposure observed in the A1 cohort at DL3 and DL4 was in the range of exposures observed in adult lung cancer patients at the 100 mg and 200 mg QD DLs, respectively. Of A1 patients evaluable for response, 1/18 had partial response (PR), 3/18 had minor responses (MR), and 4/18 had stable disease (SD). Of patients with MR, 2/3 had PR of soft tissue (ST) and 1/3 had CR by MIBG. One patient with SD had CR by MIBG of ST disease. Of A2 patients, 1/10 had CR, 3/10 PR, and 3/10 MR. Of patients with PR, 2/3 had CR by MIBG. Of patients with MR, 1/3 had PR and 1/3 CR by MIBG. Responses were seen across dose levels, ALK mutations, and in ALKi pre-treated patients. The median (range) of courses received was 2 (1-24) on A1 and 10.5 (2-28) on A2. Patients with *MYCN* amplified tumors had a mean number of 1.9 courses compared to 6.8 courses for those without *MYCN* amplification.

Summary/Conclusions

Inhibition of ALK driven neuroblastomas with Lorlatinib occurs with manageable toxicity and results in objective anti-tumor activity. These data support continued investigation of lorlatinib to incorporate this agent into up-front treatment.

Type Clinical

Presentation Preference Oral presentation

Advances in Neuroblastoma Research (ANR) Meeting
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Abstract nr. 369

The therapeutic potential of a pan-RAF Small molecule inhibitor agerafenib as a treatment option for neuroblastoma patients

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Topic Other

Background/Introduction

The mitogen-activated protein kinase (MAPK) pathway is a key driver of oncogenicity in many cancers. This pathway transmits growth and mitogen signals to the nucleus via a cascade of specific phosphorylation events involving RAS, RAF, MEK and ERK. Up to 78% of relapsed neuroblastoma samples contain mutations predicted to hyperactivate MAPK signaling. These findings underscore the significance of modulating the MAPK signaling axis for NB treatment. Agerafenib is a small-molecule pan-RAF inhibitor and assessment of agerafenib in clinical trials for treating cancer is underway. In this study, we examined the therapeutic effect of agerafenib as a treatment option for NB.

Aims

To determine the therapeutic potential of a pan-RAF Small molecule inhibitor agerafenib as a treatment option for neuroblastoma patients

Methods/Materials

We examined the effect of agerafenib on NB cell proliferation using a panel of NB cell lines including three MYCN-amplified cell lines (NGP, IMR32, and SK-N-BE(2)) and three non-MYCN-amplified cell lines (SH-SY-5Y, SK-N-AS, and LAN-6). 2.0×10^4 cells were seeded in each well of a 96-well plate and treated with serial dilutions of agerafenib for 72 hours, and cell viability was determined using Cell Counting Kit-8 (CCK-8).Then the soft agar assay was performed to test the inhibitory effect of agerafenib on NB cell anchorage-independence growth. The NB xenograft and TH-MYCN transgenic mouse models were used to determine the therapeutic effect of agerafenib on tumor growth *in vivo*. For the xenograft mouse model, an inoculum of 1×10^6 prepared NGP cells was injected into the left renal capsule. Two weeks later, mice bearing tumors with similar sizes (using bioluminescent imaging to monitor tumor growth) were randomly divided into two groups: DMSO control group and agerafenib treatment group (30 mg/kg by intraperitoneal (i.p.) injection once daily for 21 days). At the end of the treatment, all mice were sacrificed. Tumors, together with the right kidneys (control), were harvested and analyzed. For TH-MYCN transgenic NB mouse model, double transgenic TH-MYCN mice at four weeks of age, following the development of an abdominal tumor (~1 mm in diameter), were treated daily for 3 consecutive weeks with single intraperitoneal (i.p.) injections of agerafenib at 30 mg/kg or an equal volume of DMSO (controls). Three weeks later, the treatment was stopped and mice were monitored daily for symptoms. Kaplan-Meier survival data were analyzed using a logrank test.

Results

High expressions of RAF family kinases correlated with advanced tumor stage, high-risk disease, tumor progression, and poor overall survival. Targeted inhibition of RAF family kinases with agerafenib abrogated the activation of ERK MAPK pathway in NB cells. Agerafenib significantly inhibited the cell proliferation and colony formation ability of NB cells *in vitro*, and its combination with traditional chemotherapy showed a synergistic pro-apoptotic effect. Agerafenib exhibited a favorable toxicity profile, potently suppressed tumor growth, and prolonged survival in orthotopic mouse models.

Summary/Conclusions

Our preclinical data suggest that agerafenib might be an effective therapeutic agent for NB treatment, both as a single-agent and in combination with chemotherapy.

Type Translational

Presentation Preference Traditional poster

Abstract nr. 370

Replicative stress as selective force for chromosome 17q gain in neuroblastoma: embryonic stem cells as a study model

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Topic Neuroblastoma as developmental disease

Background/Introduction

Neuroblastoma (NB) presents with low mutational burden and highly recurrent segmental chromosomal aneuploidies including chromosome 17q gain in the majority of *MYCN*-amplified and non-amplified high-risk NB. A region syntenic to human 17q is often gained in murine *MYCN*-driven NBs, supporting the hypothesis that dosage effects of 17q genes are implicated in NB oncogenesis. We previously identified 17q candidate aneuploidy drivers including TBX2, a superenhancer marked core-regulatory circuit transcription factor, and BRIP1, a DNA helicase implicated in homologous DNA repair and replication fork stability. Given that human embryonic stem cells (hESCs) and embryonal cancer cells share biological similarities and that many hESC lines acquired 17q gain during culture, we hypothesized that sustained high proliferation rates and resulting replicative stress drive the selective pressure for subclones with acquired 17q gain.

Aims

We proposed to study the effect of chronic exposure to replicative stress on growth and survival of isogenic hESC cells, with and without 17q gain, as a putative model to further identify key aneuploidy drivers as possible future novel drug targets.

Methods/Materials

We co-cultured isogenic hESCs with a normal molecular karyotype (hESC17qNO) and 17q gain (hESC17q+) in equal proportion at the start of culture, either under normal growth conditions or under increased replicative stress through exposure to mild concentrations of hydroxyurea (HU). Cumulative growth was measured together with molecular analysis for chromosomal copy number using shallow whole genome sequencing and digital droplet PCR. In addition, flow cytometry based cell cycle analysis and RNA-seq based transcriptome analysis were performed to monitor effects of 17q gain on replicative stress resistance.

Results

The hESC17q+ cells had a proliferative advantage and rapidly overtook the hESC17qNO cells during prolonged culture only under replicative stress conditions. In addition, we noted 13q gain emergence. Further, hESC17q+ cells showed reduced HU-induced S-phase arrest while cells in G2/M transition increased, pointing to an attenuated replicative stress phenotype. Morphological changes were observed under HU exposure, i.e. flattened and less domed colonies, suggesting a shift towards a more primed-like hESC state. RNA-seq analysis further supported our observations: **(1)** gene set enrichment analysis identified 13q and 17q gene sets for dosage effects resulting through the copy number gains; **(2)** PRR11 was high ranked among the 17q upregulated genes under HU, indicating impact of 17q gain on control of late-S to G2/M cell cycle progression; **(3)** 13q differentially expressed genes included *ZIC2*, a well-known pluripotency marker and *SOX21*, implicated in maintenance of progenitors and inhibition of neuronal outgrowth, suggesting an ESC-context dependent effect on selection of 13q gain and further validating the biological relevance of our model system.

Summary/Conclusions

In conclusion, we provide evidence that chromosome 17q gain infers a proliferative advantage to hESC cells, only under conditions of replicative stress. We suggest that 17q gain affects gene dosage of multiple genes on 17q allowing hESCs to better adapt to replicative stress. We further hypothesize that a similar process could drive the 17q gain in high-risk NBs and that NB cell addiction to replicative stress resistance offers an entry point for novel therapeutic strategies.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 371

The cfDNA methylome as a complementary assay to classic histopathology for neuroblastoma diagnosis

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

In the clinical management of pediatric solid tumors, a biopsy remains the gold-standard to establish a diagnosis. The DNA methylation pattern of a tumor is known to correlate to the histopathological diagnosis across cancer types and thus might be of value in the diagnostic workup of tumor samples. It has previously been shown that this methylation pattern can be detected in cell-free DNA (cfDNA).
In a retrospective proof-of-concept study in children with a solid tumor we were able to correctly classify up 80% to 94% of primary diagnostic cfDNA samples according to their histopathological class, solely based on the methylation pattern of cell-free DNA. Our cohort of 60 patients included 10 neuroblastoma cases. Copy number variations could also be assessed directly from the methylation data, enabling the detection of *MYCN* status and segmental chromosomal alterations.

Aims

To explore the clinical applicability of this novel methylation profiling technique, we used it to classify a diagnostically challenging case of a three-year old girl with a small round blue cell tumor. Due to the clinical presentation of a primary tumor originating from the distal femur diaphysis with metastasis to the inguinal and pelvic lymph nodes, neuroblastoma was not considered as a diagnosis. Classical pathology and immunohistochemistry did not yield a conclusive diagnosis. The patient started treatment according to EUROEWING 08 protocol because of a suspected hard-to-classify bone tumor. SNP array analysis from the primary tumor revealed 1p deletion, *MYCN* amplification and gain of 17q consistent with neuroblastoma, however MIBG and urinary catecholamines (collected after 2 cycles of VIDE chemotherapy) yielded negative results. We aimed to further explore the possible added value of a liquid biopsy in this challenging diagnostic situation.

Methods/Materials

Cell-free DNA from a pretreatment blood sample was isolated. After quality control with capillary electrophoresis, we performed cell-free reduced representation bisulfite sequencing (cf-RRBS) and shallow WGS (sWGS), each on 10 ng of cfDNA. The cf-RRBS methylomes were decomposed according to cellular make-up using a non-negative least squares method. As reference set, publicly available methylome data from primary tumors was used. Copy number aberrations were called on the sWGS data with WisecondorX. Full methods are available on biorxiv (https://doi.org/10.1101/795047).

Results

The copy number aberrations on the liquid biopsy sample both from the sWGS and methylation profiling data are consistent with the SNP array on the primary tumor (1p deletion, *MYCN* amplification and 17q gain). Decomposition and classification of the cfDNA methylome further supports the diagnosis of neuroblastoma, with approximately 60% of the total cfDNA matching the neuroblastoma reference set. Based on these results, bone marrow of the patient was further investigated with RT-qPCR for expression of TH, PHOX2B, CHRNA3 and GAP43 further supporting neuroblastoma diagnosis.

Summary/Conclusions

In this diagnostically challenging case of a patient with a small blue round cell tumor showing little to no lineage specific markers we conclude to a neuroblastoma diagnosis based on the cfDNA copy number and methylation data. This case is an example of how liquid biopsy-based diagnosis can contribute to imaging, classical histopathology and molecular genetics.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 372

Inhibiting PHGDH counteracts chemotherapeutic efficacy against MYCN-amplified neuroblastoma

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

While directly targeting the *MYCN* oncogene remains a challenge, indirect approaches that target its binding partners or downstream effectors yield encouraging results.

Aims

We set out to identify metabolic alterations specifically associated with amplified *MYCN*.

Methods/Materials

We performed liquid chromatography mass spectrometry-based proteomics and pulsed stable isotope-resolved metabolomics (pSIRM) after labeling with ^{13}C-glucose. The *CRISPR/Cas9 technology was employed to generate single cell-derived PHGDH knockout clones.* Functional studies were performed in cell culture and patient-derived neuroblastoma xenografts in mice.

Results

We analyzed the proteome of a representative cohort of 49 primary neuroblastoma biopsies, 13 neuroblastoma cell lines and the genetically engineered clone SH-EP-Tet21/N with adjustable MYCN levels. For each cell type, ~2.000-2.300 proteins were quantified. In total, 230 proteins significantly correlated with high-level MYCN expression in the biopsies and 73 in the cell lines. Altogether, 9 proteins consistently correlated with MYCN in both models, among them phosphoglycerate dehydrogenase (PHGDH), the rate-limiting enzyme in *de novo* serine synthesis (tumor cohort, Spearman correlation coefficient r=0.86; cell line panel, r=0.71). High-level *PHGDH* mRNA expression in primary neuroblastomas correlated in two independent patient cohorts (Kocak cohort, n=476; Versteeg cohort, n=122) with unfavorable patient survival and established clinical and molecular markers for unfavorable tumor biology. We analyzed changes in PHGDH expression on transcriptional and translational levels occurring in the synthetic MYCN-inducible system, SH-EP-Tet21/N, and found that conditional MYCN expression induced PHGDH expression on mRNA and protein level. We performed ChIP qRT-PCR to test whether MYCN associated with the *PHGDH* promoter and found an enrichment to two regions previously shown to be enriched for PHGDH in existing ChIP Seq data, altogether arguing for a role of MYCN in the transcriptional regulation of *PHGDH* in neuroblastoma cells. pSIRM and absolute quantitative GC-MS analysis demonstrated a higher ^{13}C-glucose incorporation in *de novo* serine synthesis in *MYCN*-amplified cells compared to normal *MYCN* diploid cells. While serine and glycine starvation did not affect proliferation and nucleotide pools of *MYCN*-amplified cells, proliferation of normal *MYCN* diploid cells was attenuated and nucleotide pools decreased, altogether pointing towards an independence of *MYCN*-amplified cells from exogeneous serine and glycine supply. *Proliferation of 5 single-cell derived CRISPR/Cas9-mediated PHGDH knockout clones was attenuated compared to that of MYCN-amplified control cells.* Treatment of *MYCN*-amplified neuroblastoma cells with two PHGDH small molecule inhibitors likewise arrested proliferation without affecting cell viability. Treatment of NMRI-Foxn1$^{nu/nu}$ mice carrying subcutaneous patient-derived *MYCN*-amplified neuroblastoma xenografts with PHGDH inhibitors as single-agent therapy slowed down tumor growth. The 2-drug combinations PHGDH inhibitor/cisplatin revealed an antagonizing effect on chemotherapy efficacy *in vivo*.

Summary/Conclusions

Our results indicate a strong correlation between amplified *MYCN* and high-level PHGDH expression in neuroblastoma cells. PHDGH knockout and inhibition by small molecules consistently slows proliferation, but stops short of killing the cells, which then establish resistance to classical chemotherapy. Although PHGDH inhibition with small molecules has produced encouraging results in other preclinical cancer models, this approach must be considered with caution in neuroblastoma. Studies investigating potential metabolic rerouting by PHGDH inhibition are ongoing.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 373

Exploration of PHF6-RRM2 interaction as functional 'replitase' protein complex and its role in replicative stress control in neuroblastoma

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Topic Gene regulatory networks and epigenetics: from basic science to therapy

Background/Introduction

PHF6 encodes a plant homeodomain (PHD) factor containing two imperfect PHD zinc-finger domains. Insights into its multiple functions recently started to emerge. PHF6 germline mutations causes cognitive disabilities while somatic mutations are frequent in T-cell acute leukemia (T-ALL). We and others found evidence for a role in self-renewal and lineage commitment during differentiation. Recent work revealed a role in non-homologous end joining, G2 checkpoint recovery and fork restart. We identified the ribonucleotide reductase subunit M2 (RRM2) as top PHF6 interacting protein in T-ALL by IP-MS. RRM2, involved in dNTP production, plays a crucial role in DNA replication and repair and has been proposed to be part of a so-called 'replitase' that shuttles to the nucleus to secure dNTP concentrations during fork repair. In view of our discovery of RRM2 as a crucial dependency gene in neuroblastoma (NB), further insights into its regulation in NB cells could be critically important.

Aims

We aimed to expand our IP-MS analyses of PHF6 to NB cell lines and to further explore the role of the presumed PHF6/RRM2 replitase in NB.

Methods/Materials

IP-MS analysis was done on four NB cell lines. Interaction for top ranked proteins is investigated by proximity ligation assays and co-localization experiments using immunofluorescence. The

auxin-inducible degron technology was used to conditionally degrade PHF6 in the colon cancer cell line HCT116 as a model and further tested in NB. Transcriptome (RNA-seq), genome-wide open chromatin (ATAC-seq) and protein-DNA interaction mapping (CUT&RUN) is being performed upon PHF6 knock down.

Results

Additional IP-MS analysis confirmed RRM2 as top PHF6 interactor in NB and also identified topoisomerase 2B (TOP2B, implicated in transcription and chromatin architecture), two components of the nuclear pore complex (RANBP2 and ELYS, implicated in nuclear transport, DNA replication and response to replicative stress), as additional high ranked interactors. These findings suggest that PHF6 may act at the nuclear pore complex, possibly to control transcription and transcription-coupled DNA repair of highly transcribed cell identity genes. Interestingly, differential gene expression analysis after PHF6 depletion revealed an enriched gene set related to binding of PHF6 to the histone modification H3K56ac. H3K56ac is predominantly present at newly transcribed regions and is required for cell cycle re-entry after DNA repair. Based on these findings, we hypothesize that PHF6 chromatin binding and association with H3K56ac is required to shuttle RRM2 towards sites of DNA repair and to subsequently release cells from checkpoint inhibition, in line with the role of PHF6 in fork restart. In addition to the H3K56ac set, we also found enrichment for ribosome biogenesis gene sets, in keeping with previously attributed functions of PHF6. Finally, also a strong correlation with MYC and TP53 targets was observed.

Summary/Conclusions

We confirm PHF6-RRM2 interaction in NB cells and propose that PHF6 is a component of the ‘replitase’, implicated in active regulation of RRM2 function during replication and DNA repair. In addition, we propose a role for PHF6 in the gene gating process at the nuclear pore facilitating high level transcription and nuclear export for transcripts of cell identity genes.

Exploration of PHF6-RRM2 interaction as functional ‘replitase’ protein complex and its role in replicative stress control in neuroblastoma

Type Basic

Presentation Preference Oral presentation

Abstract nr. 374

Outcome of anti-GD2 immunotherapy in Dutch high-risk neuroblastoma patients

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

In high-risk neuroblastoma (HR-NBL), approximately 50% of patients suffer from recurrent disease. Anti-GD2 immunotherapy has been shown to reduce the number of recurrences in the COG cohort.

Aims

The aim of this study was to compare the outcome of a Dutch HR-NBL cohort treated with maintenance anti-GD2 immunotherapy to patients treated with single-agent isotretinoin maintenance therapy after GPOH04/DCOG04 or POG9640 induction treatment.

Methods/Materials

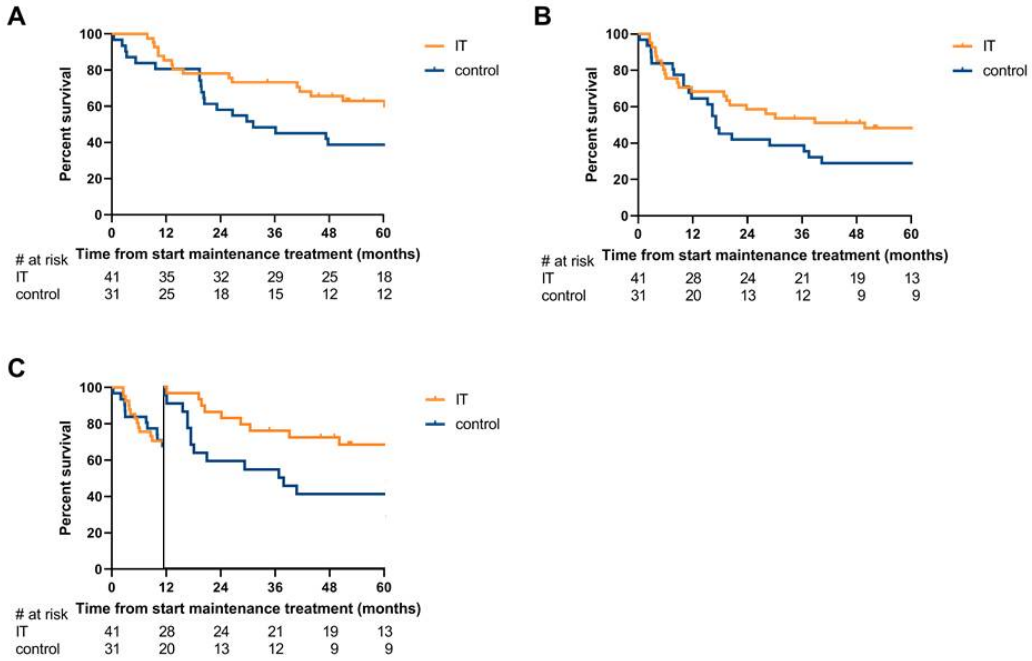
Included in the cohort were Dutch HR-NBL patients treated with immunotherapy between 2009 and 2016 according to the prospective ANBL0032 study (n=47, referred to as the immunotherapy group). They were compared to a historic control group treated with identical induction therapy between 2000 and 2015 with single-agent isotretinoin maintenance therapy (n=37, referred to as the control group). Data were collected of clinical and histological patient characteristics and response to treatment before autologous stem cell transplantation. Survival time was calculated for both groups from start of the maintenance therapy up to an event or the date of last contact. To investigate the effect of immunotherapy on late recurrences in more detail, we performed a landmark analysis at 11 months, when the event-free survival curves started deviating.

Results

The patients had a median age at diagnosis of 41 months (range 1-196). The study and control group were comparable for baseline characteristics. In this small cohort, a non-significant 14% improved 5-yr overall survival (OS) in favor of the immunotherapy group (immunotherapy 63±7%; control 49±8%; p=0.17) and a 10% improved 5-yr event free survival (EFS) (51±7% vs. 41±8%; p=0.39) were observed. For patients ≥18 months, there was a significant 24% 5-yr OS benefit (63±8% vs. 39±9; p=0.04; Figure 1A) and a non-significant 19% 5-yr EFS benefit for patients treated with immunotherapy (48±8% vs. 29±8%; p=0.12; Figure 1B). Landmark analysis showed a significantly better 5-yr EFS in the immunotherapy group compared with the control group for patients ≥18 months (68±9% vs. 41±10; p=0.03; Figure 1C), indicating the prevention of late relapses.

Summary/Conclusions

This study confirms the results of the COG study ANBL0032 that anti-GD2 immunotherapy improves the OS and EFS in patients with HR-NBL. Our small cohort, with a different induction treatment compared to the COG, had a 14% 5-yr OS benefit for patients treated with immunotherapy. We further demonstrated an immunotherapy-induced reduction of late recurrences in patients ≥18 months.



Five-year OS (A), EFS (B) and EFS with landmark at 11 months (C) for patients ≥18 months, according to treatment group. Abbreviation: IT=immunotherapy

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 375

Relationship between local control and quality assurance of radiotherapy. A report from the radiotherapy committee of the SIOPEN High Risk Neuroblastoma Trial (HR-NBL1/SIOPEN)

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

In high risk metastatic and MYCN-amplified localised neuroblastoma, radiotherapy (RT) is part of the multimodality treatment approach and plays an important role to increase local control. Since inappropriate target volume delineation or dose delivery may have a negative impact on local control in irradiated patients, radiotherapy quality assurance (RTQA) is increasingly recognized as an essential component in the management of clinical trials. A retrospective RTQA analysis of a subset of cases irradiated in the HR-NBL1/SIOPEN trial has been performed and correlated with local control data.

Aims

The aims of this analysis were 1) to perform RTQA based on imaging and RT data in high risk neuroblastoma patients and 2) to investigate and correlate local control data with the RTQA outcome results in these patients.

Methods/Materials

135 patients with complete imaging and radiotherapy data sets and local control information treated in the HR-NBL1/SIOPEN trial between 2002 and 2018 were analyzed. All patients received irradiation according to the HR-NBL1/SIOPEN trial protocol after induction chemotherapy, surgery, high dose chemotherapy, and autologous stem cell reinfusion. RTQA was performed during sessions attended by at least three SIOPEN radiotherapy committee radiation oncologists. They assessed delineation of target volume and organs at risk, target dose coverage, and dose to organs at risk for all cases. Local control data were obtained from the HR-NBL1 database and verified in the patients' source files whenever possible.

Results

The majority of the patients (70%) were treated with two-field antero-posterior or parallel oblique techniques, although from 2010 onwards an increasing number of patients were treated with more conformal techniques, including intensity modulated radiotherapy and protons. 91% of patients received the per protocol prescribed dose of 21 Gy. In 30 of the 135 patients investigated, deviations from protocol with possible negative impact were found during RTQA. A significantly increased local recurrence rate of 43% (13/30) was found when RT had not been delivered according to the protocol guidelines, compared with 22% local recurrence rate (23/105) in patients treated with the correct target volume and dose (p =. 02, chi-square test).

Summary/Conclusions

These results confirm earlier observations from the SIOPEN radiotherapy committee regarding the importance of RTQA in the treatment of neuroblastoma. Further analysis in a larger patient population is currently being performed. These data suggest the need for centralized prospective RTQA prior to RT delivery to improve protocol compliance rates and possibly local control.
The SIOPEN HR-NBL2 trial will have mandatory prospective RTQA through the SIOPE Quality and Excellence in Radiotherapy and Imaging for Children and Adolescents with Cancer across Europe in Clinical Trials (QUARTET) platform. Of note, in this trial there is a randomisation for patients with residual disease after surgery to a higher boost dose up to 36 Gy, in comparison with tumour bed irradiation only of 21.6 Gy. In this setting prospective RTQA may be even more important since delineation of residual disease is not always straightforward, and the higher dose is more likely to exceed tolerance of nearby organs at risk.

Type Clinical

Presentation Preference Rapid Fire Poster Presentation

Abstract nr. 376

Towards optimizing combination therapy in ALK-positive neuroblastoma

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

Neuroblastoma (NB) is the most common extracranial solid tumor in early childhood with a poor clinical outcome in high-risk patients, underlining the need for novel therapeutic strategies. Recently, the receptor tyrosine kinase ALK (anaplastic lymphoma kinase) has been shown to be a promising therapeutic target in neuroblastoma therapy and small-molecule inhibitors are in clinical use as monotherapies. However, an optimal integration of these therapeutics into the standard first-line chemotherapy has yet to be established.

Aims

In this study, we aim at obtaining a general understanding of the inhibitory properties of the 3rd generation ALK inhibitor lorlatinib as monotherapy as well as in the combination treatment with standard chemotherapeutics.

Methods/Materials

We determined the response to lorlatinib of a panel of NB cell lines, including ALK amplification, mutation (F1174L and R1275Q) and wild-type, by measuring cell viability in a dose dependent manner. The effect of lorlatinib on ALK downstream targets and cell cycle were determined by western blot and flow cytometry respectively. In addition, we performed RNA-sequencing on a highly sensitive cell line to gain insights into transcriptome changes at lorlatinib treatment. Finally, we tested a combination treatment of lorlatinib with two chemotherapeutics, vincristine and cisplatin, using a selection of our initial cell line panel.

Results

In our drug response assay, we observed a high sensitivity to lorlatinib in the ALK amplified cell line NB-1, whereas ALK mutated cell lines showed an intermediate response, wild-type cell lines

were non-responders. Thus, an increased ALK expression and activation is essential for an effective lorlatinib treatment which was confirmed by the analysis of the ALK downstream targets AKT and ERK1/2. Furthermore, cell cycle analysis of the responder cell line NB-1 revealed a cell cycle arrest at lorlatinib treatment. This finding was confirmed by our transcriptome analysis for early treatment time points. Finally, combination treatment of lorlatinib with vincristine or cisplatin showed a reduction in viability for the ALK-addicted cell line NB-1. We are currently investigating this combinatorial approach in a 3D spheroid context.

Summary/Conclusions

Our data suggests that ALK-addiction and the response to ALK inhibition by lorlatinib is not necessarily linked to the mutation status of ALK and that highly sensitive cells could benefit from a simultaneous combination treatment of lorlatinib with first-line chemotherapy. Further investigation will be necessary to find optimal combination schedules.

Type Translational

Presentation Preference Traditional poster

Abstract nr. 377

Epigallocatechin-3-gallate is a specific inhibitor of the LIN28B/let-7 axis in neuroblastoma

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

LIN28B is an RNA binding protein extensively overexpressed in Neuroblastoma (NB) compared to other tumour types. Its expression in the sympathoadrenergic lineage of mice reproduces the human disease, underlying the importance of LIN28B in NB pathogenesis. One function of LIN28B is to prevent the maturation of miRNAs belonging to the let-7 family, important tumour suppressors able to induce inhibition of proliferation and of terminal differentiation. Therefore, the LIN28B-let7 axis can be considered a novel therapeutic target for NB treatment.

Aims

We propose that interfering, using small molecules, with the LIN28B/let-7 miRNAs axis will lead to a block of LIN28B activity and consequently to an increase of let-7miRNA levels. This, given sufficient time, would promote cell differentiation and thus the reduction of NB aggressiveness.

Methods/Materials

We performed a screening of small molecules using the amplified luminescent proximity homogeneous assay technology (Alpha Screen). The validation of the hits was carried out using the Alpha Screen and the RNA electrophoretic mobility shift assay (REMSA). We then analysed the activity of the selected molecule on NB cell lines. We firstly constructed dose-response curves to evaluate the cytotoxicity of EGCG and choose different concentrations for the treatments.

Therefore, we evaluated through RT-PCR the variation of let-7 miRNAs after the treatment

Results

In order to identify molecules able to inhibit the LIN28B/ let-7miRNAs interaction, we screened a library of 5000 compounds. After the validation of the hits, we selected two molecules as candidate inhibitors: epigallocatechin-3-gallate (EGCG) and theaflavin-3-gallate (TFG), the last being also a metabolic derivative of the first in the liver. We synthesized some analogues of the two hits and assayed their activity *in vitro*. All the derivatives presented some variable degrees of activity, but still EGCG resulted to be the most effective compound. Therefore, we decided to validate its effect on NB cell lines. Our results showed a dose and time-dependent effects on cell viability, accompanied by the expected increase the level of let-7 miRNAs. Since it is reported that EGCG presents an unfavourable stability in solution, we attempted to improve the activity of EGCG encapsulating the molecule in PLGA-PEG nanoparticles. Using this vector we detected a significant increase in molecular activity.

Summary/Conclusions

EGCG is a well-known natural product already used in different applications, especially for its reported antioxidant activity. However, despite the promising biochemical results obtained, when we tested the molecule on NB cell lines the activity was lower than expected. This could be due to the low stability of the molecule in solution, as reported in the literature. Therefore, we enclosed the molecule in PLGA-PEG nanoparticles and assessed if its biological activity could be improved. EGCG biological effects in NB cells significantly increased when EGCG was enclosed in nanoparticles. EGCG-nanoparticles induced a higher increase of let-7miRNAs and reduction of cellular growth, even at low concentrations. In the next future, we will perform experiments aimed at investigating the activity of EGCG-nanoparticles in synergism with other drugs. We will then evaluate the efficacy of the molecule in vivo using a suitable mouse model.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 378

Evaluating combination therapy of a bispecific trifunctional antibody and checkpoint inhibition in neuroblastoma models

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

Despite advances in treating high-risk neuroblastoma that include combining the monoclonal anti-GD2 antibody ch14.18 with colony stimulating factor 2 (CSF2), interleukin 2 (IL2) or isotretinoin, 50-60% of patients still suffer relapse. Bispecific trifunctional antibodies (trAbs) are a promising innovation in the field of immunotherapies. They are heterodimeric IgG-like molecules that bind to T cells via CD3 and a tumor-associated antigen (TAA) as well as being able to activate antigen-presenting cells via their functional Fc region. A “Tri-cell complex” is created with the tumor cell, T cell and antigen-presenting cell, which induces a tumor-specific immune response. Combination therapy with checkpoint inhibitors, to prevent tumor cell immune escape, has been demonstrated to further improve trAb efficacy in a preclinical mouse model of melanoma.

Aims

We aimed to evaluate whether immune checkpoint signaling was activated by treatment with the GD2-directed trAb SUREK in neuroblastoma preclinical models, and whether a vaccination effect can be induced in a syngeneic immunocompetent neuroblastoma mouse model by simultaneous administration of irradiated murine neuroblastoma cells and SUREK.

Methods/Materials

The bispecific trifunctional antibody SUREK binds to the GD2 disialoganglioside on

neuroblastoma cells and murine Cd3. The anti-GD2 monoclonal antibody ch14.18 and the tumor-unspecific trifunctional antibody TRBs012 (alphaviurs E1 x anti-mouse-Cd3) served as controls. The murine neuroblastoma cell line NXS2 (stably transduced with a firefly luciferase) was co-cultured with bone marrow-derived dendritic cells and T cells before *in vitro* exposure to SUREK or control antibodies and/or checkpoint inhibitors. Cytotoxicity was assessed using a luciferase-based cytotoxicity assay. Cytokine release by T cells was detected via ELISA. T cell activation and expression of inhibitory receptors were assessed flow cytometrically. The immunocompetent syngeneic neuroblastoma mouse model of NXS2 cells in A/J mice was also used. We vaccinated mice intraperitoneally with 1 x 10^5 irradiated NXS2 cells and treated the animals with an intraperitoneal injection of 5 µg SUREK or control antibodies 21 and 7 days before animals were challenged with 1 x 10^6 viable NXS2 cells (subcutaneous injection). Tumor growth and survival were used to evaluate treatment efficacy.

Results

The trAb SUREK killed significantly more NXS2 cells than ch14.18 or TRBs012 *in vitro* and this cytotoxic efficacy was dependent on the presence of dendritic cells. Treatment with SUREK upregulated proteins involved in immune checkpoint signaling, including the immune checkpoint receptors programmed cell death protein 1 (Pdcd1) and lymphocyte activation gene 3 (Lag3) on T cells in co-culture with dendritic and NXS2 cells. Administration of irradiated tumor cells and treatment with SUREK prevented engraftment of subcutaneously injected tumor cells and significantly lengthened survival compared to the control groups, creating a vaccination effect.

Summary/Conclusions

Here we provide preclinical evidence that immune checkpoint signaling is induced by treatment with the trAb SUREK and for a specific vaccination effect induced by SUREK in an immunocompetent syngeneic neuroblastoma mouse model. Our results support a rationale to test combination of the trAb SUREK with checkpoint inhibitors to further improve efficacy.

Type Translational

Presentation Preference Rapid Fire Poster Presentation

Abstract nr. 379

19p loss is significantly enriched in older age neuroblastoma patients and correlates with poor prognosis

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Genomic aberrations of neuroblastoma occurring in late childhood and adolescence are uncommon and still underestimated. Deletions of chromosome 19p have been previously reported in older age neuroblastoma patients. However, to date, no large cohort has been investigated to verify whether the presence of 19p loss is observed by chance or it significantly correlates with the occurrence of neuroblastoma after a certain age.

Aims

We used copy number data of two large and independent data sets to verify if 19p loss is significantly over-represented in children and adolescents.

Methods/Materials

Public DNA copy number profiles of 556 tumors (discovery set) and of 208 tumors obtained by array-CGH assay (validation set) were used. The 19p loss occurrence was separately tested within different age groups in the discovery and validation set and the resulting P values were combined and corrected by Bonferroni’s method.

Results

In both sets, the lowest age group significantly associated with 19p loss (discovery set: 20%; validation set: 35%) was 6 years. The 19p loss was considered to be a significant marker for

overall survival in patients over 6 years of age. Relevant tumor suppressor genes (*KEAP1*, *DNM2* , *SMARCA4, SLC44A2* and *CDKN2D)* and microRNAs (miR-181c, miR-27a, and mirR-199a-1) were in 19p loss. Down-regulation of *DNM2*, *SLC44A2* and *CDKN2D* associated with poor patient outcome and older age. Among the recurrent neuroblastoma chromosomal aberrations, only 1q gain was enriched in patients older than 6 years and was mutually exclusive respect to 19p loss.

Summary/Conclusions

Our data demonstrate that 19p loss is a genomic biomarker of neuroblastoma diagnosed in older children that can predict clinical outcome.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 380

Alternative splicing in neuroblastoma generates RNA-fusion transcripts and is associated with vulnerability to spliceosome inhibitors

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Topic Gene regulatory networks and epigenetics: from basic science to therapy

Background/Introduction

The paucity of recurrent mutations has hampered efforts to understand and treat neuroblastoma. Aberrant splicing and splicing dependent RNA-fusion transcripts have been implicated in other types of cancer, however their role in neuroblastoma remains largely unexplored.

Aims

We aim to investigate the presence and possible role of aberrant splicing and splicing dependent RNA-fusion transcripts in neuroblastoma. In addition, we attend to establish whether the spliceosome can be targeted to treat neuroblastoma.

Methods/Materials

Bioinformatic analysis of RNA-sequenced neuroblastoma datasets was performed to elucidate risk-associated changes in expression levels of spliceosome factors and to identify novel RNA-fusion transcripts. Comparative analysis with other types of tumors and non-transformed tissue was executed to confirm neuroblastoma specificity of identified fusion candidates. One candidate was selected for functional characterization in an *in vitro* assay of neuronal differentiation. Splicing

activity was attenuated in neuroblastoma cells by the spliceosome inhibitor, Pladienolide B. PCR with primers spanning fusion junctions was performed to elucidate the impact of attenuated splicing on the expression of fusion transcripts compared to their wild type cognates. To understand if targeting the spliceosome can reduce tumor growth in a preclinical model, we treated mice that had been xenografted with neuroblastoma cells with the Pladienolide B spliceosome inhibitor.

Results

Elevated expression of splicing associated factors was a strong predictor of high-risk neuroblastoma and reduced patient survival. We identified >900 primarily intrachromosomal RNA-fusion transcripts generated by genes in close proximity. Fusions were enriched in chromosomal regions gained or lost in neuroblastoma and included well-known neuroblastoma oncogenes. As a proof-of-principle that a gene product with altered properties can be produced by these fusions, we characterized the *ZNF451-BAG2* fusion which generates a truncated BAG2-protein capable of inhibiting retinoic acid-induced differentiation. A majority of fusions contained canonical splicing sites and a subset exhibited increased sensitivity to spliceosome inhibition resulting in loss of expression after treatment. This effect was accompanied by selectively induced apoptosis in neuroblastoma cells *in vitro* as well as reduced growth of neuroblastoma xenografts upon treatment with the spliceosome inhibitor Pladienolide B.

Summary/Conclusions

Our findings elucidate a splicing dependent mechanism able to generate altered gene products, relevant for neuroblastoma pathogenesis and representing possible novel drug targets. In addition, we show that targeting the spliceosome is a potential strategy to treat neuroblastoma.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 381

Combined targeting of the p53 and pRb pathway in neuroblastoma

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

Clinical complete remission is achieved in many stage 4 neuroblastoma patients, but the high risk of relapse and the accompanying treatment-resistant nature of these tumors is still a challenge. We have previously identified higher frequencies of mutations that affect both the p53 and the pRb pathway, such as the homozygous loss of *CDKN2A* or co-amplification of *MDM2*and *CDK4*. In addition, both cyclin D1 and MDM2 overexpression is a common characteristic of primary neuroblastoma. These genes act upstream of Rb and p53, respectively, and hence play a major role in cell cycle regulation.

Aims

Our aim was to study whether the aberrations in the p53 and pRb pathway render cells more sensitive to CDK4 or MDM2 inhibition. Moreover, we were interested in whether combined inhibition of CDK4 and MDM2 would be of added value.

Methods/Materials

We exposed a series of 11 cell lines to the most promising CDK4 (ribociclib, palbociclib and abemaciclib) and MDM2 (SAR405838, HDM-201 and idasanutlin) inhibitors for 72 hours. We selected cell lines with well-defined mutational properties: homozygous *CDKN2A* deletion, co-amplification of *CDK4* and *MDM2*, *TP53* mutation and/or *MYCN* amplification. Moreover, CHP134 cells with inducible overexpression of CDK4 and/or MDM2 were generated to test our findings in an isogenic system. The effect of single compound and combined inhibition on cell viability and the cell cycle was studied using 10 different concentrations of abemaciclib and idasanutlin. This combination was also tested in an *in vivo* neuroblastoma xenograft model.

Results

Sensitivity for the MDM2 inhibitors inversely correlated with *TP53* mutation status, but surprisingly, it did not correlate to other p53 pathway aberrations (*MDM2* amplification/*CDKN2A* deletion). Similarly, there was no clear correlation seen for the CDK4 inhibitors with G1 checkpoint aberrations (*CDK4* amplification/*CDKN2A* deletion). Induced overexpression of CDK4 and/or MDM2 in CHP134 also did not increase sensitivity to CDK4 or MDM2 inhibitors. Bliss Independence values, calculated as a measure of synergy after combined treatment with abemaciclib and idasanutlin, revealed no synergistic effect. Instead, we saw an additive to slightly antagonistic effect in most cell lines. This adverse effect was supported by cell cycle analyses, which showed a lower apoptotic fraction, as well as PARP and caspase 3 cleavage, after combined treatment, as opposed to MDM2 inhibition alone. The *in vivo* study supported these results and showed reduced tumor growth after abemaciclib or idasanutlin treatment. Although combined treatment resulted in stable tumor volumes, the combined effect was not synergistic.

Summary/Conclusions

As was previously suggested in the first clinical trials with CDK4 inhibitors, neither *CDK4* nor *CDKN2A* status are clear biomarkers for CDK4 inhibitor sensitivity. Our results suggest that *MDM2* and *CDKN2A* status also fail as biomarkers for MDM2 inhibitor sensitivity. Further testing is necessary to identify (other) biomarkers for these inhibitors. Moreover, the logical rationale to combine CDK4 and MDM2 inhibitors in neuroblastoma patients with both pRb and p53 pathway disturbances, should be taken with precaution.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 382

Combined Loss of 1p36 Gene KIF1Bbeta and NF1 Causes Neuroblastoma, Pheochromocytoma and Composite Tumors in Mice

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Topic Neuroblastoma as developmental disease

Background/Introduction

We recently discovered a tumor suppressor gene KIF1Bβ located on 1p36 that is required for sympathoblast differentiation and developmental apoptosis during NGF competition. Thus, we generated conditional gene targeting of KIF1Bb in the mouse sympatho-adrenal lineage and generated crosses with mice expressing Cre-recombinase under the control of the gene for dopamine beta hydroxylase (DBH-Cre). We discovered that KIF1Bβ is required for nerve growth factor (NGF)-dependent neuronal differentiation through anterograde transport of the NGF receptor TRKA and that pathogenic KIF1Bβ mutations identified in neuroblastoma impair TRKA transport. Although the expression of neuronal differentiation markers is ablated in KIF1Bβ-deficient mouse neuroblasts, our KIF1Bfl/fl;DbhCre mice did not develop any symapthoadrenal malignancies. We predicted that loss of KIF1Bb alone might not be sufficient to cause neuroblastoma in the KIF1Bβfl/flCRE mice. Recently, a novel pathogenic KIF1Bb missense mutation together with a NF1 loss of function mutation was identified in a patient diagnosed with neural crest tumors and loss of NF1 has been associated with neuroblastoma and pheochromocytoma. We therefore asked if loss of KIF1Bb together with loss of NF1 will mirror neuroblastoma and pheochromocytoma development.

Aims

Developing novel neuroblastoma and pheochromocytoma mouse models for identifying cancer cell of origin.
Profiling tumor heterogeneity and immaturity on single cell resolution of mouse neuroblastoma and pheochromocytoma tumors.

Methods/Materials

We generated homozygous KIF1BβdbcKO mice and used tumors for histological, immuno-histochemical, and cytogenetic studies to characterize proliferation and differentiation status. We perfused and dissected tumors and adrenal glands, FACS-sorted approximately 400 single cells per tumor/gland and performed single cell sequencing. We performed targeted in situ sequencing (ISS), allowing the generation of targeted, multiplexed expression profiles within fixed tumor tissues at single-cell resolution.

Results

We observed sympathetic ganglion (SCG) hyperplasia in the first month after birth and tumors arising at the adrenal gland of adult double cKO and NF1cKO mice. Double-cKO mice developed neuroblastoma, pheochromocytoma and composite tumors originating from immature chromaffin cells as were control breeding have not resulted in such tumor promoting phenotype. We profiled tumor heterogeneity on single cell resolution. Single cell analysis of the tumors are currently analyzed with respect to regulatory patterns identified in early or late embryonic mouse sympathoblast /crest populations.
Single-cell transcriptomics requires isolation of discrete cells and information about the original location within tissue is lost. Therefore, it is not possible to draw cellular maps of the tumor architecture and their positioning in relation to other cells. In order to map the cells within their physiological, morphological, and anatomical context and space, we performed in situ transcriptomics in these tumors, a method that involves targeted in situ sequencing (ISS).

Summary/Conclusions

Our analysis of our double-cKO mice that develop both, pheochromocytoma and neuroblastoma, provokes the question if NB and PCC might have a common progenitor or if these are parallel events during sympatho-adrenal development.

Monika

Bedoya-Reina

Plescher

Combined Loss of 1p36 Gene KIF1Bbeta and NF1 Causes Neuroblastoma, Pheochromocytoma and Composite Tumors in Mice

Type Basic

Presentation Preference Oral presentation

Abstract nr. 383

Inhibition of the CD47/SIRP-alpha interaction to increase myeloid cell destruction of neuroblastoma

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

CD47 is a cell surface protein that binds SIRP-α on macrophages and dendritic cells to provide a “don’t eat me” signal inhibiting phagocytosis. CD47 is overexpressed in cancers, presumably to counteract pro-phagocytic signals such as tumour-specific antigens and cell surface stress markers. Antibodies targeting CD47 have shown efficacy in syngeneic mouse models of leukaemia, lymphoma and breast cancer. However, some solid tumours, such as melanoma, are resistant to phagocytosis even after CD47 blockade, and healthy erythrocytes are an antigen sink. Hence combining CD47 blockade with agents that induce immunogenic “eat-me” signals (chemotherapy and/or anti-GD2 antibody) may increase the efficacy of CD47 based therapies in neuroblastoma.
TTI-628 is a fusion of a mutant human SIRP-α protein that has increased CD47 affinity, fused with murine IgG2a Fc. A fully human equivalent with minimal erythrocyte binding is currently in Phase I trials in adults

Aims

To assess the ability of TTI-628 to induce phagocytosis of murine neuroblastoma cells *in vitro* and to synergise with temozolomide and anti-GD2 antibody therapy *in vivo*.

Methods/Materials

Transgenic Th-MycN$^{+/-}$ Th-ALK$^{F1174L/-}$ mice were backcrossed 6 generations from the parental strain 129X1/SvJ to C57BL/6. Spontaneous abdominal tumours were harvested, dissociated and

implanted subcutaneously to generate *in vivo* passaged tumours. These tumours were maintained *in vitro* as tumourspheres and injected into further mice for therapeutic trials with temozolomide and anti-GD2 antibody (14G2a) with or without TTI628. Flow cytometry was used to assess the GD2 and CD47 expression of tumourspheres, and the leukocyte infiltration of engrafted tumours. To assess phagocytosis, bone marrow derived macrophages were co-cultured for 2 hours with either pHrodo labelled Staph Aureus bioparticles, or CFSE labelled dissociated tumourspheres from the same strain. The proportion of either pHrodo or CFSE positive macrophages was assessed by flow cytometry.

Results

Tumourspheres were weakly positive for GD2 and strongly positive for CD47. In the absence of either 14G2a or TTI-628 no phagocytosis was observed *in vitro*. A dose dependent increase in phagocytosis was seen for both 14G2a and TTI-628 monotherapies. No difference was observed in Staph Aureus phagocytosis of bone marrow-derived macrophages from 129X1/SvJ and C57BL/6 strains. However preliminary data suggest C57BL/6 macrophages are more effective in antibody mediated phagocytosis than 129X1/SvJ ones. Syngeneic C57BL/6 tumours from pilot *in vivo* experiments reveal variable leukocyte infiltration, including macrophages ($CD45^{+}CD11b^{+}F4/80^{+}$). Survival studies are ongoing.

Summary/Conclusions

CD47 and GD2 based therapies induce phagocytosis of neuroblastoma by syngeneic macrophages *in vitro*. Tumour allograft studies using Th-MYCN-ALK mice in 129X1/SvJ and C57BL/6 backgrounds provide immune competent platforms with different myeloid cell properties for evaluating a novel approach to chemoimmunotherapy using trial-ready reagents.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 384

A multicentric retrospective study on local control in children with high risk neuroblastoma treated with radiotherapy in Belgium

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

External radiotherapy is part of the multi-modality treatment of high risk neuroblastoma. Although most patients with high-risk neuroblastoma die from uncontrolled metastatic disease, improvement of local control may also improve overall survival.

Aims

The aim of this retrospective study is to analyse the local control and overall survival obtained in the Belgian cohort of patient treated for a high risk neuroblastoma.

Methods/Materials

We identified 109 patients treated in Belgium between May 2002 and September 2018, of whom 92 could be analysed. 75 did not progress during the preceding treatment with induction chemotherapy, surgery, high dose chemotherapy, and autologous stem cell infusion and were treated with radiotherapy to a dose of 21 Gy on the tumour bed. We retrospectively analysed the medical records and treatments of all these patients, in collaboration with the 5 Belgian paediatric radiotherapy centres.

Results

With a median follow-up of 3,6 years [0,0-16,3 years] of the global cohort of 92 patients, the 5-year overall survival (OS) was 52,6% ± 5,4% and the 5-year event free survival (EFS) was 50,8% ± 5,3%. Amongst the 75 irradiated patients, the 5-year local recurrence (LR) was 12,3% ± 3,8%, with a median follow up of 4,4 years [0,8-16,3 years]. LR was significantly influenced by the resection status with a LR of 3,5% and 32,7%, respectively for patients with or without macroscopic complete resection (p=0,001). This translates into a significant difference in the 5-year OS of 59,2% and 27,3% for completely and incompletely resected patients respectively. Equally, OS was significantly influenced by the LR with a 5y-OS and mean OS of 62,2% and 10,08 years for patient without LR and 0% and 2,17 years for patient with LR (p<0,0001).

Summary/Conclusions

In our cohort of Belgian patients, overall survival in high-risk neuroblastoma is influenced by local control. Since radiotherapy can improve local control, there is a rational to use it to improve the

overall survival, potentially even more in patients with residual disease after surgery. Efforts should be made to enrol patients in prospective randomised trials in order to improve our knowledge on the benefit of radiotherapy in high-risk neuroblastoma and the best regimen to use. Of note, the future SIOPEN HR-NBL2 trial has a randomisation for patients with residual disease after surgery to a tumour bed dose of 21.6 Gy followed by a boost dose up to 36 Gy, compared to tumour bed irradiation only of 21.6 Gy.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 385

Paternal pre-conception stress as a risk factor for neuroblastoma.

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Topic Neuroblastoma as developmental disease

Background/Introduction

While neuroblastoma (NB) appears to have a genetic background, its etiology cannot be explained solely by genetics. Yet, the role of environment in its development remains understudied. Consequently, there are no established risk factors for NB aside from rare genetic predisposition and it is not clear what determines its malignancy. NBs arise due to defects in sympathetic neuron (SN) differentiation. We hypothesize that pathological states disrupting this process lead to accumulation of neuroblasts, which in conjunction with preexisting or new genetic aberrations promotes NB formation. Since these defects in SN differentiation occur in utero, environmental factors triggering them are most likely to be prenatal or pre-conception exposures. Indeed, maternal stress during pregnancy induces neurodevelopmental defects in offspring. In line with this, we have previously shown that in a mouse model of NB, prenatal stress interferes with SN differentiation in offspring, leading to increased frequency and malignancy of NB. This effect was driven by activation of maternal sympathetic nervous system, which causes uterine vasoconstriction and transient fetal hypoxia. Strikingly, paternal stress prior to conception causes neurodevelopmental and behavioral changes in the offspring that are very similar to those caused by maternal stress during pregnancy. These paternal effects were associated with epigenetic changes in sperm and offspring, which are known to regulate both neuronal differentiation and tumorigenesis. The role of paternal exposures in NB has also been suggested by epidemiological data correlating increased risk of this disease with certain occupations in fathers.

Aims

The goal of the current study was to determine the impact of paternal stress prior conception on NB development and phenotype.

Methods/Materials

Hemizygous TH-MYCN males were subjected to 6 weeks of chronic unpredictable stress (CUS) prior to conception and then mated with wild type females. Sperm epigenetic profiles, placenta

morphology and NB frequency and phenotypes in TH-MYCN offspring were compared between the control and stress groups.

Results

CUS triggered changes in non-coding RNA content of the germline, including miRNAs and tsRNAs. Paternal stress decreased also the rate of successful pregnancies from 75% in controls to 37.5% and increased frequency of death in utero from 5.5% to 29.2%. The male TH-MYCN offspring of stressed fathers exhibited accelerated NB development and higher tumor frequency, as well as a more metastatic phenotype. In contrast, in female offspring, NB development remained unaffected by paternal stress, yet the tumors appeared to be more metastatic. In line with these data, we have observed morphological abnormalities in placentas of the stressed fathers' offspring, which suggested insufficient perfusion of the labyrinth tissue, the main site of the oxygen exchange between maternal and fetal blood. These changes were more profound in male fetuses, where they associated with increased area of the junctional zone of the placenta, which stained positively for hypoxia markers.

Summary/Conclusions

Our results indicate that paternal pre-conception stress affects placental development via epigenetic mechanisms, which in turn impairs SN differentiation and promotes development of aggressive NB in a sex-specific manner. Moreover, our data implicate fetal hypoxia as a common mechanism underlying impact of parental stress on NB risk.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 386

The BRIP1 17q dependency gene in neuroblastoma: from fork stability to translation

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Neuroblastoma (NB) is an aggressive childhood cancer marked by low mutational burden and highly recurrent DNA copy number aberrations, including 17q gain in most high-risk cases. We previously identified *BRIP1* (17q23.2), as a candidate non-mutated dependency gene based on integrated transcriptome, copy number and survival data. BRIP1 is a multifunctional protein with DNA helicase activity for unwinding complex DNA structures (including G-quadruplexes). It binds to BRCA1, TopBP1 and MLH1 and is implicated in replication fork dynamics and DNA repair. In addition, a recent study suggested a role in control of gene expression through binding of a regulatory long noncoding RNA.

Aims

We aimed to (1) independently validate our initial bioinformatic approach for identification of copy number driven dependency genes, using a new and more extensive transcriptome and copy number data set and to (2) explore BRIP1 dependency using *in vivo* modeling and knock down and overexpression in NB *in vitro* cellular models.

Methods/Materials

We applied an integrative computational analysis to perform network inference analysis on a large transcriptome and copy number data set of 219 NB patients. We performed *in vitro* functional assays, immunoblotting for DNA damage and response and DNA combing to measure replicative stress upon BRIP1 knock down.

Results

The computational analysis confirmed BRIP1 as co-driver in NB. In addition to extensive *in vitro* validation of BRIP1 dependency, pRPA and pCHK1 staining and DNA combing revealed strong effects on replication fork stalling while yH2AX and 53BP1 staining showed increased DNA damage following BRIP1 knock down in NB cells. For *in vivo* testing of dependency, we crossed a stable transgenic *dβh-BRIP1;dβh-mCherry* overexpressing zebrafish line with the *dβh-eGFP-MYCN* line and observed strong increased tumor penetrance ($p<0.0001$). Bulk and single cell RNA-sequencing were performed to gain insight into the transcriptional impact of BRIP1 overexpression in early tumor lesions (6 weeks) versus late (10 weeks or more) full blown tumors. Similar experiments are ongoing with LSL-*dβh-BRIP1* and LSL-*dβh-MYCN mice.* To assess which aspect of BRIP1 function drives dependency, we treated NB cells with forced BRIP1 overexpression with several compounds affecting DNA repair (triapine, talazoparib, ATRi, and CHK1i) and DNA (TYMPyP4) and RNA G-quadruplex (silvestrol) stability. Strong rescue effects were observed upon BRIP1 overexpression in TYMPyP4 and silvestrol treated NB cells, suggesting that a combined DNA and RNA helicase effect of BRIP1 could mediate tumor dependency. The latter, represents a hitherto largely unstudied novel property of BRIP1. Finally, we are currently testing a BRIP1 helicase inhibitor in a panel of NB cell lines and patient derived NB organoids and have initiated efforts towards the synthesis of a BRIP1 degrading drug (PROTAC).

Summary/Conclusions

Our *in vitro* and *in vivo* data support BRIP1 as a 17q copy number driven dependency factor in NB. We provide evidence that both DNA and RNA helicase function of BRIP1 is implicated in NB cell fitness, at least partly through controlling MYCN induced replicative stress and translation. We are testing targeted inhibition or degradation of BRIP1 as a novel option for drugging high-risk NB.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 387

ASPM is a regulator of neuroblastoma cell proliferation and differentiation

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Topic Neuroblastoma as developmental disease

Background/Introduction

ASPM is a microtubule-associated protein involved in mitotic spindle assembly during cell division. Biallelic mutations in *ASPM* are the most common cause of autosomal recessive primary microcephaly (MCPH), a neurodevelopmental disorder characterized by intellectual disability and reduced brain volume ensuing from defective neural stem cell proliferation. In the other extreme, elevated ASPM levels during development are associated with hyperproliferation and may, therefore, have a causative role in neuro-embryonal malignancies.

Aims

Due to their role in regulating proliferation in neuronal progenitor cells, we hypothesized that genes associated with MCPH, and particularly *ASPM*, might drive neuroblastoma cell proliferation. We aimed to evaluate whether *ASPM* overexpression constitutes an independent risk factor in neuroblastoma.

Methods/Materials

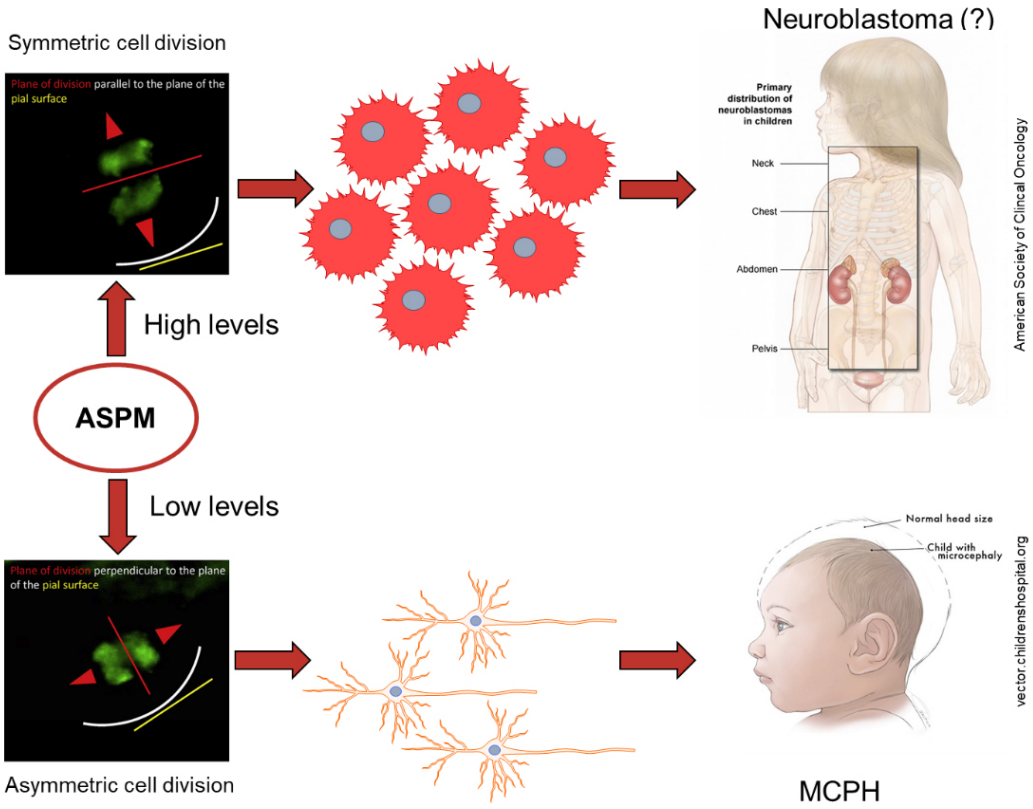
Expression of all genes associated with MCPH was analyzed in 25 publically available data sets from tumors arising in either adults or children/adolescents and normal tissues. The association of *ASPM* expression levels with clinical covariates and outcome was investigated in a large, representative neuroblastoma cohort (n=498). Event-free and overall survival in patients with tumors expressing high or low *ASPM* levels were compared using Kaplan-Meier analyses. Multivariate regression analyses based on Cox proportional hazard models were performed to assess the value of *ASPM* expression as a prognostic factor in the context of established neuroblastoma risk criteria (*MYCN* amplification, INSS stage, age, *TERT* expression). The effect of siRNA-mediated *ASPM* knockdown on cell viability, proliferation, cell death and differentiation was investigated.

Results

Comparisons of multiple microarray gene expression data sets revealed the highest *ASPM* expression levels to be in neuroblastomas and other neuro-pediatric tumors in contrast to tumors arising in adulthood and normal tissues. Elevated *ASPM* levels were significantly associated with adverse clinical outcome and unfavorable clinical covariates, indicating a functional role of ASPM in aggressive, undifferentiated neuroblastoma biology and a crucial function for proliferation. Multivariate regression analysis revealed that high *ASPM* expression constitutes an independent risk factor in addition to established neuroblastoma risk factors. *ASPM* knockdown in the NGP neuroblastoma cell line reduced cell viability and proliferation. Features of neuronal differentiation also appeared four days following ASPM knockdown, such as neurite-like outgrowths, cytoskeletal changes and increased expression of differentiation markers, including microtubule associated protein 2 (MAP2), tubulin beta 3 class III (TUBB3) and the gene encoding the neuronal migration protein, doublecortin (DCX). Differentiation-associated apoptosis was also flow cytometrically detected that accompanied reduced cell proliferation.

Summary/Conclusions

Our data are in accordance with ASPM playing a functional role in maintaining the undifferentiated phenotype of highly aggressive neuroblastoma cells. ASPM function in the spindle apparatus suggest spindle-checkpoint and spindle-assembly inhibitors may be potential treatment options for neuroblastomas with high-level *ASPM* expression.



Is the microtubule-associated protein ASPM a possible link between microcephaly and neuroblastoma?

Type Translational

Presentation Preference Oral presentation

Abstract nr. 388

A single cell atlas of the developing murine adrenal gland

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Topic Neuroblastoma as developmental disease

Background/Introduction

Neuroblastoma is the most common extracranial solid tumor, and contributes to ~15% of pediatric cancer related deaths. The exact cell of origin for this embryonal malignancy has yet to be elucidated, but the dogma of the field is that it is neural crest derived. About half of primary neuroblastomas arise in the adrenal gland.

Aims

The aim of this study is to describe mouse adrenal gland development at single cell level and to identify the different cell types present. This will provide insight on which signaling pathways are active in these cells, and what the microenvironment these cells are exposed to.

Methods/Materials

In this study we investigated the developing murine adrenal gland with single cell RNA (scRNA) sequencing, to identify the different cell types that are present as well as their gene expression signatures in an unbiased manner. The timepoints analyzed are E13.5, E14.5, E17.5, E18.5, P1, and P5. RNAscope, a single molecule fluorescent *in situ* hybridization technique, was used visualize the individual populations in adrenal gland tissue.

Results

Five main population of cells, originating from medulla, cortex, endothelial, stroma and immune cells, make up the mouse adrenal gland during development. The medulla population consists of six sub populations, the cortex of five sub populations and the stroma, endothelial and immune populations each having four, six and four sub populations, respectively. Two of the medulla populations are of special interest, a population of cells expressing markers well established for migrating neural crest cells (Sox10 and Erbb3), which we term the NC/SCP population, while a second population cycles actively and highly expresses Ccnd1 (the neuroblast populations). Both populations were the only medullar population found in every developmental time point analyzed. RNAscope shows that the NC/SCP cells co-localize with their progeny.

Summary/Conclusions

Investigating the adrenal gland with an unbiased approach of scRNA sequencing provides insight into the organ as a whole. In the developing adrenal gland, 5 main cell populations/clusters are present. The adrenal medulla is made up of 6 sub populations, with two populations of note, namely the NC/SCP and neuroblast population. This dataset will be a resource for future neuroblastoma research.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 389

Single cell RNA-sequencing analysis of tumor heterogeneity and dynamic adaptive transcriptome changes in a MYCN driven zebrafish model.

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

Neuroblastoma (NB) is a pediatric tumor of the sympathetic nervous system with poor survival for high-risk patients. High-risk NB often presents with segmental copy number alterations including *MYCN* amplification in half of the cases. Analysis of molecular events during early NB development in NB model systems are typically hampered by the limited accessibility and low number of tumor cells. To resolve this issue, we established a protocol for isolation of fluorescence based flow sorting of zebrafish NB cells using dβh*:eGFP* and dβh*:MYCN;*dβh*:eGFP* lines enabling the collection of a sufficient number of cells for bulk and single cell RNA-sequencing.

Aims

We aimed to explore the MYCN driven zebrafish NB model to unravel early transcriptional adaptive and dynamic changes in tumor cells and microenvironment during MYCN driven tumor formation using single cell RNA-seq technology.

Methods/Materials

eGFP-sorted cells were obtained from tg(dβh*:eGFP*) and tg(dβh*:MYCN;* dβh*:eGFP*) zebrafish at different time points. Cells were collected 6 days (early detectable sympathoblasts) and 6 weeks (putative hyperplastic lesions) post fertilization by eGFP based FACs sorting. For the MYCN overexpressing line, cells were also collected from established full-blown tumors. Single cell RNA-

seq was performed using the Chromium technology (10X Genomics). Initial analysis was performed by preprocessing with Cell Ranger, data was mapped using Alevin. Samples were integrated using SCIintegration and subsequent filtering and data analysis was performed using both Seurat and SCANpy.

Results

Zebrafish single NB cells showed a gene signature highly reminiscent of human MYCN amplified neuroblastoma which dynamically evolved over the three time points analyzed. This dynamic flux is currently under detailed study to identify key alterations occurring during tumor progression. Remarkably, we found two distinct tumor cell populations with enrichment for MYCN/MYC gene sets but with either high human MYCN transgene or zebrafish endogenous mycn expression, thus revealing an unexpected and hitherto not reported aspect of tumor heterogeneity in this model system. We also analyzed eGFP negative tumor associated cells representing tumor microenvironment derived cells. A comparative analysis with single cell RNA-seq data obtained from unsorted MYCN driven zebrafish tumors and human high-risk human NB samples is underway. We conclude that FACs sorting of tumor cells improved the analytical power of single cell analysis to more accurately map early tumor forming transcriptome alterations by avoiding dilution of signatures through non-tumor cell types.

Summary/Conclusions

We present for the first time a single cell based transcriptome landscaping of zebrafish MYCN driven neuroblastoma tumor heterogeneity and use this resource to model the dynamic and putative adaptive transitions in NB transcriptomes during the formation of established tumors. Using this data source, we will investigate the cell of origin and capture early genetic co-drivers.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 390

Application of minimally invasive surgery techniques in treatment of neuroblastoma

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

Major goals of surgical treatment of neuroblastoma are completion of resection and safety of patient. Simultaneously minimally invasive techniques are broader used in modern surgery. Application of minimally invasive techniques may fulfill both goals of treatment in neuroblastoma.

Aims

Analysis of single center experience in treatment of neuroblastoma with minimally invasive surgery techniques.

Methods/Materials

Between 2010-2019, 85 children underwent surgery for neuroblastoma. 12 of these were operated with usage of minimally invasive surgery techniques (9 with laparoscopy and 3 with thoracoscopy). These techniques were mostly used in infants and children under age of 4 with low grading of primary lesion up to 4cm of size, without IDRF, mostly situated in suprarenal area. After resection tumor was removed by mini-laparotomy or mini-thoracotomy.

Results

There were no intraoperative or post operative complication. Resections were macroscopically complete. Histopathology showed two cases of ganglioneuroma and ten cases of neuroblastoma. N-myc amplification was detected in one case. In 6 patients surgery was the only treatment, the follow up is 6 months up to 5 years. One patient (7%) occurred with local recurrence. One patient (7%) sufferd of metastatic disease. One patient (7%) with a tumor in the chest and abdomen, a

local recurrence appeared in the second body cavity

Summary/Conclusions

Minimally invasive surgery techniques are safe. Their effectiveness in treatment of neuroblastoma is still matter of reaserch but they seem to be used with success in some cases.

Type Clinical

Presentation Preference Traditional poster

Abstract nr. 391

Non-invasive treatment monitoring of neuroblastoma patients using copy number and methylation profiling of cfDNA

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

The genetic work up of neuroblastoma (NB) patients is currently based on the examination of tissue biopsies that only reflect a spatially limited snapshot of the primary tumor. Given that most tumors are heterogeneous, subclones may be overlooked. Furthermore, additional surgical sampling during therapy to monitor disease evolution is not possible for NB patients. Non-invasive sampling of blood (liquid biopsy) can resolve these issues and opens new possibilities for more accurate genetic work up and serial sampling for therapy response monitoring.

Aims

In this study, we aimed to investigate the DNA copy number and methylation profiles of cfDNA retrieved from plasma samples both at diagnosis and during follow-up of NB patients, in order to investigate the potential of cfDNA to measure therapy response and to detect early relapses.

Methods/Materials

We have collected cfDNA from diagnostic and follow-up samples from 7 patients and performed shallow whole genome sequencing (sWGS) as reported by Van Roy, *et al.* (2017). For methylation profiling, we applied a recently published method by De Koker, *et al.* (2019), that enables reduced representation bisulfite sequencing of cfDNA (cfRRBS). Patient 1, 2, 3 and 5 were sampled at

diagnosis and during treatment; patient 4, 6 and 7 were sampled at diagnosis and at progression.

Results

DNA copy number alterations were successfully detected in the tissue and the liquid biopsy, except for 1 patient. Using cfRRBS, a strong decrease in the estimated tumor fraction was observed during treatment in patients 1, 2, 3 and 5. cfRRBS detected for all cfDNA samples a small tumor fraction, however this fraction was lower than 10% in half of the cases and further investigation in order to define a reliable cut-off is needed. Shallow whole genome sequencing did not identify DNA copy number aberrations in 6 out of 10 of these follow-up samples.In patient 2, a small increase in tumor signal was noted, which might be indicative of poor therapy response. In the patients where both samples at diagnosis and at progression were available (pt 4, 6 and 7), a high tumor fraction was still detectable around 250 days after diagnosis. Furthermore, CNAs were detected at tumor progression of patient 4 and 7.

Summary/Conclusions

Our study shows that sWGS and cfRRBS can be applied on cfDNA as non-invasive approach to monitor therapy response and relapse prediction in NB patients, providing complementary information. We are currently expanding our cohort of patients and samples to further explore the power and accuracy of these methods.

Type Translational

Presentation Preference Traditional poster

Abstract nr. 392

The neuroblastoma specific lincRNA NESPR controls noradrenergic cell identity and neuroblastoma cell survival

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Topic Gene regulatory networks and epigenetics: from basic science to therapy

Background/Introduction

Neuroblastoma tumor cells are defined by a noradrenergic or mesenchymal state. Transcription factors composing the core regulatory circuitries (CRCs) that define these states are well described and include PHOX2B, ISL1, GATA3 and HAND2 for the noradrenergic state and FOSL1, FOSL2 and JUN for the mesenchymal state. The role of long non-coding RNAs (lncRNAs) in these CRCs remains poorly understood despite the fact that these molecules are emerging as transcription regulators.

Aims

By studying the role that lncRNAs occupy in determining neuroblastoma cell identity and their relationship with protein-coding transcription factors we hope to paint a more accurate and comprehensive picture of core regulatory networks.

Methods/Materials

We performed a pan-cancer lncRNA expression analysis comparing over 35 different tumor types to identify neuroblastoma-specific lncRNAs. Antisense oligonucleotides (ASOs) and siRNAs were used to silence the identified neuroblastoma-specific transcript. Treated cells were then subjected to colony formation, cell growth and apoptosis assays followed by RNA-sequencing. We then used 4C-sequencing to chart potential interactions between relevant loci. Finally, Chromatin Immunoprecipitation by RNA-Pulldown (ChIRP) sequencing was performed to identify DNA binding regions of the identified lncRNA.

Results

Pan-cancer expression analysis identified the neuroblastoma-specific lncRNA NESPR (NEuroblastoma Specific Phox2B Regulatory RNA). NESPR is located in the PHOX2B super-enhancer region and, unlike many lncRNAs, NESPR is abundantly expressed, efficiently spliced, highly conserved in mammals and present in both the nucleus and the cytoplasm as observed with fractionation and single-cell expression data. In neuroblastoma, high NESPR expression is associated with high-stage disease, MYCN amplification, and poor patient survival. Interestingly, NESPR expression is confined to noradrenergic neuroblastoma cells and downregulated upon de-differentiation to the mesenchymal state. Knockdown of NESPR significantly decreased colony formation and cell growth and lead to an increase in apoptosis. Of note, these effects were strictly dependent on the nuclear fraction of NESPR. RNA-sequencing of NESPR-silenced neuroblastoma cell lines revealed a significant reduction in the expression of PHOX2B and other components of the noradrenergic CRC, suggesting that NESPR may be involved in regulating PHOX2B expression and, as such, defining noradrenergic cell identity. 4C-sequencing demonstrated a long-range interaction between the NESPR locus and the PHOX2B promoter in noradrenergic cell lines but not in mesenchymal cell lines. Moreover, ChIRP-sequencing revealed binding of NESPR to DNA elements confined within the PHOX2B insulated neighborhood, suggesting NESPR regulates PHOX2B expression in cis. Strikingly, ChIRP-sequencing revealed binding of NESPR to various regions outside the PHOX2B locus. Motif analysis of these NESPR binding sites revealed a significant enrichment of GATA3, ISL1, and HAND2 binding motifs, suggesting a potential trans function for NESPR in the noradrenergic CRC.

Summary/Conclusions

Experiments are ongoing to further validate these findings. Overall, our results have uncovered a key regulatory function for the lncRNA NESPR in the noradrenergic CRC and demonstrate its role in neuroblastoma cell survival. To further elucidate the molecular mechanism underlying NESPR function, we are identifying its protein binding partners and potential influence on the chromatin environment of its target genes. Finally, we have established a NESPR knock-out mouse model to further study NESPR function in vivo.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 393

Fluorescence-guided tumor detection in Neuroblastoma using the organoid methodology: Preclinical validation.

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

Neuroblastoma surgery is considered a big challenge for the pediatric surgeon. Total resection remains the goal of surgery, however it is difficult to distinguish between tumor- and healthy-tissue. Tumor-specific fluorescent imaging agents for different targets are upcoming and recently introduced for clinical use.

Aims

To evaluate the use of fluorescence navigation during debulking surgery for neuroblastoma, multiple imaging targets were tested on a high throughput screening platform using the organoid methodology.

Methods/Materials

Comparing flowcytometry and 3D multiplex confocal imaging, differential expression between 9 different patient derived neuroblastoma organoid lines were compared. Results were validated *in vivo* in xenografts by conjugating the probes of interest to a near-infra red fluorophore and using a real time intraoperative detecting system. Dose escalation-, blocking-, pharmacokinetic-, and biodistribution studies were performed.

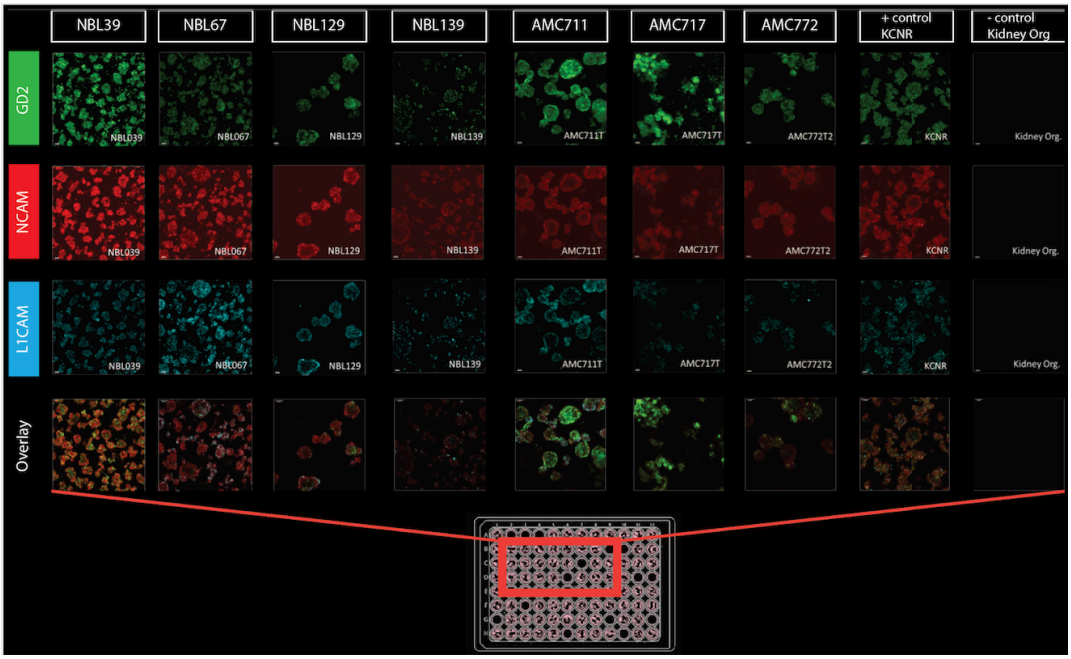
Results

The tested targets on our platform for this study were GD2, NCAM and L1CAM. Expression diferred per line for FACS and 3D imaging. Target GD2 was validated *in vivo* and imaged between 24 - 128 hours. The optimal dose for fluorescence tumor detection bij targeting GD2 was 1nmol

with optimal imaging between 72 – 96 hours after administration. This dosage corresponds with a dose of 10mg/m^2 in children.

Summary/Conclusions

This study describes for the first time the organoid platform as a screening tool for fluorescence-guided surgery probes and the development and evaluation of anti-GD2-IRDye800CW for the use in a clinical trial.



Type Translational

Presentation Preference Oral presentation

Abstract nr. 394

Neuropeptide Y/Y5 receptor axis as a hypoxia-inducible pro-metastatic pathway in neuroblastoma.

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

As tumors originating from precursors of sympathetic neurons, neuroblastomas (NBs) expresses neuronal proteins, such as neuropeptide Y (NPY) and its receptors. NB cells constitutively express Y2 receptor (Y2R), which maintains their proliferation and tumor vascularization. However, pro-apoptotic conditions additionally induce expression of Y5 receptor (Y5R), which acts as a survival factor. High Y5R expression was also observed in angioinvasive NB cells surrounding blood vessels, suggesting a role for this receptor in NB dissemination. Indeed, in NB patients, high serum levels of NPY associated with the presence of metastases. Furthermore, our studies in other tumor types implicated that Y5R can be up-regulated by hypoxia – a known stimulator of the metastasis.

Aims

The goal of this study was to identify mechanisms that may underlie potential pro-metastatic effects of NPY in NB.

Methods/Materials

Expression of NPY and its receptors in NB cells, SK-N-BE(2) and SK-N-AS, with or without exposure to hypoxia (0.1% oxygen for 72h) was assessed by RT-PCR, Western blot and immunocytochemistry. The effect of NPY on migration of NB cells was tested using transwell assay, while interactions with RhoA pathway were measured by RhoA pull-down and proximity ligation assay. To determine the effect of hypoxia on NB metastasis, SK-N-BE(2) and SK-N-AS cells were pre-exposed to hypoxia for 72h and injected into adrenal fat pads of SCID/bg mice.

Results

We have found that NPY, acting via Y5R, stimulates NB cell motility and acts as a chemotactic

factor for these cells in a concentration-dependent manner. Hypoxia increased expression of Y5R in NB cells and their migratory response to NPY. This increase in NPY-driven migration observed in hypoxic NB cells was fully blocked by Y5R antagonist. Notably, SK-N-BE(2) cells pre-exposed to hypoxia had also increased metastatic potential, as evidenced by higher infiltration of lung tissues by NB cells upon their orthotopic injections. The effect of NPY/Y5R pathway on NB cell migration was associated with marked cytoskeleton remodeling, manifested by an increase in the number of fiolopodia. In migratory cells, the active fraction of Y5R present on the cell surface was located in the leading and trailing edges of NB cells, and co-localized with RhoA-GTP, a known cytoskeleton regulator. RhoA-GTP pull-down assay confirmed NPY-induced RhoA activation in NB cells, while proximity ligation assay revealed direct interactions between Y5R and RhoA-GTP triggered by NPY stimulation.

Summary/Conclusions

Altogether, our data indicate that NPY/Y5R/RhoA pathway stimulates overall motility of NB cells and their directional migration toward NPY. This effect is exacerbated in hypoxia. Further studies are required to determine the contribution of these processes to NB metastasis.

Type Basic

Presentation Preference Traditional poster

Abstract nr. 395

Combined blood and bone marrow cell-free DNA and disseminated tumor cell detection for sensitive response monitoring and early relapse detection in high-risk neuroblastoma patients

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

To monitor treatment response and detect the occurrence of minimal residual disease (MRD) during therapy and a possible relapse early, minimally invasive liquid biopsy methods are increasingly applied. As more than 95% of high-risk neuroblastoma patients show disseminated tumor cells (DTCs) in the bone marrow (BM) at diagnosis, detection of DTCs in BM using automated immunofluorescence plus FISH (AIPF) is used for highly sensitive MRD detection. However, in case of relapses outside the BM, disease detection in this compartment may fail.

Aims

We therefore combined DTC detection with *MYCN* and *ALK* analysis in cell free DNA from blood and bone marrow plasma and investigated (1) response at mid and end of induction therapy, (2) the feasibility as combined approach for MRD monitoring and (3) whether relapse/progression can

be diagnosed early.

Methods/Materials

Hotspot mutations of the *ALK* gene, *MYCN* amplification and other copy number changes were investigated by ddPCR in total 358 cell free DNA (cfDNA) samples from blood and 218 cfDNA from bone marrow aspirate plasma samples of 19 high-risk neuroblastoma patients. On average 19 sequential blood cfDNA and 11 BM cfDNA samples were analyzed per patient. ddPCR results were combined with the corresponding BM DTC state as determined by AIPF using GD2 and CD56, and compared to clinical response parameters assessed at the same timepoint.

Results

Our study showed a significant reduction of cfDNA markers in response to therapy in blood and BM mid and end of induction therapy. Parallel DTC analysis showed similar response kinetics. Further, we determined robust cut-offs for each ddPCR assay and detected positive signals in 24 out of 90 cfDNA samples. In 5 patients tumor markers were only found in blood and BM cfDNA and BM aspirates were free of DTCs. Vice versa in 14 of 90 samples, MRD was exclusively detected by DTC analysis, however these cases presented with DTC infiltration below 500 cells per million. At time points where clinical response evaluation is available metastatic as well as complete response will be correlated with the response in liquid biopsies. However, we were able to detect 9 of 15 relapses/progressions in blood cfDNA at or even before the clinical manifestation. Remarkably, in one case of local relapse without BM involvement, elevated *MYCN* copy numbers (10 copies/reference) were detected in blood cfDNA six weeks before clinically confirmed relapse.

Summary/Conclusions

The combined analysis of genetic markers and drug targets in blood and bone marrow plasma cfDNA and DTC enumeration is a sensitive approach for monitoring minimal disease and allows early detection of relapse. cfDNA analysis is especially crucial in patients with localized relapse or when clinical response evaluation fails to detect the onset of disease progression. Thus, liquid biopsy analysis will be an important component of NB diagnostics.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 396

Systematic target actionability reviews of preclinical proof-of-concept papers to match targeted drugs to paediatric cancers – A pilot study on MDM2 and TP53

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Topic Other

Background/Introduction

Children with cancer are in urgent need of new therapies, as approximately 25% of patients experience a relapse and 20% succumb to their disease. Repurposing of targeted agents developed for adult indications could provide novel therapeutic options for paediatric cancer patients.

Aims

To prioritise targeted drugs for paediatric clinical development and efficiently guide clinical development for novel targeted agents, we applied systematic review methodology to develop a Target Actionability Review (TAR) strategy. These TARs assess the strength and completeness of published preclinical proof-of-concept (PoC) data by structured critical appraisal of and summarizing the available scientific literature for a specific target (or pathway) and the associated drugs in paediatric tumours.

Methods/Materials

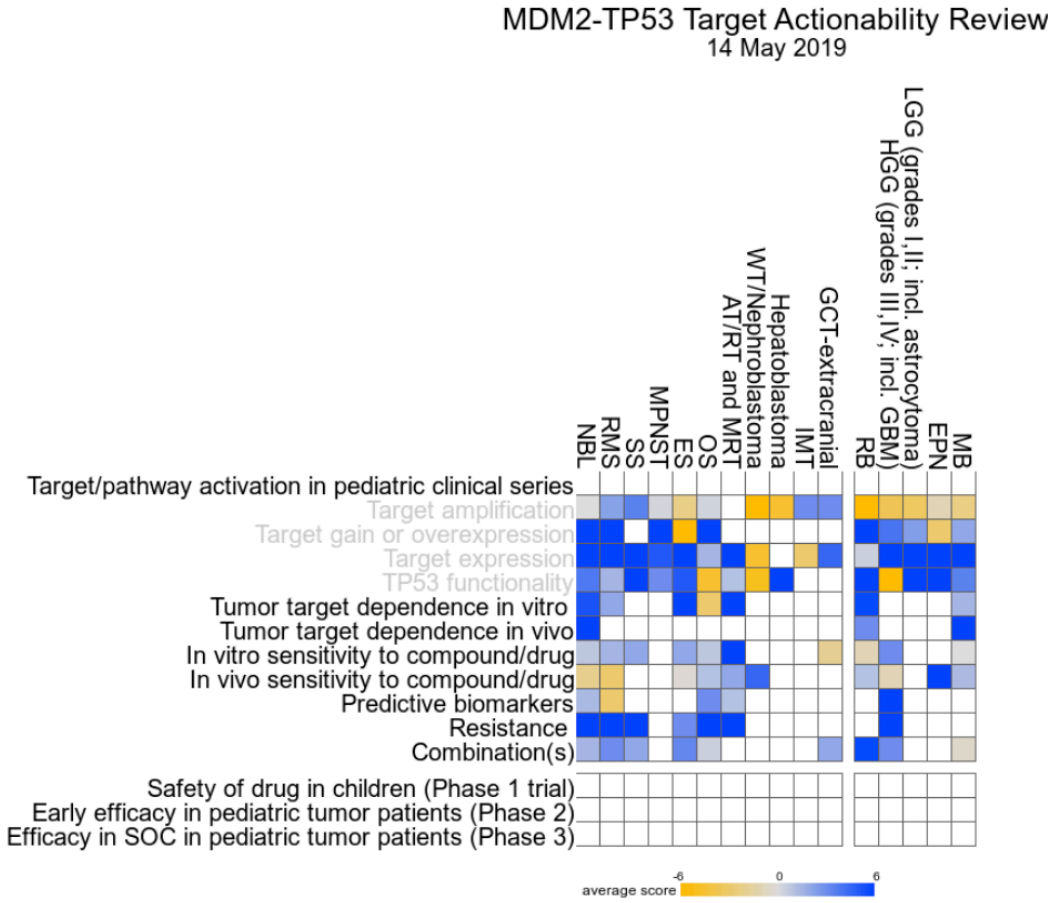
The TAR strategy involves a sensitive literature search in PubMed and the selection of relevant papers. For each paper, the individual experimental findings are extracted, marked for paediatric tumour type, and categorised into nine separate PoC data modules. Each experimental finding is scored for experimental outcome and quality independently by two reviewers; discrepancies are assessed by a third reviewer and resolved by adjudication. Scores corresponding to one PoC module are merged for each tumour type and visualised in a heatmap matrix in the publicly available R2 data portal [r2.amc.nl].

Results

To test our TAR methodology, we conducted a pilot study on MDM2 and *TP53*.
The search terms ["*paediatric cancer type" AND (MDM2 OR HDM2)*] and [*"paediatric cancer type" AND nutlin*] identified 726 unique papers, 161 of which met our inclusion criteria. Another 12 next-generation sequencing studies were added to assess the mutational status of *TP53* in paediatric cancer, as *TP53* mutations generally lead to resistance to MDM2 inhibitors. *MDM2* amplifications were frequently found in rhabdomyosarcoma, whereas they seem to be absent in paediatric brain tumours. Gain or overexpression of MDM2 occurs across all studied tumour types. *TP53* mutations are frequent in osteosarcoma, Wilms tumour and glioblastoma. *In vitro* inhibition of MDM2 was relatively effective in all included tumour entities. However, most *in vivo* studies revealed merely reduced tumour growth and only a few reported stable disease or complete response. Long-term nutlin treatment was studied in neuroblastoma, rhabdomyosarcoma and osteosarcoma and resulted in acquired *TP53* mutations. Moreover, this pilot TAR reveals that it may be beneficial to combine MDM2 inhibitors with chemotherapy or other drugs.

Summary/Conclusions

The heatmap generated from analysis of 173 publications on MDM2 and *TP53* can be used to identify mechanism-of-action-based matches between MDM2 inhibitors and specific cancer subtypes. This TAR supports clinical evaluation of MDM2 inhibitors in neuroblastoma, rhabdomyosarcoma and high grade gliomas. Our systematic review also revealed a lack of studies investigating the role of MDM2 in paediatric tumourigenesis and predictive biomarkers as well as resistance mechanisms in most indications. Furthermore, our review highlights tumor types (such as malignant peripheral nerve sheath tumours, rhabdoid tumours, hepatoblastoma and inflammatory myofibroblastic tumours) where preclinical data is incomplete or lacking and for which additional preclinical testing is advisable. The TAR strategy is now the standard for systematic reviews on target actionabilty within the ITCC-P4 consortium.



MDM2/TP53 TAR heatmap

Type Translational

Presentation Preference Oral presentation

Abstract nr. 397

Neurologic and Orthopedic Manifestations of Spinal Cord Compression in Intermediate-risk Neuroblastoma: A report from the Children's Oncology Group (COG) study ANBL0531

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

Background: Epidural or intradural tumor extension occurs in 5-15% of patients with neuroblastoma (NB), and may occur with or without neurologic impairment. Neurologic outcome may be impacted by degree of disability at diagnosis and clinical management.

Aims

Aims: The ANBL0531 Phase 3 trial included a secondary objective to examine the clinical symptoms and neurologic/orthopedic outcome of intermediate-risk NB patients with paraspinal or intraspinal tumors.

Methods/Materials

Methods: Between 2007 and 2011, 404 evaluable patients were enrolled on study, including 138 with paraspinal +/- intraspinal tumors. Baseline assessment included duration of neurologic symptoms, degree of motor deficit (Grade 1= mild, Grade 2= moderate, Grade 3= severe), sensory deficit, pain, and orthopedic abnormalities. Follow-up neurologic and orthopedic evaluations were obtained at 6, 12, 24, and 36 months off therapy and then annually.Treatment was biology- and response-based, as previously described. For patients with intraspinal tumors, the criteria to stop therapy included both the protocol-defined treatment endpoint (partial response of the primary tumor) as well as sufficient resolution of the intraspinal tumor component to allow for observation with minimal risk of neurologic compromise. In this circumstance, additional cycles of chemotherapy, rather than surgical decompression with laminectomy, was recommended.

Results

Results: The median age for all 138 patients with any paraspinal/intraspinal tumor involvement at diagnosis was 9.1 months (range= 0.2-86.2 months) and was no different than for the 54 patients with symptomatic cord compression at diagnosis. Disease stages included n=20 (INSS stage 2A/2B), n=72 (stage 3) n=42 (stage 4) and n=4 (stage 4S). Tumor biology features included n=125 with favorable histology, n=112 with hyperdiploidy, and n=18 with either 1p36 and/or 11q23 loss of heterozygosity. Symptomatic cord compression was reported at baseline in 54 patients as Grade 1 (n=11, 20.4%), Grade 2 (n=15, 27.8%), or Grade 3 (n=28, 51.9%) motor deficit. Bladder or bowel dysfunction were reported at baseline in 31 of the 54 symptomatic patients (57.4%). Sensory deficits were noted in 24 patients (44.4%) and 13 (24.1%) reported pain. Orthopedic abnormalities were reported at baseline in 15 of the 54 (27.8%) symptomatic patients, with scoliosis in 8 patients (14.8%) and 1 (1.9%) with kyphosis. At diagnosis, 8 of the 138 patients underwent >90% tumor resection, 15 underwent ≥50% to 90% resection, and 111 had a biopsy only or <50% resection of the tumor; surgical data was not available for 4 patients. Fifty-eight patients (42.0%) had a second operation. Surgical complications at any timepoint were reported in 34 patients (24.6%), 18 of which were present at baseline and 16 resulting following surgery. Fifty-five patients (39.9%) received more chemotherapy cycles than initially assigned. Overall response at end of therapy was complete response in 11 (8.4%), very good partial response in 49 (37.4%), partial response in 54 (41.2%), and stable disease in 11 (8.4%).

Summary/Conclusions

Conclusion: Excellent tumor response was achieved using a biology- and response-based treatment algorithm for patients with non high-risk NB presenting with paraspinal+/-intraspinal disease extension. The role of additional cycles of chemotherapy, type of surgical intervention, and neurologic and orthopedic outcomes will be reported.
Clinical trial information: NCT00499616.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 398

Schwann cell plasticity regulates neuroblastic tumor cell differentiation

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

Schwann cells develop along neuroblasts from trunk neuronal crest stem cells in embryonic development. The remarkable plasticity of Schwann cells is essential for nerve regeneration but also contributes to neuropathies and cancer. Stromal, tumor-associated Schwann cells are characteristically found within ganglioneuroma, where the presence of stromal Schwann cells is associated with a favorable tumor behavior and increased patient survival, whereas aggressive subtypes of neuroblastic tumors lack stromal Schwann cells. It has not been investigated yet whether the adaptive potential of Schwann cells is manifested in ganglioneuroma.

Aims

This enigma prompted us to investigate (1) the molecular wiring and functional state of stromal versus injury associated repair Schwann cells and thereby determine whether the presence of stromal Schwann cells in benign neuroblastic tumors is an expression of Schwann cell plasticity, and (2) how Schwann cell signals could be leveraged as therapeutics.

Methods/Materials

We here performed transcriptome and high-resolution mass spectrometry profiling of human neuroblastic tumors, rich and poor in Schwann cell stroma, as well as human injured nerves, rich in repair Schwann cells. Further we established a novel direct and trans-well co-culture model, recapitulating some aspects of ganglioneuroma development *in vitro* which was combined with functional and biochemical assays.

Results

We provide evidence that stromal Schwann cells in ganglioneuroma exhibit a repair Schwann cell characteristic gene expression signature. In turn, primary repair Schwann cells had a pro-differentiating and anti-proliferative effect on neuroblastoma cell lines and this was mediated by soluble factors. Within the pool of secreted stromal/repair Schwann cell factors, we identified EGFL8, a matricellular protein with so far undescribed function, to induce neuronal differentiation of aggressive neuroblastoma cells. EGFL8 expression further correlated with favorable tumor stage and increased patient survival.

Summary/Conclusions

Our findings suggest that stromal Schwann cells exert nerve repair associated functions in the tumor-environment and underline the therapeutic value of Schwann cell-derived factors for aggressive, Schwann cell stroma-poor neuroblastoma.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 399

1RG-CART, a CRUK-sponsored phase I trial to evaluate a novel chimeric antigen receptor-transduced autologous T cells in children with neuroblastoma

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

The re-programming of a patient’s immune system through genetic modification of the T cell compartment with chimeric antigen receptors (CARs) has led to durable remissions in chemotherapy refractory B cell cancers expressing CD19 antigen. CARs cause redirection of T cell specificity and rely upon identification of a tumour cell surface antigen absent from critical normal tissues. Successful targeting of solid cancers by CAR T cells has been limited by the requirement for infiltration and expansion within the inhibitory tumour microenvironment.

Aims

Disialoganglioside GD2 is abundant on the surface of most neuroblastomas but absent from most normal cells outside the CNS. To avoid the theoretical inhibitory effects of antibodies against the 14.18 antibody previously administered to many relapsed patients, we devised a second generation (CD28-zeta) anti-GD2 CAR, which incorporates a novel humanised single chain antibody (huk666). We have evaluated safety and feasibility of manufacturing and administering patient-derived CAR-transduced T cells in the setting of relapsed or refractory neuroblastoma

Methods/Materials

The target cell dose was successfully manufactured in 15 out of 15 heavily pretreated patients undergoing apheresis, and 9 patients have received single infusions of either 10 transduced cells/m^2 (n= 6; 4 without conditioning, 1 x cyclophosphamide (Cy) alone, 1 x Cy and fludarabine (Flu)), or 10/m² (n=3 with Flu/Cy) or $10^9/m^2$ (n=3 with Flu/Cy).

Results

No patients receiving 10/m² showed CAR T cell engraftment or clinical response. Of three patients receiving 10 /m² T cells, one patient with high burden disease, experienced grade 2 cytokine release syndrome necessitating therapeutic reversal with IL-6 antagonist tociluzumab, sustained low level CAR-T cell engraftment, improvement in performance status, widespread necrosis of previously heavily infiltrated bone marrow, and partial resolution of bone metastatic disease. New complete clinical outcome data on the final cohort of patients receiving $10^9/m^2$ will be presented at the meeting. No patients experienced dose limiting toxicity.

Summary/Conclusions

Manufacture of greater than 10/m² 1RG-CART cells is feasible and no dose limiting toxicity is found in any patients. Current research efforts are focussed on understanding and boosting persistence.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 400

The molecular aetiology of ATRX aberrations in neuroblastoma

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

A subgroup of high-risk neuroblastoma patients with very poor clinical outcome is characterised by genetic aberrations in the chromatin-remodeler ATRX. The majority of these tumours have in-frame ATRX deletions while some rare cases show ATRX stop-codons. ATRX is known to be involved in the removal of G-quadruplexes, DNA repair, H3.3 incorporation, inhibition of macroH2A1 disposition and indirectly in H3K27 tri-methylation. ATRX aberrations are known to cause alternative lengthening of telomeres (ALT), in which the telomeres are elongated by a homologous recombination-like mechanism resulting in C-circles, elongated telomeres and ALT-associated PML bodies (APBs). There is limited knowledge of the role of (in-frame deleted) ATRX in neuroblastoma.

Aims

The aim for this project is to create models of ATRX alterations to study the molecular aetiology of ATRX aberrations in neuroblastoma and to identify effective therapeutic interventions for this subgroup of patients.

Methods/Materials

Currently, we have one cell lines (CHLA-90) and one organoid line (AMC772T2) with different ATRX deletions. For both mutant lines and an ATRX wildtype cell line (CHP134) we performed ATRX immunoprecipitation (IP) followed by mass spectrometry to identify the common lost or gained ATRX protein-binding partners.

To expand the number of neuroblastoma models with ATRX alterations we used CRISPR-Cas9 to create isogenic cell line and organoid models with ATRX knock-out (KO) mutations and different

patient-specific in-frame ATRX deletions. We also created a PiggyBac doxycycline-inducible wildtype ATRX plasmid and a PiggyBac doxycycline-inducible in-frame deleted ATRX plasmid.
We used the C-circle assay, telomere southern blotting and an APB FISH/IF to investigate ALT characteristics in these models. We also performed high-throughput compound screens of AMC772T2 and CHLA-90 and will extend this to the newly created models.

Results

We identified that the ATRX deletions in CHLA-90 and AMC772T2 result in in-frame deleted ATRX protein products. The in-frame deleted ATRX protein from CHLA-90 is able to interact with DAXX, a known ATRX interaction partner, while this interaction seems lost in AMC772T2. We obtained mass spectrometry results from the ATRX IPs of CHLA-90, AMC772T2 and CHP134 (ATRX wild-type). These data are further analysed and (the absence of) interaction partners will be validated.
In addition, we have generated isogenic ATRX KO GIMEN clones and isogenic ATRX KO SKNAS clones. These clones were negative for ALT characteristics in the C-circle assay, telomere southern and ABP FISH/IF. Since these clones are still telomerase dependent, we will additionally inactivate/reduce telomerase activity to enable them to switch to ALT. Furthermore, we created models with inducible ATRX overexpression in CHLA-90 and AMC772T2 and models with ATRX deletions. We will investigate ALT characteristics in these lines.
The isogenic ATRX KO and overexpression models will be used in high-throughput compound screens and will be compared with the compound sensitivity data of CHLA-90 and AMC772T2 to identify possible therapeutic targets.

Summary/Conclusions

We have created models of ATRX KO, in-frame deletions and overexpression in neuroblastoma cell lines and organoids. These models will be used to increase the understanding of the molecular aetiology of neuroblastoma tumours with ATRX aberrations and to identify effective therapeutic interventions.

Type Translational

Presentation Preference Oral presentation

Advances in Neuroblastoma Research (ANR) Meeting
ANR 2020
Amsterdam
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Abstract nr. 401

Clinical and Biological Factors associated with Refractory disease in UK patients with High Risk Neuroblastoma

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

Despite advances in neuroblastoma (NB) therapy, around 20% of high risk cases will develop primary refractory disease, and fail to achieve an adequate response to induction chemotherapy to allow surgical resection of the primary tumour, consolidation with myeloablative therapy, radiotherapy and minimal disease therapy. Patients in the UK diagnosed with high risk NB, from 2002-2017 were treated on the European HR-NBL1/SIOPEN trial (NCT01704716).

Aims

To describe the clinical and biological factors associated with primary refractory disease in high risk NB patients defined as patients who came off the SIOPEN HR-NBL-1 trial due to insufficient response, the treatment received, subsequent clinical course and outcome.

Methods/Materials

This is a retrospective study, which links epidemiological, clinical, genetic, and treatment data of refractory patients identified from 21 Paediatric Oncology Principal Treatment centres, in the UK and Ireland. Refractory patients were defined as those who came off the HRNBL-1 trial because of insufficient response, defined as > 3 positive bone lesions on mIBG scan or a positive bone marrow aspirate / trephine, after first line therapy with COJEC or modified N7 chemotherapy and up to 2 cycles of topotecan, vincristine and doxorubicin (TVD). The clinical, biological and treatment factors are described and chi-square and fisher-exact tests used to compare the refractory cases characteristics with 166 high risk UK NB HRNBL-1 trial cases, identified for a genetic study using high-density single nucleotide polymorphism arrays.

Results

In this interim data analysis from 10/21 centres, 37 refractory disease cases were identified so far. All were stage 4, 24% *MYCN* amplified, 12/20 (60%) 11q deleted, median age at diagnosis 3.6 years (range 1.4 – 20.4 years, mean = 5.1 years; 3% aged <18 months, 68% 18 months-5 years and 29% >5 years). In comparison, there were 19% of 166 high risk NB cases <18 months, 69% 18 months-5 years, 12% >5 years (P<0.01); 48% *MYCN* amplified (P=0.01) and 40% had 11q deletions (P<0.01). Treatment for refractory disease included additional cycles of TVD in 25/37 (68%) cases (range 1-4 cycles), temozolomide alone or with irinotecan in 13/37 (35%) cases, BEACON trial treatment (NCT02308527) in 8/18 (44%) cases diagnosed after 2013 (24%). Response to second line or subsequent treatments for refractory disease assessed by the International Neuroblastoma Response Criteria (INRC) was 2/37 (5%) complete response, 8/37 (22%) very good partial response, 7/37 (19%) partial response, 5/37 (14%) mixed response, 7/37 (19%) stable disease and 7/37 (19%) progressive disease (1 unknown). Subsequently 11/37 (30%) patients received myeloablative therapy and 10/37 (27%) patients immunotherapy. The median follow-up period was 3.4 years (IQR 1.8 – 4.5 years) with 10/37 (27%) alive disease free, 8/37 (22%) alive with disease and 19/37 (51%) cases have died from disease.

Summary/Conclusions

Refractory patients are more likely to be non-*MYCN* amplified and older at diagnosis compared

with standard high risk patients. These early data show that some refractory patients can achieve subsequent responses and complete standard treatment for high risk NB. Once completed, this study will form a useful historical comparator group for forthcoming clinical trials for refractory NB such as the VERITAS trial (NCT03165292).

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 402

Clonal heterogeneity before treatment underlies spatial and temporal evolution in neuroblastoma

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

Patients with high-risk neuroblastoma (HR-NB) frequently present with widely metastatic disease with tumors harboring recurrent *MYCN*, *TERT* and *ATRX* alterations. Most genomic studies to-date, however, have focused on single diagnostic samples. Consequently our understanding of the clonal and genetic evolution of disease progression and metastasis remains limited.

Aims

We set out to deliver a detailed characterization of clonal architecture of HR-NB during disease evolution and identify the biological pathways important for progression.

Methods/Materials

We studied 203 sequential and spatially separated samples from 101 patients with informed consent and IRB approval (Clinicaltrials.gov, NCT00588068): 96 pre-treatment, 22 on-therapy, 33 relapse and 29 late-relapse samples (1-17 samples/patient). We performed WGS in 203 and RNAseq in 168 samples. We mapped somatic alterations (substitutions, indels, rearrangements and copy-number changes) and characterized underlying clonal phylogenies. Analyzing genome-wide mutational signatures, we identified the prevalent mutational processes at diagnosis and during therapy. We studied mutagenic effects related to chemotherapeutic agents used in HR-NB therapy in PDX models. Superimposing mutational signatures onto subclonal structures, we determined the timing and pattern of spread amongst primary and metastatic sites. We overlaid these findings onto gene expression profiles, focusing on tumor mutational burden (TMB) and immune infiltrates.

Results

We identified 1,193,405 mutations in total. TMB was on average 0.6/Mb (range=0.06-2.6Mb) at diagnosis but significantly increased at relapse (average=2.3/Mb, range=0.3-7Mb), accompanied by a shift in mutational patterns. C>A transversions are dominant at diagnosis (COSMIC signature SBS18), but with different prevalence amongst tumors with distinct driver alterations. At progression there is strong enrichment for C>T and T>C transitions linked to platinum and temozolomide exposures. PDX modeling shows that SBS18 is continuously present whereas chemotherapy-induced mutations are transient.

We identify complex clonal structures in 86% of patients showing oncogenic clonal diversification in the primary site frequently presenting as multiple metastatic lineages (met) even before treatment. Once treatment started, patterns of met-to-met seeding was frequently observed with evidence of progression after a period of dormancy at local as well as distant sites.

Disease progression was associated with subclonal expansions harboring distinct mutations affecting the same pathways RAS-MAPK (22/40) and *TP53* (3/40) as well as continuous rearrangements altering *ATRX* and *TERT* (13/40). On chemotherapy pre-existing clones with an increased TMB (3-13x) became dominant. However, this was not associated with any change in the immune infiltrate as determined by RNAsee.

Summary/Conclusions

By analyzing sequential HR-NB tumors, we show that complex genetic trajectories present at diagnosis undergo independent evolution at the primary and metastatic sites but nevertheless converge on the same biological pathways. This also provides insights on evolutionary processes important for disease progression showcasing the need for comprehensive genomic profiling to inform basic and translational research and clinical care.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 403

MEK1/2 IS A TARGET OF PRIMA-1MET-INDUCED NEUROBLASTOMA CELL DEATH

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

Neuroblastoma has a low frequency of *TP53* mutations, and its downstream pathway is usually intact. We previously demonstrated the efficacy of PRIMA-1^{MET}, thought to be p53 activator, against neuroblastoma, either alone or in combination with common clinical chemotherapeutics.

Aims

The present study assessed p53's involvement in PRIMA-1^{MET} action and which other cellular pathways are implicated in the response.

Methods/Materials

CellTiter 2.0 was used to determine IC50. pcDNA3.1(+) plasmid was used to over-express p53 in LA1-55N (MNA, p53-null cells). JQ1 was used to decrease the levels of MYCN and p53 in LA1-55N and CLB-GA (non-MNA) cells, respectively. A Proteome Profiler Array Human Phospho-Kinase Array Kit was used to assess activation of the most common intra-cellular signaling pathways. Subsequently, western blot analysis was used to confirm and detail these results.

Results

LA1-55N cells expressing p53 were significantly more resistant to PRIMA-1^{MET} and contained significantly more GSH than normal LA1-55N cells. JQ1 decreased the expression of both MYCN (LA1-55N) and p53 (CLB-GA), and it decreased intracellular GSH. JQ1 synergized with PRIMA-1^{MET} in LA1-55N (p53-null cells) and in CLB-GA (non-MNA) cells. Analysis of cellular signaling pathways demonstrated the involvement of the MEK/ERK pathway. PRIMA-1^{MET} inhibited

MEK1/2 signaling. Co-treatments with PRIMA-1^{MET} and several MEK/ERK pathway inhibitors in BE-2C, CHP212, NBL-S, NGP and SK-N-DZ, demonstrated good synergies for Crizotinib (ALKi) and trametinib (MEK1/2i), and a mixed response for U0126 (MEK1/2i) and SCH772984 (ERK1/2i).

Summary/Conclusions

Previous and current results suggest that p53 is indirectly involved in PRIMA-1^{MET}-mediated cell death. This occurs through p53's modulation of GSH levels rather than direct interaction between PRIMA-1^{MET} and p53. We identified the MEK/ERK pathway as a major target for PRIMA-1^{MET} as it is a potent inhibitor of MEK1/2. Synergistic action with other inhibitors of the MEK/ERK signaling pathway was confirmed, and this could present a novel and efficient option for neuroblastoma treatment in the future.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 404

Vaccination of high-risk neuroblastoma patients with anti-id antibody mimicking GD2 to maintain protective immunity after passive immunotherapy with anti-GD2 antibody ch14.18/CHO

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

Disialoganglioside GD_2 is an established tumor associated antigen with abundant expression on neuroblastoma (NB) and restricted expression on normal cells. As it is a suitable target for immunotherapy, human/mouse chimeric anti-GD_2 antibodies ch14.18 (dinutuximab) and ch14.18/CHO (dinutuximab beta) were developed and are marketed in US and Europe, respectively, for passive immunotherapy of high-risk NB.
Active immunization based on GD_2 following antibody therapy may induce a long-lasting protective immunity to extend the therapeutic benefit of NB antibody immunotherapy. It has been shown recently that overall survival is improved in NB patients who generated an immune response to ch14.18/CHO, which indicates the induction of immunity via an internal idiotypic network cascade.
Anti-idiotypic antibody ganglidiomab to ch14.18 was raised in mice and shown to carry an internal image of GD_2 in vitro and in murine models. Here we describe results of a compassionate need program in NB patients vaccinated by intramuscular injection of alum adsorbed ganglidiomab, following ch14.18/CHO immunotherapy.

Aims

To evaluate tolerability, immune response and outcome of vaccination with ganglidiomab in a compassionate use program.

Methods/Materials

Between 2012 and 2014, six high-risk NB patients (2-8 y); 4 in CR, 2 with disease following multimodal therapies including stem cell transplant and ch14.18/CHO immunotherapy were repeatedly treated under compassionate need with 0,5mg alum adsorbed ganglidiomab by subcutaneous vaccination (5-10 vaccinations every 2 weeks). Blood for serum measurements was withdrawn at various timepoints. Antibody responses to ganglidiomab, to its idiotype and to GD_2

by ELISA as well as complement lysis to a NB cell line were investigated. Last patient follow-up was in 2019.

Results

Vaccinations were well tolerated with mild transient local reactions only. In immune sera of all patients a marked induction of antibodies to ganglidiomab and to its variable regions was detected. In 3 of 6 patients also an induction of antibodies to GD2 was found. Induction of cytolytic activity via complement of GD2+ LAN1 NB cells was seen in 2 patients. All six patients were alive without evidence of disease at their last follow up in 2019, i.e. approx. 5 years after start of vaccinations.

Summary/Conclusions

Repeated s.c. injections of alum-adsorbed ganglidiomab may evolve as additional well tolerated active immunotherapy modality after ch14.18 antibody treatment to maintain a long-lasting immunity for prevention of progression or re-occurrence of disease, thereby improving overall survival. A clinical trial program is warranted to explore this hypothesis.
Supported by a grant of AWS (Austria).

Type Clinical

Presentation Preference Oral presentation

Advances in Neuroblastoma Research (ANR) Meeting
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Abstract nr. 405

A Precision Medicine Biology Trial for patients with refractory or relapsed neuroblastoma: A New Approaches to Neuroblastoma Therapy Consortium (NANT) Study

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Limited data exists regarding the genetic and immunologic predictive biomarkers in recurrent or refractory neuroblastoma (rNB). Understanding rNB mechanisms of resistance through in depth genomic or tumor microenvironment (TME) alterations is critical to devising novel targeted therapies.

Aims

This study describes the feasibility of performing next generation sequencing, assessing the landscape of genomic and immunologic alterations of rNB tissues, and feasibility of obtaining

genomic data from tumor enriched bone marrow aspirates.

Methods/Materials

Patients with rNB were enrolled on the IRB-approved NANT 15-01 protocol. Tumor from bone, bone marrow or soft tissue disease, as well as germline blood were analyzed using Ashion's GEM 600-gene panel or GEM Extra Platform with the latter platform including full transcriptome (RNASeq) and whole-exome (WES) sequencing coupled with germline subtraction. In addition, soft tissue specimens were evaluated by immunohistochemistry (IHC) for C-MYC and MYCN expression, CD163 (tumor associated macrophages (TAM)) and PDL-1. The generated report on samples with adequate genomic material for testing was returned back to the treating physician to aid in clinical decision making. Bone marrow aspirate specimens were enriched for tumor cells using positive selection for GD2 and analyzed pre- and post-enrichment by flow for GD2, CD45, NCAM and Phox2B.

Results

To date, 83 biopsy specimens from 85 enrollments (77 patients) have been submitted, 52 (68%) of which completed all study analyses and generated a genomic report. Of the 83 specimens, 32/41 (78%) were from soft tissue, 14/29 (48%) bone, and 6/13 (46%) bone marrow. 46/77 (61%) had a report generated from the first specimen submitted. Thirty tumor specimens had insufficient quality for sequencing. Of those, 8 (27%) patients submitted a second specimen (re-enrolled) with 6/8 (75%) resulting in a report. Physicians who received a complete genomic report, were asked to fill out a one year follow up survey, which showed 12/45 (27%) utilized the genetic results to enroll patients on targeted therapies. From the 52 reports generated, actionable genetic variants were identified in 43/52 (83%), 7/52 (13%) identified non-actionable genetic variant, and 4/52 (8%) identified no genetic variant. The most common mutations identified included ALK mutations (33%), which is higher than previously reported for relapsed patients. Additional alterations in MYCN (25%), ATRX (15%), RAS/MAPK pathway-related genes (11%), and CDKN2A (8%) were identified. Soft tissue samples were also analyzed for TME markers, showing heavy infiltration of TAMs in 17/32 (53%) including 7/8 (88%) refractory specimens. PDL1 ($\geq$5%) was detected in 5/32 (16%) of soft tissue samples. 12/46 (26%) bone marrow aspirate samples submitted for enrichment were found positive for neuroblastoma (>1% GD2+ by flow). 9/12 (75%) were enriched to >30% GD2+ cells for genetic sequencing.

Summary/Conclusions

A multi-institution clinical genomics study in neuroblastoma is feasible with high chance of success in obtaining useful samples with guided biopsies, including bone metastases. Enrichment of bone marrow aspirates was feasible in a subset of these patients. A substantial proportion of children with rNB have actionable alterations with significant ALK enrichment in this multiply recurrent patient cohort. Approximately half of rNBs show evidence of an active tumor microenvironment.

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A Precision Medicine Biology Trial for patients with refractory or relapsed neuroblastoma: A New Approaches to Neuroblastoma Therapy Consortium (NANT) Study

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Shamirian A

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 406

Quantitative and highly sensitive detection of neuroblastoma minimal residual disease based on MYCN amplicon breakpoints

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

More than half of the patients with high-risk neuroblastoma relapse and succumb to their disease despite initial remission in response to intensive treatment, implying the survival of neuroblastoma cells as minimal residual disease (MRD). *MYCN* amplification is a major driver of high-risk neuroblastoma, and a molecular criterion for treatment classification. Since the *MYCN* amplicon is

stable during progression, the genomic breakpoints of the *MYCN* amplicon will largely persist in all tumor cells.

Aims

PCR-based assays to detect MRD levels are already incorporated in the clinical routine for patients with leukemia. We hypothesized that risk stratification for patients with neuroblastoma would benefit from individual PCR-based MRD assays utilizing the *MYCN* amplicon chromosomal breakpoints. Here we show that PCR-based tracking of unique genomic *MYCN* amplicon breakpoints can be used to sensitively and specifically detect MRD in *MYCN*-amplified neuroblastoma.

Methods/Materials

We employ a custom neuroblastoma hybrid capture-sequencing approach for high-coverage sequencing (>1000x) of the *MYCN* amplicon sequence from formalin-fixed, paraffin-embedded and fresh-frozen primary tumor material. Patient-specific PCR primers are designed from the *MYCN* amplicon chromosomal breakpoints identified by panel sequencing. After successful validation of the *MYCN* amplicon breakpoint via classical PCR and Sanger sequencing, the personalized MRD assay applies dual-labeled fluorescent hydrolysis probes within RQ-PCR or droplet digital PCR techniques.

Results

As a proof of concept, we performed targeted sequencing in seven *MYCN*-amplified neuroblastoma cell lines and successfully recovered all identified *MYCN* amplicon breakpoints (n=20) by classical PCR and Sanger sequencing. To estimate the detection limits of our MRD assay, eight *MYCN* breakpoints in cell lines underwent further analysis by RQ-PCR and droplet digital PCR. *MYCN* chromosomal breakpoint PCR amplicons were detectable in single tumor cells among up to 10^6 reference cells. Targeted sequencing of 13 primary tumor samples revealed 35 *MYCN* amplicon breakpoints that were also validated for eight patients. *MYCN* breakpoints detected in primary neuroblastomas remained stable in relapsed neuroblastomas. In total, patient-individual RQ-PCR and digital droplet PCR assays were developed for all 13 patients with the sensitivity to detect a single tumor cell in 10^4 to 10^6 normal cells. Using our strategy for personalized MRD monitoring, we could track tumor cells with *MYCN* amplicon breakpoints in bone marrow aspirates at diagnosis and during therapy, which was feasible within the clinical treatment schedule and revealed MRD persistence until relapse and good response to the first treatment course. Moreover, the MRD assay detected bone marrow infiltration below the detection limits of the currently used cytology and GD2 immunohistochemistry assays.

Summary/Conclusions

PCR-based tracking of patient-specific *MYCN* amplicon breakpoints can be used as a highly sensitive and specific assay detecting neuroblastoma MRD in high-risk cases. Robust MRD testing has the potential to support MRD-based therapy adaptations and pave the way for analyzing clinical significance of changes in neuroblastoma MRD levels in clinical trials. Particularly future trials testing when to integrate particular immunotherapies, would benefit from integrating MRD testing and MRD-based criteria in patient trials.

Targeted hybrid-captu
NB-MRD

proces
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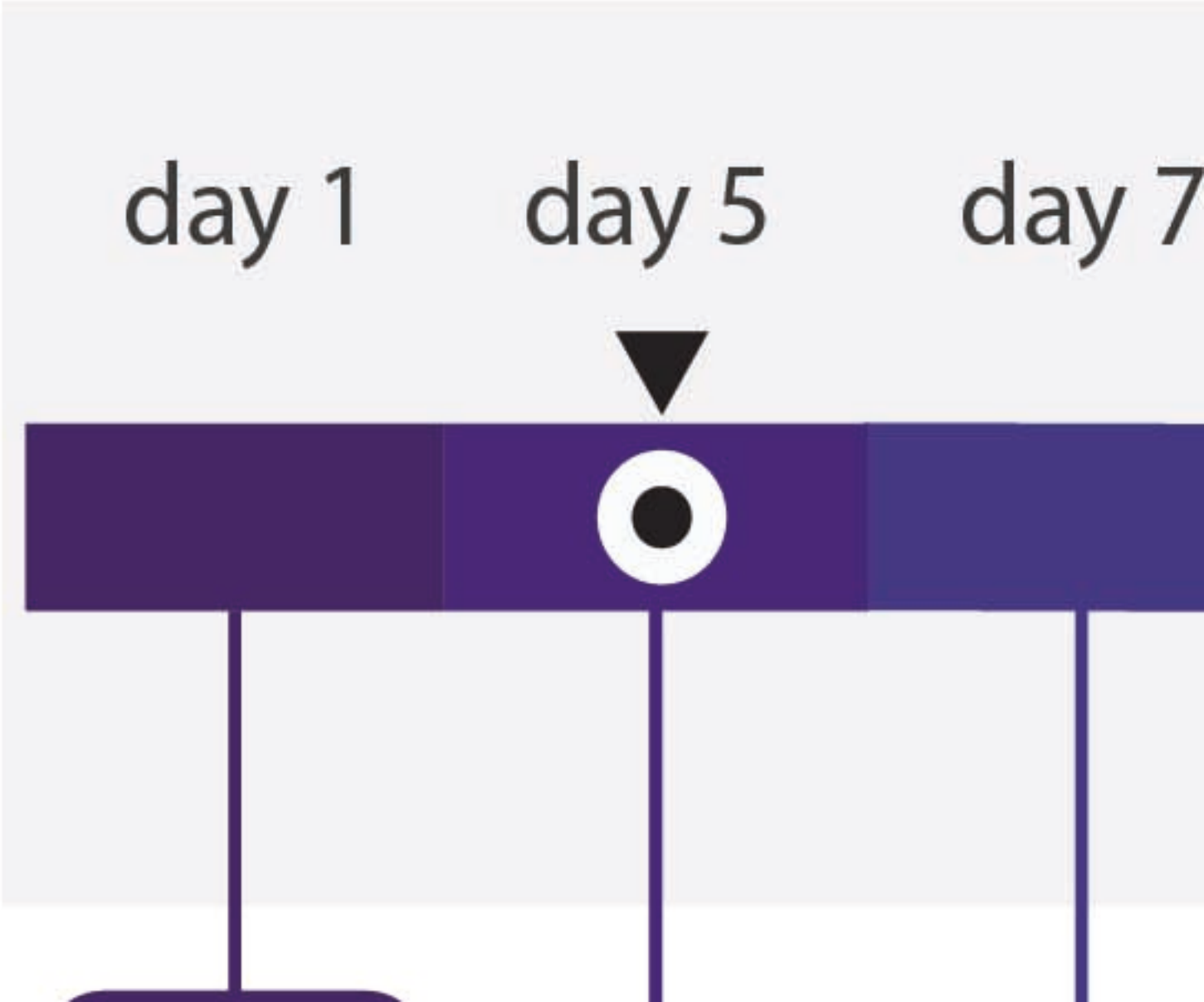

Outline of the clinical implementation of ‘Neuroblastoma-MYCN-breakpoint-MRD’ detection.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 407

Differential Expression of Macrophage-Associated Oncology Markers in Drug-Resistant Neuroblastoma Model.

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

Aggressive neuroblastoma often displays an immunosuppressive tumour microenvironment (TME) which promotes the polarisation of tumour-associated macrophages (TAMs) to the M2 functional phenotype. These M2 cells display pro-tumour affects by stimulation of tumour cell migration, invasion, intravasation and angiogenesis, as well as remodelling of the extracellular matrix and suppression of adaptive immune responses. Hence, it is critical to understand tumour interactions with these immune cells in the TME and how these interactions are influenced by the expression of several immuno-oncology markers.

Aims

This study aims to investigate how common variations in drug sensitivity may contribute to differential expression of immune regulatory markers and therefore immunosuppression in neuroblastoma.

Methods/Materials

Cisplatin-sensitive KellyLuc and cisplatin-resistant KellyCis83Luc cell lines were injected via the tail vein of mice to establish a metastatic xenograft disease. Metastases were resected after 40 days and were formalin-fixed, paraffin-embedded. H&E staining identified tumour cell enriched regions which were microdissected for RNA-seq analysis using the HTG EdgeSeq ImmunoOncology panel of 549 markers. Markers with significant expression variation between the cisplatin-sensitive and resistant xenografts were analysed via bioinformatics and literature review to assess involvement in macrophage function. These were validated via RT-qPCR analysis on the xenograft samples and on the 2D-grown neuroblastoma cell lines: Kelly, KellyCis83, SH-SY5Y, NB1691, SKNBE and CHP212.

Results

Cisplatin-resistant xenografts had an average of 11.4 metastases per mouse vs 6.3 for cisplatin-sensitive, almost a 2-fold increase. 29 of the immuno-oncology markers were differentially expressed in sensitive vs resistant xenografts. 4 markers from this group: Triggering Receptor Expressed on Myeloid Cells 1 (TREM-1), Chemokine Ligand 15 (CCL15), Macrophage Stimulating 1 Receptor (MST1R) and Suppressor of Cytokine Signalling 3 (SOCS3), were found to influence macrophage function in various cancers. SOCS3 was detected in all cell lines tested, with highest expression in CHP212 cells and lowest in Kelly, NB1691 and SKNBE cells, suggesting that these cell lines may create a more immunosuppressive TME if grafted into the murine model. SOCS3 and CCL15 had significantly lower expression in cisplatin-resistant xenografts when analysed via RT-qPCR analysis. Conversely, CCL15 was significantly upregulated in cisplatin-resistant xenografts when analysed via HTG EdgeSeq. TREM-1 and MST1R were not detected in any of the samples tested through RT-qPCR.

Summary/Conclusions

Understanding how tumour cells interact with the immune system in the TME is critical to developing new immunotherapies for the treatment of neuroblastoma. It is well known that traditional 2D cell culture is insufficient for assessing these interactions due to poor presentation of immune antigens. In this study, discrepancies were evident upon comparing samples from the xenograft model vs 2D-grown cell lines, highlighting the need to develop more reproducible 3D models for studying immunosuppression in the neuroblastoma TME *in vitro*. SOCS3 and CCL15 have demonstrated potential as novel targets for neuroblastoma immunotherapy due to their differential expression and impact on macrophage-mediated immunosuppression.

Type Translational

Presentation Preference Traditional poster

Abstract nr. 408

Translation addiction as a novel therapeutic target in neuroblastoma

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Translational control relies on the orchestrated activity of the ribosomal complexes as well as initiation and elongation machineries. In addition, also inherent properties of the RNA-sequence and structures commonly located in the untranslated regions (UTR) of the transcript, such as G-quadruplexes (G4s), also play a key role. Recent work increasingly supports a role for translational control as a hallmark for cancer. The role of adaptive changes in protein synthesis during tumorigenesis is supported by the identification of recurrent mutations affecting both components of the translation machinery itself, mRNA *cis*-regulatory elements as well as multiple oncogenic pathways activating translation. While these specific cancer dependencies related to translation have emerged as innovative entry points for precision oncology, they have not been explored in depth in neuroblastoma.

Aims

To perform phenotypic and molecular evaluation of pharmacological eIF4A inhibition in neuroblastoma and scrutinize the mode-of-action through a comprehensive and integrated proteome profiling approach.

Methods/Materials

We tested the response of a panel of 7 neuroblastoma cell lines to the eIF4A inhibitor CR31B, a synthetic silvestrol analogue. Next, shotgun proteome profiling of IMR32 cells treated with CR31B (or DMSO as a reference) in a time series (3h, 6h, 12h, 24h, 48h and 72h) was performed followed by the identification of patterns of differential protein expression.

Results

The eIF4A inhibitor CR31B treatment had strong effects on cell viability within low nanomolar concentration range in all cell tested cell lines. Comparative proteomics analysis before and after CR31B exposure in neuroblastoma cells revealed 600 significantly differentially expressed proteins from a total of 4452 quantified proteins. Further filtering towards hits displaying at least a 2-fold absolute fold change in expression level, lead to the discovery of TPX2 as one of the top targets, with TPX2 downregulation upon CR31B treatment confirmed by western blot analysis. While TPX2 is a crucial regulator of Aurora kinase A (AURKA, a critical factor of MYCN stability in neuroblastoma and mitotic regulator) and acts as spindle assembly factor, recent evidence also points towards a moonlighting function for TPX2 in DNA repair and replication fork stability, in concert with AURKA. Shotgun proteome profiling will be further complemented with ribosome profiling (ribo-seq) and total RNA-seq. Initial knock down of TPX2 in neuroblastoma cells support its role as dependency factor. We will also perform *in vivo* experiments to scrutinize the functional role of TPX2 in replicative stress resistance and evaluate the pharmacological potential of CR31B using the *dßh-eGFP-MYCN* driven zebrafish neuroblastoma model.

Summary/Conclusions

Our data point at TPX2 as a putative key downstream effector of eIF4A driven translational addiction in neuroblastoma and should stimulate further investigation of this druggable vulnerability.

Type Translational

Presentation Preference Rapid Fire Poster Presentation

Abstract nr. 409

Anti-B7-H3 Antibody MGA271 Induces Antibody-Dependent Cellular Cytotoxicity with Ex Vivo Activated Natural Killer Cells against B7-H3 Positive Neuroblastoma and Tumor Microenvironment Cells and Enhances Immunotherapy of Neuroblastoma Tumors with Adoptively Transferred aNK Cells Combined with Anti-

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

Standard treatment of high-risk neuroblastoma (NB) with the anti-disialoganglioside (GD2) monoclonal antibody (mAb) dinutuximab after myeloablative chemotherapy has increased event-free and overall survival of patients. However, 40% of patients still relapse during or after this treatment. Using a second mAb that targets a different antigen on NB and/or tumor microenvironment cells concomitantly with dinutuximab has the potential to improve therapy.

Aims

B7H3 (CD276), a member of the B7 family of molecules, is expressed on many tumors, including neuroblastoma. We determined if MGA271, a humanized anti-B7-H3 mAb, can induce antibody-dependent cellular cytotoxicity (ADCC) with *ex vivo* activated natural killer (aNK) cells against NB and tumor microenvironment cells and so increase the anti-NB efficacy of aNK cells combined with the anti- GD2 mAb dinutuximab.

Methods/Materials

Expression of B7-H3 on eight MYCN-amplified (MYCN-A) and three non-amplified (MYCN-NA) neuroblastoma cell lines, on cultured mesenchymal stromal cells (MSCs) from the bone marrow of neuroblastoma patients, and on cultured human endothelial cells (ECs) was determined with flow cytometry. ADCC mediated by aNK cells (1:1 effector:target cell ratio) with MGA271 (Macrogenics) against NB cells, MSCs, and ECs was assessed *in vitro*. The efficacy of aNK cells (10^7 IV, 2x/week x 4 weeks) with dinutuximab (15 µg IV, 2x/week x 4 weeks) without or with MGA271 (100 µg IV, 2x/week x 4 weeks) against established intra-renal NB tumors formed by MYCN-A, luciferase-labeled cell lines SMS-KCNR or CHLA-136 in immunodeficient NOD-scid gamma (NSG) mice was evaluated by quantifying tumor growth and by mouse survival.

Results

B7-H3 was highly expressed, as demonstrated by flow cytometry, on all of eleven NB cell lines, including LA-N-6 and CHLA-90 which do not express GD2, on MSCs, and on ECs. MGA271 efficiently induced ADCC with aNK cells against NB cell lines, including LA-N-6 and CHLA-90 which were not sensitive to ADCC mediated by dinutuximab. MGA271 also induced ADCC with aNK cells against MSCs and ECs in a dose-dependent manner. NB tumors formed in NSG mice after intra-renal injection of SMS-KCNR or CHLA-136 cells were most effectively treated with aNK cells and dinutuximab when MGA271 was added as evaluated by both tumor growth and mouse survival.

Summary/Conclusions

B7-H3 is expressed on NB cells, MSCs, and ECs, and MGA271 induces ADCC with aNK cells against these B7-H3 positive cells. Treatment efficacy of dinutuximab with aNK cells against NB tumors in NSG mice is significantly increased by addition of MGA271.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 410

Molecular diagnostics and targeted therapy of neuroblastoma in light of intratumour heterogeneity

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

Intratumour heterogeneity (ITH) constitutes a major problem for molecular diagnostics and molecular targeted therapies leading to cancer progression and treatment failure. We had previously investigated ITH in neuroblastoma by performing a multi-region whole-exome sequencing (WES) and transcriptome sequencing (RNA-Seq) of 51 spatially separated tumour samples and 10 normal controls derived from 8 primary and 2 relapsed neuroblastomas. The WES data had identified a high rate of spatial heterogeneity with 22 % single-nucleotide variations that were present in only one region of a tumour. Spatial heterogeneity was also apparent for mutations in known cancer-related genes in high-risk neuroblastomas whereas low-risk and intermediate-risk tumours lacked such mutations.

Aims

Our project aims to longitudinally analyse the changes of clinically relevant molecular markers during the disease progression and to decipher the impact of chemotherapy on tumour evolution.

Methods/Materials

Following the WES analysis, we established an ultra-deep targeted re-sequencing of 1,479 SNVs in 150 samples from the same 10 patients with a median coverage of 2500 x. Next to the 61 samples already analysed by WES, we included 89 several additional samples in the targeted sequencing cohort, for which only limited or FFPE material had been available. For 8 of the 10 patients, the samples profiled by targeted sequencing covered at least two time points of the course of the disease.

Results

The targeted re-sequencing approach confirmed the absence of mutations in cancer-related genes in the low-risk and intermediate-risk neuroblastomas as well as in tumour samples from high-risk patients that were in complete remission for more than 3 years. Aberrations in cancer-related genes were present in 5 out of 10 patients of our cohort. About 30% (0-60%) of the mutations in cancer-related genes were found to be clonal, i.e., every sample from the same patient showed clear evidence of this mutation. These clonal mutations included activating mutations in *HRAS*, and *ALK* and deleterious mutations in *ATM*. Other driver mutations like *FGFR1* (N577K), *BRAF* (V600E) and *CCND3* were solely observed in single biopsies or in some but not all tumour regions from the same neuroblastoma, indicating spatial ITH. Importantly, the most prominent genetic heterogeneity was prevalent between different time points in the course of the disease, *e.g.* before and after therapy. One high-risk patient showed mutations in *BRCA2* and *PWWA2A* together with a *MYCN* amplification which were restricted to the time of the tumour resection after therapy but undetectable in earlier samples, while an *ALK* mutation remained clonally stable over time. For another high-risk neuroblastoma, the *ALK* mutation was detectable in all initial samples and essentially disappeared after standard therapy. To trace evolutionary trajectories under the influence of the therapy, we performed a clonality analysis based on the targeted re-sequencing data. This analysis revealed both linear as well as branched evolution in neuroblastoma.

Summary/Conclusions

Our data indicate that the decision for second line targeted therapies e.g. ALK inhibitors, should incorporate precise molecular diagnostics covering multiple biopsies in order to address both spatial and temporal ITH in neuroblastoma.

Type Translational

Presentation Preference Oral presentation

Advances in Neuroblastoma Research (ANR) Meeting
ANR 2020
Amsterdam
11-14
May
www.anr2020.org

Abstract nr. 411

Analysis of immune checkpoints in patients with high-risk neuroblastoma treated with dinutuximab beta with and without IL-2.

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

Checkpoint inhibitors have emerged as a promising treatment option in cancer. However, in pediatric oncology, checkpoint blockade is not standard of care yet and clinical trials are mainly focused on inhibition of PD1/PD-L1- and CTLA-4/CD80-CD86 pathways.

Aims

To evaluate the role of checkpoint inhibitors in immunotherapy, we analyzed a set of soluble immune checkpoint molecules and T-cell activation markers in the serum of high-risk neuroblastoma patients treated with the anti-GD_2 antibody (Ab) dinutuximab beta (DB) and isotretinoin with and without IL-2.

Methods/Materials

In a SIOPEN Phase II clinical trial (EudraCT 2009-018077-31) 160 patients were randomly

assigned to receive up to 5 cycles of 100 mg/m^2 DB-LTI (d8-17) and 160 mg/m^2 oral isotretinoin (d19-32) without (81 pts, DB arm) and with 6x10^{6}IU/m^2 scIL-2 (d1-5; 8-12) (79 pts, DB and IL-2 arm). Serum concentration of T-cell activation marker ((soluble (s) CD25 (sIL-2r), 4-1BB, sCD27) and immune checkpoints (LAG-3, CTLA-4, CD86, PD-1, PD-L1, PD-L2, TIM-3 and Galectin-9) were determined in the first cycle prior to the treatment (baseline) and during the Ab-infusion with or without IL-2 (day 8 of Ab-infusion) using a bead based immunoassay. Finally, pts were grouped according to the median checkpoint concentration (into high and low level) and correlations between these groups and the 2-year progression-free survival (PFS) were evaluated.

Results

Pts of the DB and IL-2 arm showed a strong elevation of the T-cell activation markers sCD25, sCD27 compared to the baseline (4.7 and 1.8 fold increase, respectively). Importantly, in pts of the DB arm sCD25 was also increased, whereas sCD27 remained stable (2.3-fold increase and 1-fold increase compared to the baseline, respectively). Compared to the base line, the costimulatory T-cell receptor 4-1BB was significantly increased in the DB arm (1.5 fold) but not in the DB and IL-2 arm (1.1-fold). Notably, we observed a significantly improved survival in pts with high 4-1BB serum concentrations during the Ab-infusion solely in the DB and IL-2 arm (P = 0.0497). The PFS was 74% (95% CI [0.58; 0.89]) and 58% (95% CI [0.42; 0.74]) for high and low level pts, respectively.
Immune checkpoints PD-1, PD-L1 and TIM-3 were similarly elevated in the DB arm and the DB and IL-2 arm (1.2-, 1.4-, 2.2- and 1.3-, 1.4-, 1.7-fold increase, respectively). In contrast, PD-L2 was significantly decreased during the Ab-infusion compared to the baseline in both cohorts (0.8. and 0.9-fold increase, for the DB- and the DB and IL-2 arm, respectively).
Importantly, the TIM-3 ligand Galectin-9 was elevated in the DB and IL-2 but not in the DB arm (2.0- and 1.0-fold increase compared to the baseline, respectively).
Survival analysis did not reveal a direct negative impact of checkpoint serum concentration and survival. Surprisingly, DB pts with high levels of TIM-3, showed significantly prolonged PFS of 74% (95% CI [0.6; 0.89]) compared to 47% (95% CI [0.32; 0.63]) for low level pts.

Summary/Conclusions

DB with and without IL-2 resulted in increased immune checkpoint and T-cell activation marker concentrations. Therapy induced immune checkpoints were not associated with poor outcome.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 412

ROCK inhibition induces changes in the neuroblastoma tumor microenvironment

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

We have shown that every fourth neuroblastoma patient presents with at least one mutation or structural aberration associated with the Rho/Rac signaling pathway; a pathway implicated in neural crest differentiation and migration. These mutations promote a more active state of Rho, which is believed to result in an increased downstream Rho-associated kinase (ROCK) activation. ROCK has previously been identified as a promising drug target to inhibit cancer growth and metastatic spread. Furthermore, Rho/ROCK signaling pathway effects on cytoskeleton arrangement and cell shape have been suggested to also be involved in tumor promoted modulation of the tumor microenvironment (TME).

Aims

Here we have explored ROCK as a therapeutic target in neuroblastoma with emphasis on its' influence on the TME and immune response.

Methods/Materials

We evaluated two different ROCK inhibitors, RKI-1447 and HA-1077, in a 2D cell viability assay (WST-1) and in a multi-cellular tumor spheroid (MCTS) model with co-cultured fibroblasts and tumor cells. We also performed immunohistochemistry (IHC) staining on cells within the TME after treatment with HA-1077 in tumors from the transgenic neuroblastoma model TH-*MYCN* and quantified infiltrating cells.

Results

Using two different ROCK inhibitors, we demonstrated that ROCK inhibition suppressed neuroblastoma growth both in a 2D cell viability assay and in a MCTS model with co-cultured fibroblasts and tumor cells. The MCTS 3D tumor model is preferred for better modeling of physiological drug distribution, cell-to-cell interactions, and the TME. In the MCTS model we did not observe an effect of the two ROCK inhibitors on the fibroblasts at concentrations used in this study, but noted evident repressed tumor cell growth. In addition, we monitored populations of T cells ($CD3^+$, $CD4^+$ and $CD8^+$), macrophages ($F4/80^+$) and fibroblasts (α-SMA^+) within ROCK inhibitor, HA-1077, treated and untreated tumor tissue from homozygous TH-*MYCN* mice. Treatment with HA-1077 significantly inhibited tumor growth in homozygous TH-*MYCN* mice. Through quantifying the number of infiltrating cells in ROCK inhibitor treated tumor tissue compared to untreated tumor tissue we observed a significant increase in $CD3^+$ and $CD8^+$ T cells following ROCK inhibition.

Summary/Conclusions

This shift in $CD8^+$ T cells indicates a shift toward a cytotoxic T cell immune response following ROCK inhibition in our neuroblastoma tumor tissue samples. Further experimentation is necessary to verify these results; investigations are ongoing. We hope that these studies can reveal new insights into the Rho/ROCK signaling pathway and involvement of the TME in neuroblastoma.

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ROCK inhibition induces changes in the neuroblastoma tumor microenvironment

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Type Translational

Presentation Preference Rapid Fire Poster Presentation

Abstract nr. 413

Second high-dose chemotherapy with autologous stem cell rescue in relapsing high risk neuroblastoma patients

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

The prognosis of progressing or relapsing high risk neuroblastoma is still poor. Often, further progression is already observed during second-line induction chemotherapy. However, part of the patients respond to second-line induction chemotherapy and are candidates for consolidation treatments such as second high-dose chemotherapy with autologous stem cell rescue (ASCR).

Aims

To determine outcome of patients receiving second high-dose chemotherapy with autologous stem cell rescue after first relapse or progression.

Methods/Materials

Data of high risk neuroblastoma patients registered in the prospective national trials NB97 and NB2004-HR were analyzed. Inclusion criteria were INRG high risk criteria and age < 21 years at initial diagnosis, presenting with relapse/progression after previous high-dose chemotherapy with ASCR, response or stable disease to second line relapse induction therapy, and consolidated with second ASCR between 2007 and 2018. Only patients treated for first relapse or progression were included. Event-free-survival (EFS) was calculated from second ASCR to subsequent relapse, death or last follow up, overall survival (OS) was calculated from second ASCR to death or last follow up.

Results

A total of 21 patients met the inclusion criteria and were included in the analysis. At first diagnosis, 18 were stage 4 > 18 months and three were stratified high risk by MYCN amplification.

Patients presented at a median age of 2.7 at initial diagnosis (range 0.6-6.3 years) and of 5.8 years at second ASCR (range 3.2-12.6 years). Seven patients were treated for isolated locoregional relapse and 14 for systemic or combined relapse.
At second ASCR, five patients presented in complete remission, eleven in partial remission and one patient with stable disease. The remission status was not available in four patients. High-dose regimens used were heterogeneous and treosulfan-based in eleven patients and busulfan-based in six patients. In eleven patients the second high-dose chemotherapy was combined with mIBG-therapy.
One patient died from transplant-associated toxicity, 13 patients experienced relapses 2.6 to 35 months after the second ASCR, 9 of them died. Seven patients are currently alive without further progression. At three years after second ASCR, EFS was 0.32 ± 0.10 and OS 0.57 ± 0.11.

Summary/Conclusions

In this retrospective analysis of a small cohort of patients with relapse of high-risk neuroblastoma responding to second-line induction chemotherapy, consolidation with second high dose chemotherapy and ASCR seemed to be of benefit. This approach warrants further investigation in prospective trials.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 414

The Composition, States and Dynamics of Microenvironmental Landscape of High-Risk Neuroblastoma Revealed by Single-Cell RNA Sequencing

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

Studying the tumour microenvironment (TME) enables the identification of the immunosuppressive strategies utilized by tumours to evade the host immune system, the development of novel therapeutic targets and strategies. Yet, the TME of high-risk neuroblastoma is poorly characterized.

Aims

Here, we used state-of-the-art single-cell sequencing technology to shed light on the composition, states and dynamics of microenvironmental landscape of high-risk NB after treatment with high dose chemotherapy.

Methods/Materials

Single-cell RNA sequencing (RNA-Seq) has been applied on fresh surgical specimens of 12 high-risk neuroblastoma patients mostly after debulking surgery. We extensively optimized the workup protocols and samples are now mildly dissociated to the single-cell level and sorted by flow cytometry. Single cell suspensions were subsequently subjected to single cell RNA-Seq using the CEL-seq2 platform. Results were interpreted and compared to reference datasets using the bioinformatic platform R2. To validate our results, we generated co-culture systems with fluorescent GFP labeled organoids and immune cells.

Results

We characterized the cellular identities of nearly 9000 cells that passed quality controls and split them into a nonimmune and immune cell compartment. We observed large amounts of immune cell infiltration in post-treatment tumour biopsies. Within the heterogeneous microenvironment of pretreated neuroblastoma, T and NK cells make up a considerable part of the immune infiltrate. Neuroblastoma cell clusters in particular downregulate of MHC-1 genes (HLA-A, HLA-B, HLA-C and B2M), a previously known reported tumour escape mechanism from T lymphocytes described in many human tumours of different origins. We also observed a large cell subsets of CD8+ T cells showing signs of ‘‘exhaustion’’, as characterized by the downregulation of specific genes associated with effector functions (INFG, TNF, FGFBP2, and CX3CR1) or proliferation (KI67) or the upregulation of inhibitory checkpoint genes which are linked to the dysfunctional state (PD-1, CTLA4 and TIGIT). To investigate the potential interactions between neuroblastoma and NK and T cell subsets, we utilized a previously published computational pipeline of immune-related ligand-receptor interaction to gain insights into the immunomodulatory relationships among these cell clusters.

TIGIT was identified as consistently highly expressed in tumour-infiltrating lymphocytes across multiple NB patients. Interestingly, TIGIT expression correlated with NK cell subsets. Hence, we propose that blockade of TIGIT might prevent or reverse NK and CD8+ T cell exhaustion and promoted T and NK cell–dependent neuroblastoma immunity. To evaluate of the contribution of TIGIT immunomodulation on neuroblastoma exvivo, we deployed a new 3D co-culture system of NK cells from normal adult peripheral blood mononuclear cells (PBMC) donors with patient-derived neuroblastoma organoids. To assess the amount of viable tumour cells left after CD8+T and NK cell encounter, GFP-LUC expressing neuroblastoma organoids were co-cultured with whole PBMC or PBMC depleted of NK cells or CD8 T cells. Immune cell mediated neuroblastoma killing is currently being tested in combination with various checkpoint inhibitors.

Summary/Conclusions

This work provides a unique insight into the cellular composition of neuroblastoma tumours after treatment. We used scRNA-Seq to infer the transcriptional states of NB cells and the tumour-infiltrating immune cells in 12 high-risk post-treatment neuroblastoma patients. Coupling scRNA sequencing with functional validation systems such as our 3D neuroblastoma organoid co-culture platform might reveal novel immunotherapeutic interventions to be implemented to the standard of care to improve the clinical results of high-risk neuroblastoma patients.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 415

Alk mutations - a promessing future in target therapy

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

A vast array of oncogenic variants has been identified for anaplastic lymphoma kinase (*ALK*). Therefore, there is a need to better understand the role of *ALK* in cancer biology to optimize treatment strategies.

In tumors where the full-length *ALK* itself is mutated, such as neuroblastoma, the picture regarding the role of *ALK* as an oncogenic driver is less clear. The discovery of germline missense point mutations in the intact *ALK* oncogene related to the etiology of familial neuroblastoma, and of somatically acquired mutations that predict for inferior outcome in patients with the most aggressive form of this disease, has positioned *ALK* as the most important mutated oncogene

tractable for targeted therapy in this disease.
We described a case of 4-year old child with stage 4 neuroblastoma, amplified *MYCN*, with an at least uncommon fusion *ALK-MYC* and *OTOF–ALK* which may have conferred an extremely aggressive disease. The patient died after 6 months of diagnosis, despite the use of Crizotinib.

Aims

To describe an aggressive fusion of *ALK* mutation which may be suitable as a target therapy once it is found in the tumor sample.

Methods/Materials

A sample obtained from a cervical lymph node metastasis was analyzed by NGS method using the kit for detection OptiView DAB IHC and amplification OptiView.

Results

After diagnosis of Neuroblastoma, patient received chemotherapy as high risk protocol, with continued aggressive progression of disease even after alternative schedules. As soon as the NGS results were available, Crizotinib was started. Unfortunately, the child died with progressive disease after 1 month.

Summary/Conclusions

Neuroblastoma is the most common cancer diagnosed during the first year of life. Many clinical, biological, genetic and biochemical factors are associated with worse prognosis. This case addresses many of the current issues surrounding the role of *ALK* mutations and neuroblastoma and discusses the prospects for clinically effective targeted treatments based on mutated *ALK* inhibition.

Type Translational

Presentation Preference Traditional poster

Advances in Neuroblastoma Research (ANR) Meeting
ANR 2020
Amsterdam
11-14
May
www.anr2020.org

Abstract nr. 416

The neuroblastoma dependency factor RRM2 is regulated during sympathoblast differentiation and represents a synergistic drug target for high-risk neuroblastoma

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

DNA replication is a tightly controlled process which is essential for cell survival. Oncogene-

induced replicative stress increases fork stalling and collapse, resulting in double strand breaks which can drive tumor initiation. Further increased replicative stress can become a threat to cancer cells due to excessive DNA damage, imposing cells to adopt a replicative stress resistor phenotype. RRM2, a subunit of the ribonucleotide reductase, is critically involved in control of low dNTP levels in early S-phase, which represents a potential vulnerability for cancer cells. The role of RRM2 as a novel dependency factor and options for targeted therapy have not been studied in neuroblastoma.

Aims

We aimed to study the role of RRM2 in neuroblastoma oncogenesis, its control during normal development and under the MYCN induced replicative stress adaptive regulatory network. Further, we also aimed to explore options for combination therapies targeting RRM2 both *in vitro* and *in vivo*.

Methods/Materials

An integrated *in silico* analysis was performed using genomic and transcriptome data of more than 200 neuroblastoma cases. Functional assays were performed upon RRM2 knockdown and overexpression. Drugging assays were done in neuroblastoma cell lines and organoid cultures. Zebrafish *dßh-RRM2* and *dßh-MYCN* stable transgenic lines were generated. CasID technology was used to pull down RRM2 promotor bound proteins.

Results

Computational analysis confirmed RRM2 as top-ranked putative co-driver in neuroblastoma. Strong RRM2 dependency was shown *in vitro* through knockdown in neuroblastoma cells, with transcriptome analysis revealing enrichment of expected FOXM1, cell cycle and TP53 gene signatures. Transgenic zebrafish lines expressing *RRM2* in immature sympathoblasts were crossed with a *dßh-MYCN* transgenic zebrafish line. RRM2 overexpression dramatically increased tumor penetrance from 20% to nearly 100%, accelerating neuroblastoma formation with tumors formed as early as 5 weeks of age in the double positive fish ($p<0.0001$). To gain insight into which factors control RRM2 expression, we used CasID to identify RRM2 promotor bound proteins and identified NurD and PAF chromatin factors. The catalytic NurD subunit CHD4 is strongly upregulated during neuroblastoma formation in mice. Further, CHD5 is a commonly deleted presumed tumor suppressor located on 1p36 and known to replace CHD4 in NurD complexes during neuronal differentiation. We further mapped RRM2 expression levels in single cell RNA-seq trajectories in developing mouse sympathoblasts and noted highest expression levels of RRM2 in the so-called "bridging population", a highly proliferative population that interconnects Schwann cell precursor cells with the mature chromaffin cells. These findings suggest that, in addition to adaptive regulation of RRM2 to replicative stress, control of RRM2 expression levels may play a role in sympathoblast differentiation. Importantly, adaptive responses regulating RRM2 expression are also controlled by ATR/CHK1 and WEE1 cell cycle checkpoint kinases, as shown by pharmacological inhibition. We provide *in vitro* and *in vivo* data showing that RRM2 inhibition with triapine strongly sensitizes neuroblastoma cells to ATR/CHK1 and WEE1 inhibition.

Summary/Conclusions

RRM2 is a critical dependency factor in high-risk NB which is also strictly regulated during sympathoblast differentiation. We propose RRM2 as a *bona fide* target for exploring novel synergistic drug combinations through phase I clinical trials.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 417

Validation of a standardized diagnostic workflow for analyzing circulating tumor DNA in Neuroblastoma

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

The minimal invasive nature of liquid biopsies and their application as a potential biomarker for cancer diagnosis and monitoring has already been widely demonstrated in various research studies. Especially in neuroblastoma extensive tumor heterogeneity has been observed and could lead to biases in diagnostic tumor profiling. Therefore the additional analysis of circulating tumor DNA (ctDNA) could be beneficial for the use in clinical diagnostics; however, a fully validated standard protocol is not yet available.

Aims

Our aim was to establish a diagnostic workflow by (1) investigating pre-analytical conditions including blood collection tubes, transport and storage, (2) comparing manual and automated cell-free DNA extraction methods and (3) validating the molecular analysis of neuroblastoma-specific tumor markers by digital droplet PCR (ddPCR).

Methods/Materials

Healthy donor blood was collected in standard blood collection tubes (EDTA, Heparin) and leucocyte stabilizing tubes (PAXgene ccfDNA, Roche cfDNA and Streck BCT) and cfDNA was extracted from plasma after storage of 0-48 hours at RT or +4°C, respectively. Extracted cfDNA

was analyzed for yield and fragment size distribution (genomic DNA contamination) by TapeStation (Agilent, USA), Qubit fluorometry (ThermoFisher, USA) and applied on down-stream applications (NGS, ddPCR). The most commonly used QIAamp circulating nuclear acid kit (Qiagen, Germany) was compared to the fully automated extraction protocol Maxwell® RSC ccfDNA plasma kit (Promega, USA), and the quality and quantity of the extracted cfDNA analyzed with the methods described above. ddPCR with Bio-Rad's (USA) wet-lab validated ddPCR Assays was applied for the detection of *MYCN* amplification, *ALK* amplification and *ALK* hotspot mutations (*ALK* p.F1174L and p.R1275Q) in cfDNA reference standards (Horizon, UK) and spike-in samples.

Results

The cfDNA extracted from widely used EDTA-samples show good results for liquid biopsy analyses if stored for a maximum of 4 hours at room temperature or for up to 24 hours at +4°C. For longer storage or transportation of blood samples leucocyte stabilizing tubes are recommended. Furthermore, using Streck BCT or EDTA blood samples, stored for maximum 24 hours at +4°C, leucocytes and low amounts of tumor cells can be isolated and analyzed in addition to the ctDNA.
Concerning the extraction methods and ddPCR we were able to meet the required quality criteria in order to implement the workflow for analyzing circulating tumor DNA from neuroblastoma blood samples in a diagnostic laboratory. This includes partial automation which allows us to analyze more samples in a standardized manner, leading to fast turnaround times.

Summary/Conclusions

Taken together, we established a validated partially automated diagnostic workflow including guidelines for pre-analytical conditions adapted to small blood volumes for processing of peripheral blood and cfDNA extraction for reliable downstream ctDNA analysis compliant with real-life conditions in clinics and diagnostic labs. By using the ddPCR we implemented a highly sensitive and specific detection method for *MYCN* and *ALK* alterations in ctDNA of neuroblastoma patients. These additional genetic results supplied by molecular diagnostics could improve disease stratification, therapeutic target detection and monitoring and allow researchers to study tumor heterogeneity and evolution in the future.

Type Clinical

Presentation Preference Rapid Fire Poster Presentation

Abstract nr. 418

ALK mutation type and frequency in circulating tumor DNA correlate with response to lorlatinib and predict disease progression

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

Tumors evolve under the selective pressure of therapy, and resistant subclones, either pre-existing at diagnosis or acquired during treatment, lead to relapsed and progressive cancer. Tissue biopsy samples are not always feasible in children, do not lend themselves to serial monitoring, and are inadequate representations of entire tumor bulk. Adult oncology studies have shown that assays of circulating tumor DNA (ctDNA) can track the evolutionary dynamics and heterogeneity of tumors and detect early emergence of therapy resistance, minimal residual disease, and relapse. This is of particular value in high-risk neuroblastoma, where relapsed disease harbors novel genomic variants and the mechanisms of resistance, including to ALK inhibition, are not understood.

Aims

Through our ongoing Phase 1 trial of lorlatinib, a next-generation potent and specific ALK inhibitor for children with relapsed/refractory ALK-driven neuroblastoma, we sought to explore the utility of ctDNA for predicting response and/or resistance to lorlatinib and for detecting disease recurrence before it is apparent clinically in patients with relapsed/refractory *ALK* mutated neuroblastoma.

Methods/Materials

Relapsed/refractory patients >12 months, with *ALK* mutations or amplification were eligible; prior ALK inhibitor treatment was allowed. Lorlatinib was orally administered once daily in 28-day courses. Four dose levels (45, 60, 75, 95 mg/m^2/day) were assessed and DL5 (115 mg/m^2/day) is currently enrolling. Blood samples for ctDNA profiling were obtained at baseline and then temporally matched with radiologic restaging. Samples were subjected to the FoundationOne

Liquid platform. We analyzed the trajectory of the known *ALK* mutant variant allele frequency (VAF) and its correlation with clinical response, as well as the emergence of novel variants that suggest evolution of resistance and disease progression.

Results

Of the 32 patients currently enrolled, 24 have ctDNA results from more than one timepoint, with clinical evaluations available on 21 of the patients. One patient harbored a germline *ALK* mutation, and maintained an *ALK* R1275 VAF of ~0.5 with near complete radiologic response to therapy. Four patients never had *ALK* mutation detected in serial ctDNA sampling, despite harboring known *ALK* clonal mutations prior to trial enrollment. Of the remaining 15 patients, the majority had trajectories in mutant *ALK* VAF that correlated with disease burden and clinical response. Only 5 patients had changes in *ALK* mutation VAF that did not reflect their clinical response, with decreasing *ALK* VAF despite disease progression. Six of the 15 patients showed the emergence of novel *ALK* mutations during lorlatinib therapy that correspond with disease progression. These mutations include the I1171T, L1196M, G1202R, and D1203N, which, when position is known from sequencing data, occur in *cis* with the original somatic ALK mutation. Each of these has been described as single resistance mutations to earlier generation ALK-inhibition in non-small-cell lung cancer, but are predicted to be sensitive to lorlatinib.

Summary/Conclusions

Prospective evaluation of ctDNA allows for monitoring of disease burden during ALK inhibition therapy in patients with high-risk neuroblastoma. Emergence of new ALK mutations can predict disease progression, and the mechanism of resistance when two individually sensitive mutations occur in *cis* is being investigated and will be reported.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 419

Low DLG2 gene expression, a link between 11q-deleted and MYCN-amplified neuroblastoma, causes forced cell cycle progression, and predicts poor patient survival.

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Topic Gene regulatory networks and epigenetics: from basic science to therapy

Background/Introduction

There are two groups of aggressive neuroblastomas, one with *MYCN* amplification, and another with 11q chromosomal deletion; these chromosomal aberrations are generally mutually exclusive. The *DLG2* gene resides in the 11q-deleted region, thus makes it an interesting neuroblastoma candidate tumor suppressor gene.

Aims

The aim of this study was to evaluate the possibility of *DLG2* to be a tumor suppressor gene, important for the biology of aggressive neuroblastoma.

Methods/Materials

We evaluated the association of *DLG2* gene expression in neuroblastoma with patient outcomes, stage and *MYCN* status, using online microarray data combining independent neuroblastoma patient data sets. Functional studies were also conducted using neuroblastoma cell models and the fruit fly.

Results

Using the array data we concluded that higher *DLG2* expression was positively correlated to patient survival. We could also see that expression of *DLG2* was inversely correlated with MYCN status and tumor stage. Cell proliferation was lowered in both 11q-normal and 11q-deleted neuroblastoma cells after *DLG2* over expression, and increased in 11q-normal neuroblastoma cells after *DLG2* silencing. Higher level of *DLG2* increased the percentage of cells in the G2/M phase and decreased the percentage of cells in the G1 phase. We detected increased protein levels of Cyclin A and Cyclin B in a fruit fly model when over expressing *dMyc* or silencing *dmDLG* , indicating that both events results in enhanced cell cycling. Induced *MYCN* expression in neuroblastoma cells caused lowered *DLG2* gene expression, this was confirmed in the fly, when *dMyc* was over expressed, the dmDLG protein level was lowered, indicating a link between Myc over expression and low dmDLG level.

Summary/Conclusions

We conclude that low *DLG2* expression level forces cell cycle progression, and that it predicts poor neuroblastoma patient survival. The low *DLG2* expression level could be caused by either *MYCN*-amplification or 11q-deletion.

Low *DLG2* gene expression, a link between 11q-deleted and *MYCN*-amplified neuroblastoma, causes forced cell cycle progression, and predicts poor patient survival.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 420

Human stem cell based models of neuroblastoma provide a complementary system to address critical biological questions in neuroblastoma.

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Current human models of neuroblastoma rely on PDX and human cell lines. While these models can provide important insight into how specific mutations impact the biology of neuroblastoma, the mutation being investigated is influenced by the array of other mutations present in the genome. In addition, these tumors have already been transformed and it is difficult to determine which mutation initiated tumorigenesis. We propose a human stem cell based model of neuroblastoma would provide a complementary system to investigate these important questions in neuroblastoma biology.

Aims

The aim of this study is to utilize human stem cell based models of neuroblastoma to identify how various MYC-driven tumors differ molecularly in driving tumorigenesis.

Methods/Materials

Human induced pluripotent stem cells (iPSC) derived integration free from healthy human adults were transduced with inducible $MYCN^{WT}$, $MYCN^{P44L}$ and CMYC. $MYCN^{WT}$ were also transduced with either empty vector of constitutive expression of ALK^{F1174L}. iPSC were differentiated toward trunk neural crest cells implanted into the renal capsules of immunocompromised mice. Mice were euthanized when tumors grew to 2cm in diameter. Nucleic acids were extracted from flash frozen tumor tissue and submitted for RNA-seq and ChIP-seq analysis.

Results

ALK/MYCN tumors had lower latency than MYCN tumors. Differential gene expression analysis revealed ALK/MYCN tumors, compared to MYCN tumors, upregulated genes involved in integrin signaling including ITGB1 and ITGB5.

$MYCN^{P44L}$ and CMYC tumors were more penetrant than $MYCN^{WT}$ tumors. RNA and DNA have been extracted and submitted for RNA-seq and ChIP-seq analysis, respectively, to identify potential differences in genes driven by each transcription factor.

Summary/Conclusions

Here, we show that human stem cell based models of neuroblastoma can identify important biological differences in how specific genetic mutations influence neuroblastoma tumorigenesis. In the case of ALK/MYCN tumors, our study suggests blocking integrin signaling may represent a new therapeutic strategy to treat tumors, particularly in tumors resistant to ALK inhibitors.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 421

Haddad Syndrome and Neuroblastoma: a case report

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

Haddad Syndrome (HS) is a very rare disease including Congenital Central Hypoventilation Syndrome (CCHS) and Hirschsprung disease (HSCR). We report a case of HS complicated by neuroblastoma.

Aims

Methods/Materials

Case description: Second child born at full-term with perinatal asphyxia. During hospitalization, she presented respiratory failure, needing assisted ventilation and tracheostomy. Genetic studies showed a de novo heterozygous mutation (C.780dupT) of PHOX2B gene, confirming CCHS. At 1 month of age HSCR was diagnosed, with subsequent segment resection and coloanal anastomosis. The presence of CCHS and HSCR led to the diagnosis of Haddad Syndrome. As CCHS and HSCR may be associated with Peripheral Neuroblastic Tumor, a close follow up was performed. At 13 months of age a right surrenalic mass was found, associated with an increment of urinary VanillylMandelic Acid (25.94 mg/gr urinary creatinine). Magnetic Resonance Imaging showed a voluminous thoraco-abdominal mass, from D5 to L2, with predominant left paravertebral thoracic development, inhomogeneously hyperintense in TR sequences and positive in DWIBS (mm 108 x 36 x 37); IDRF were present. MIBG-Scintigraphy confirmed the presence of the tumor in the thoraco-abdominal region. The ultrasound-guided ago-biopsy showed fragments with proliferation of neuronal cellular elements with maturation phenomena and calcifications, low MKI and MYCN-non-amplified. Findings were compatible with neuroblastic tumors not further classifiable. Bone marrow biopsy were negative. During hospitalization, the patient presented recurrent life-threatening event with hypoglycemia, hyperglycemia and metabolic acidosis. Therefore, because of the complex clinical presentation of the baby and her metabolic instability, in agreement with the family, we chose a wait and see approach.

Results

Summary/Conclusions

We highlight the importance of a multidisciplinary follow up for CCHS patients in order to early detect associated illnesses such as HSCR and tumors derived from the neural crest and to plan the best treatment for the patient.

Type Clinical

Presentation Preference Traditional poster

Abstract nr. 422

Dynamic Contrast-Enhanced Perfusion MRI and Diffusion-Weighted Imaging as an imaging biomarker for neuroblastic tumors.

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Neuroblastoma (NB) is the most frequent solid extracranial cancer in childhood. Its diagnosis, prognosis and monitoring are based on the information provided by multiparametric magnetic resonance (MR) and MIBG images.

Aims

Our focus in this work is to explore the utility of diffusion and perfusion changes in NB as an early biomarker of diagnosis, using diffusion weighted (DWI) and dynamic contract enhanced (DCE) MRI.

Methods/Materials

Multiple MR imaging real-word sequences, available within the H2020 PRIMAGE project, were used from 50 patients. Volumes-of-interest were calculated and transferred to DCE perfusion and apparent diffusion coefficient (ADC) maps. Histogram analysis and clustering based unsupervised machine learning algorithms were used to determine the values of mean and standard deviation (SD) of initial area under the curve at 60 seconds (IAUC60) and ADC for automatic differentiation of neuroblastic tumors. The resolution of voxel intensity was estimated and the IAUC60 and ADC data was smeared accordingly to allow us to identify and remove the noise and low-quality voxels (defined as those voxels with an uncertainty larger than 10%).

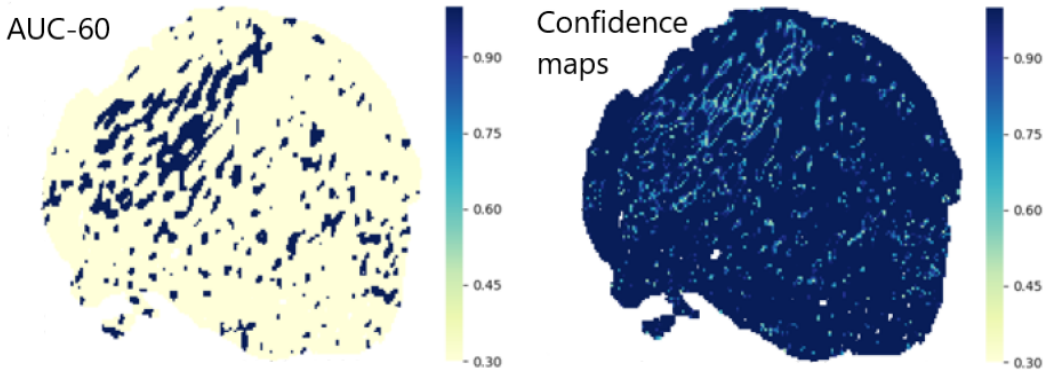
Results

Significant differences in mean ADC (expressed in 10^{-3} mm^2/s) were found for neuroblastic tumors: 1.2 for ganglioneuroma, 0.74 for ganglioneuroblastoma, and 0.54 for neuroblastoma, with an uncertainty of 37%, 43% and 20%, respectively. This result improves tumor differentiation with respect to state-of-the-art voxel-by-voxel methodologies, which were found to be: 1.6 for ganglioneuroma, 1.7 for ganglioneuroblastoma, and 1.3 for neuroblastoma, with an uncertainty of 45%, 63% and 23%, respectively. Mean IAUC60 was found to have a value of 43 (and 14%

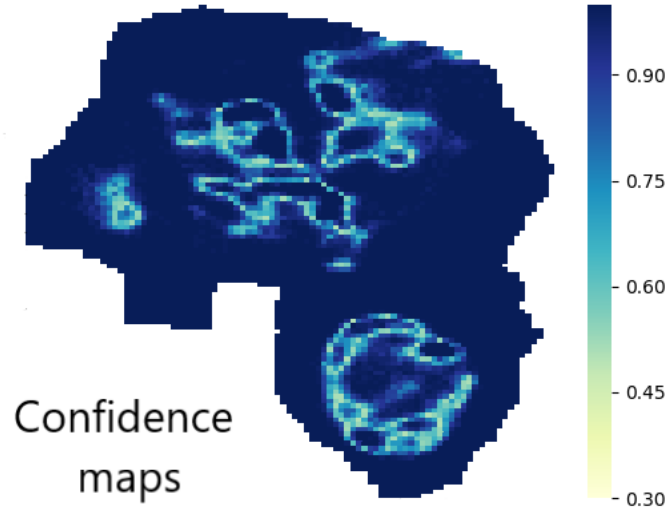
uncertainty) for neuroblastoma, as opposed to a value of 17 (and 17% uncertainty) with state-of-the-art voxel-by-voxel methodologies.

Summary/Conclusions

The proposed novel technique to determine IAUC60 and ADC parameters holds promise for differentiating benign and malignant neuroblastic tumors. This work lays the foundation for future advances in reproducible imaging biomarkers for paediatric solid tumor cancers.



Confidence maps based on the Apparent Diffusion Coefficient (ADC) to account for neuroblastic intra-tumor heterogeneity.



Representation of neuroblastic tumor habitats (left) and confidence maps based on the initial area under the curve after 60 seconds (iAUC-60) (right).

Type Translational

Presentation Preference Oral presentation

Abstract nr. 423

WHOLE TRANSCRIPTOME PROFILING OF LIQUID BIOPSIES FROM TUMOR XENOGRAFTED MOUSE MODELS ENABLES SPECIFIC MONITORING OF TUMOR-DERIVED RNA

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Liquid biopsies enable disease diagnosis and treatment response monitoring. While the use of cell-free DNA is well established, extracellular RNA has particular advantages, mostly because of its dynamic nature. Besides free-floating (circular) RNA, RNA is also present in extracellular vesicles, lipo-protein complexes and platelets. More recently, the concept of tumor educated platelets was established whereby tumor-derived RNA ends up in platelets and the overall splicing pattern of platelet RNA is altered. In this study, we used tumor xenografts as an elegant model system, to assess to which extent tumor (human) RNA ends up in different blood (plasma) fractions. This will support the decision to select the fraction which is best suited to probe tumor-derived extracellular transcriptomes. Based on proof-of-concept data, we anticipate that monitoring through liquid biopsies will enable the assessment of therapeutic effects on tumor cells.

Aims

We aim to determine in which blood-derived plasma fraction (from tumor educated platelets to platelet-free plasma) resides the highest tumor-derived RNA concentration using xenograft model systems and to establish standardized experimental and computational procedures for plasma preparation and sequencing-based profiling of extracellular messenger RNA (mRNA).

Methods/Materials

We examined the circulating transcriptome of extracellular mRNA in two different subcutaneous xenograft models, i.e. SK-N-BE(2C) cells and the BRC016 breast cancer PDX model, as well as in

non-tumor bearing control mice. From 5 mice per group, blood was collected via cardiac puncture using an EDTA tube, followed by platelet, platelet-rich, platelet-poor and platelet-free plasma preparation. Using 60 µl input volumes, an established total RNA sequencing workflow to profile extracellular mRNA (Everaert, Helsmoortel et al., 2019) with synthetic spike-in RNA for calibration purposes, and a dedicated data processing pipeline to unambiguously distinguish human (tumor) from murine (host) mRNA, we charted the extracellular transcriptomes.

Results

Total RNA sequencing of small volumes of biofluids with (partially) degraded RNA is technically challenging. The use of synthetic spike-in RNA and downsampling of sequencing libraries to equal depth (1 or 5 million reads, depending on the model) enable robust data comparison.
Despite the high RNA concentration variability among individual mice, we were able to detect human RNA in all plasma fractions in a ratio that was on average ten to hundred times lower than murine RNA. In line with the large RNA cargo in platelets, the murine RNA concentration in plasma increased with increasing platelet concentration. In contrast, human RNA concentrations were relatively constant. As such, we did not find supporting evidence for preferential loading of tumor RNA in platelets.
Using small biofluid input volumes and low (down-sampled) sequencing depth, we detected up to 1500 human tumor-derived protein-coding genes and over 7000 murine genes. Functional exploration of the resulting transcriptomes revealed various enriched gene sets and pathways, indicative of biological signal. We are currently conducting neuroblastoma xenograft studies with targeted therapeutic agents, in order to assess whether on-target activity of the drug on the tumor cells can be monitored in plasma.

Summary/Conclusions

We provide a firm proof-of-concept for the RNA-based detection of specific tumor signal in plasma from xenografted mice. We conclude that platelet-free plasma is the preferred liquid biopsy type for therapeutic response monitoring.

*Vanessa Vermeirssen and Jill Deleu contributed equally to this work.
*All authors are also affiliated to the *Cancer Research Institute Ghent (CRIG).*

Type Translational

Presentation Preference Rapid Fire Poster Presentation

Abstract nr. 424

Lutetium-177-DOTATATE treatment for relapsed/refractory neuroblastoma patients

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

Despite improved outcomes for high-risk neuroblastoma (NB) patients, survival is still low for patients with refractory and/or relapsed disease, and additional treatments are needed. Since most neuroblastoma tumors express somatostatin receptor-2 (SSTR-2), SSTR-2 analogues such as DOTATATE can be used for imaging and targeted therapies. Lutetium-177-DOTATATE is FDA approved for treatment of gastrointestinal neuroendocrine tumors in adults which also express SSTR-2 and can be considered for treatment of relapsed/refractory NB patients.

Aims

To describe Lu-177-DOTATATE treatment in patients with relapsed/refractory NB.

Methods/Materials

Patients with relapsed/refractory NB were identified for possible treatment with Lu-177-DOTATE based on the following parameters: Gallium-68-DOTATE PET scan avidity, major organ toxicities ≤grade 2, adequate bone marrow function (platelets >50K, ANC >500/ul), and availability of stored autologous peripheral blood stem cells. Treatment was administered in the outpatient clinic following radiation safety guidelines. Patients received pre-medication of antiemetics followed by an infusion of renal protective amino acids (2.5% Lysine + Arginine) at the rate of 250 mL/hour starting at least 30 minutes prior to Lu-177-DOTATATE. Lu-177-DOTATATE was infused over 30 minutes using a Graseby syringe pump. Patients and their families received verbal and written instructions regarding radiation safety restrictions. Up to four doses of Lu-177-DOTATATE were administered at 8-10 week intervals.

Results

Five patients with multiply relapsed NB, (median 4 relapses and 7 prior therapies), ages 4.5, 6, 10, 11 and 38 years, received 9 doses of Lu-177-DOTATATE. One patient received 4 doses, another 2 doses and the other 3 patients received 1 dose each. Administered dose ranged

between 100- 200 mCi/administration. The infusions were well tolerated with grade 1 nausea with one of 9 treatments and no episodes of emesis. Weekly laboratory evaluations did not reveal changes in renal function up to the last follow up (3 months – 1.5 years). There was no ≥grade 2 organ or hematologic toxicity except grade 2 thrombocytopenia noted after 3rd dose in the patient who received 4 doses of Lu-177-DOTATATE. One patient had significant clinical improvement (less pain and less pain medication usage) after initial dose with Ga-68-DOTATATE PET scan that was stable disease (SD) by International Neuroblastoma Response Criteria (INRC) yet had >40% reduction of soft tissue masses and decrease in extent and avidity by SUV of all osseous lesions. However, this patient had disease progression after the 4th dose. One patient had a mixed response after the first dose and progression after the second dose. Two patients had progression of disease clinically and on scans after the first dose. The 5th patient had a clinical response with decrease of pain and has yet to have a follow up scan.

Summary/Conclusions

Lu-177-DOTATATE is a feasible outpatient treatment with minimal toxicities for patients with relapsed/refractory NB. Further treatments on phase I trials are necessary for dose discovery in children with NB.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 425

The development of a human embryonic stem cell derived differentiation model to study normal and neuroblastoma development.

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Topic Neuroblastoma as developmental disease

Background/Introduction

Neuroblastoma (NB) is a clinically and genetically heterogeneous embryonal cancer thought to arise from sympatho-adrenergic progenitors (SAPs) derived from the neural crest. NB can be defined as a developmental disorder resulting from a disruption of normal differentiation. This is a complex process guided by environmental cues and executed through gene regulatory networks controlled by multiple transcription factors (TFs) such as PHOX2B, SOX11 and TBX2. Many aspects of human neural crest differentiation remain poorly understood; defining the normal differentiation process is therefore essential to improve our limited understanding of the pathogenesis of NB and developing more effective therapies while minimizing side effects. Currently, accurate human model systems for normal and aberrant SAP development are lacking. Using human pluripotent stem cells (hPSCs), we optimised and characterised an *in vitro* differentiation model to generate SAPs, thereby allowing research into normal SAP development and NB oncogenesis.

Aims

Using hPSCs we optimised and validated an *in vitro* differentiation model to investigate normal and aberrant SAP development.

Methods/Materials

Utilizing a modified dual-SMAD inhibition differentiation protocol developed by the Studer laboratory at the Memorial Sloan Kettering Cancer Center, we performed *in vitro* differentiations of hPSCs into SAPs. Over the course of the 40-day differentiation process, cells were harvested for RNA isolation and processed for polyA-selected bulk RNA sequencing at specific time-points. The

transcription profiles were evaluated using multi-dimensional analysis including Principal Component Analysis (PCA), hierarchical clustering and both gene and gene-set enrichment analysis (GSEA). For more in depth analysis and validation of our system, the expression profiles of specific neurodevelopmental markers such as PAX6, SOX10, ASCL1, PHOX2B, GATA2/3 and HAND2 were assessed. Comparison with three previously sequenced laser capture micro-dissected foetal adrenal neuroblasts from 13-16 week gestational age human embryos and 15 NB tumours is in progress.

Results

Reproducibility of our experiments was indicated by PCA and hierarchical clustering where biological replicates grouped together at each time-point. During the differentiation to multipotent neural crest cells (NCCs), from day 0 to day 16, we could confirm the development of truncal NCCs based on the transient expression of SOX9 and SLUG, combined with an increase in the expression of SOX10 and HOX genes, including A7, B8 and B9, as well as the absence of PAX6 expression. Further differentiation of truncal NCCs into SAPs showed downregulation of SOX10 expression and upregulation of TFs including ASCL1, PHOX2B, GATA2/3 and HAND1/2, consistent with development of lineage-committed SAP cells. Comparison of these gene expression profiles with NB tumours and foetal adrenal neuroblasts is currently on-going.

Summary/Conclusions

We developed a hPSC-derived, *in vitro* differentiation model to study normal SAP differentiation and NB development. This novel platform is now being used to explore NB transformation *in vitro* using genetically-modified and patient-specific hPSCs. Thus, this human model system will provide key insights into early NB oncogenesis and serves as a platform for future mechanistic and therapeutic studies in NB.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 426

Recurrent rare high level amplification of the BRISC and BRCA1-A complex member 2 (BABAM 2) gene in high-risk neuroblastoma

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Topic Other

Background/Introduction

Neuroblastoma (NB) is a childhood cancer characterized by recurrent copy number aberrations and a low mutational burden. Around 25% of the tumors, usually high stage, present with high level amplification of the *MYCN* gene, one of the most important genetic markers in terms of prognosis and treatment impact, together with other rare but recurrent amplifications. The prognosis of children with NB tumors carrying amplified genes is unfavorable. Previous studies from our lab and others have shown that rare recurrent amplicons harbour genes critically involved in NB, including *MYC, LIN28B, ALK, MDM2* and *CDK4*.

Aims

Unraveling the nature of a novel rare recurrent amplicon encompassing the *BABAM2* locus in neuroblastoma

Methods/Materials

Shallow whole genome sequencing was performed to map copy number imbalances. Whole genome DNA and RNA sequencing were done respectively to unravel the nature of the amplicon and to determine RNA expression level and identify alternative or fusion transcripts. In search for additional cases with focal *BABAM2* amplification, TARGET and TCGA databases were mined.

Results

A first case of NB with high level amplification of the exons 4-5 of the *BABAM2* coding region was identified in a high stage neuroblastoma of a 7-month old infant diagnosed presenting with liver metastases, but without bone marrow involvement. Shallow whole genome sequencing of the tumor revealed the presence of typical segmental aberrations including *MYCN* amplification, 1p deletion, 2p gain (including *ALK*), 17q gain in addition to whole chromosome imbalances (losses of whole chromosome 9, 16, 19 and X and gain of chromosome 22). Whole genome sequencing was performed to study the *BABAM2* amplicon in further detail and revealed an integration of *BABAM2* exon 4 into the *MYCN* amplicon. RNA sequencing showed an overexpression of *BABAM2*. Comparison of the RNA sequencing profile of this case with >500 other NB cases led to the identification of an extra NB case with an amplicon encompassing *BABAM2* and *ALK*. In addition, database mining revealed another NB case with *BABAM2* amplification and one case of pheochromocytoma/paraganglioma. The BABAM2 protein acts as an adapter in several protein complexes, including the BRCA1-A complex and the BRISC complex, involved in DNA double strand break repair and mitotic spindle assembly, thereby providing new therapeutic opportunities. The *BABAM 2* gene is upregulated in several types of cancer, including ovarian cancer and in AML with 11q23 rearrangements and t(8;16), where BABAM2 was found to be overexpressed due to intragenic transcription initiation.

Summary/Conclusions

In this study, we describe the first neuroblastoma cases presenting with amplification of the *BABAM2* gene. A systematic search revealed this alteration to be rare but recurrent strongly suggesting a tumor driving role in NB.

Type Translational

Presentation Preference Rapid Fire Poster Presentation

Abstract nr. 427

Pleuroparenchymal fibroelastosis: a rare complication of neuroblastoma therapy

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Topic Other

Background/Introduction

Non-infectious pulmonary complications after chemotherapy and/or bone marrow transplant are well described. Pleuroparenchymal fibroelastosis (PPFE) is a rare interstitial lung disease, first reported as an idiopathic pneumonitis in adults in the 1990s. The disease is characterized by pleural thickening and fibrosis, parenchymal fibrosis predominantly in the upper lobes, with peripheral and subpleural elastic deposition. PPFE, as described in adults, has also been associated with prior bone marrow transplantation, lung transplantation, infection, toxic exposures/pollutants, chemotherapy, and in autoimmune disease. Patients with PPFE are at risk for progressive respiratory failure, refractory pneumothorax. Currently, the only known therapeutic option is lung transplant. We present a case series of patients with history of neuroblastoma, referred for lung transplant evaluation for end stage lung disease secondary to PPFE.

Aims

To describe the association of prior history of neuroblastoma to secondary lung fibrosis, specifically PPFE, occurring years after treatment.

Methods/Materials

The electronic medical records of eight patients with a history of neuroblastoma referred to Texas Children's Hospital for lung transplant evaluation from 2012-2019 were retrospectively reviewed. Clinical information, including symptoms, age at diagnosis of lung fibrosis, lung function testing and body mass index at time of presentation to the lung transplant service, along with treatment regimen, were extracted. Radiologists with an expertise in chest imaging and experience in diagnosis of PPFE reviewed chest CT scans, and pathology (either via lung biopsy or explant after lung transplant) was reviewed, if available, for confirmation of PPFE.

Results

The mean age of diagnosis of neuroblastoma was 3.3 years (range 1.8-4.7 years). The mean time between diagnosis of neuroblastoma and presentation of PPFE was 9.4 years (range 6.25-

11.75 years). All 8 patients received chemotherapy according to the following protocols: ANBL0532, ANBL00P1, and A3973. Furthermore, all patients received radiation therapy (not to the chest however), along with autologous bone marrow transplant, preceded by varying conditioning regimens. All patients presented with progressively worsening dyspnea and dry cough; four of the eight patients presented with pneumothorax, with persistent air leak. Lung function testing demonstrated a severe restrictive defect, and the mean body mass index z-score was -2.9. Six patients presented with severe malnutrition, which became challenging to manage despite enteral and sometimes parenteral supplementation. Six of the eight patients have been lung transplanted: 4 are alive and doing well >2 years post-transplant; 1 patient was recently transplanted; and 1 patient died 6 years after transplant. Two patients were not eligible for transplant: one patient received talc pleurodesis, which is viewed as a contraindication in most lung transplant centers; the other was removed from the list for non-adherence.

Summary/Conclusions

Pleuroparenchymal fibroelastosis is a rare but underrecognized and important complication, seen in some patients years after neuroblastoma treatment. We speculate on cause of the progressive lung disease—whether it is an association of certain chemotherapeutic regimens (as thought in adults), or are certain patients predisposed due to other factors (eg, possible telomere shortening or genetic predisposition). Our future work will include further investigation into those predisposing factors that could lead to PPFE in this population.

Type Clinical

Presentation Preference Rapid Fire Poster Presentation

Abstract nr. 428

Angiogenic potential of small extracellular vesicles in drug resistant neuroblastoma

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Topic Other

Background/Introduction

Neuroblastoma is a paediatric cancer of the sympathetic nervous system and the most common solid tumour of infancy which accounts for approximately 15% of all paediatric oncology deaths. The main challenge in treating high risk neuroblastoma is metastatic spread and development of chemotherapeutic resistance. Extracellular vesicles are membrane bound vesicles containing bioactive molecules including RNAs and proteins that play an important role in cancer through cell-cell communication. We hypothesis that the development of cisplatin resistance in neuroblastoma cells increases the angiogenic potential and alters the proteome of their small extracellular vesicles (sEV).

Aims

Methods/Materials

The cell lines Kelly (cisplatin sensitive) and KellyCis83 (cisplatin resistant) were cultured in high density bioreactors with sEV depleted media, which was harvested weekly for sEV isolation by differential centrifugation. sEV were characterised by Western blot analysis for positive protein markers HSP70, TSG101 and CD9, and the negative marker VDAC-1. Nano-particle tracking analysis (NTA) and transmission electron microscopy (TEM) was performed to characterise sEV size range and morphology, respectively. To assess angiogenic potential, human umbilical vein endothelial cells (HUVEC) were incubated with Kelly and KellyCis83 sEV on Matrigel and tubule formation and length were measured at 8 and 24 hours.

Results

Kelly and KellyCis83 sEV were positive for HSP70, TSG101 and CD9 and negative for VDAC-1 by Western blot. TEM revealed spherical membrane bound particles with a NTA consistent size profile between 80-200nm. There was a significant increase in the number and length of tubules formed in HUVECs treated with KellyCis83 sEV compared to Kelly sEV and untreated HUVECs.

Summary/Conclusions

Our data indicates that the development of cisplatin resistance results in the secretion of sEV that

bear an increased angiogenic potential compared to cisplatin sensitive cells. In future studies we aim to investigate the sEV proteome of Kelly and KellyCis83 cells to identify which proteins are involved in driving this angiogenic potential.

Type Basic

Presentation Preference Traditional poster

Abstract nr. 429

New diagnostic tool based on label-free Tomographic Phase Microscopy to detect circulating neuroblastoma cells

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Topic Other

Background/Introduction

Circulating tumor cells (CTCs) serve as tumor biomarkers in many types of cancer and their clinical utility is under investigation. Recent evidence suggests that CTCs are detectable in neuroblastoma (NB) and may be useful as predictive biomarkers in early-phase clinical trials of response to chemotherapy. In addition, genome characterization of CTCs will offer the unique potential to design a precise targeting therapy to avoid disease recurrence. Despite the large number of approaches proposed to detect and classify CTCs, e.g. by examining their physical and/or biological properties, the accurate identification of CTCs still remains an unaccomplished goal.

Aims

Achieving affordable and accurate liquid biopsy for NB clinical use has so far proven difficult due to two main problems, i.e. technological issues in development of practical devices because of very small number of CTCs with respect the other components of the bloodstream (e.g., red blood cells, white blood cells and platelets) and lack of reliable biomarkers. The development of a method based on label-free microscopy to detect a set of all-optical and morphological features will be useful for NB CTCs detection in blood.

Methods/Materials

A new technology is proposed, based on the combination of tomographic phase microscopy (TPM) and microfluidics. TPM is an imaging technique able to supply three-dimensional phase contrast images at single cell level that will be applied on cells that rotate into engineered microfluidic channels in order to accurately control their rotation. The method allows to discern the inner structures of a cell without labelling but measuring the refractive indexes and thicknesses of the cell organelles as nucleus, nucleolus, mitochondrion etc. To provide reference values for nuclei and mitochondria, NB cell lines (SHSY5Y, SKNSH, CHP134, IMR32, SKNBE) were evaluated by using Amnis ImageStream^X Mk II flow cytometer. Cells previously stained with DAPI (nuclear stain) or MitoTracker (mitochondrial stain) were evaluated by IDEAS software combining fluorescence intensity (DAPI or MitoTracker) and morphometric measures (darkfield or brightfield). In particular, distinct cells subpopulations in the same cell lines were observed based nuclei diameter (DAPI; intensity darkfield) or mitochondria number (mitotracker; area brightfield).

Results

Five NB cell lines were evaluated by TPM while rolling into microchannels and by flow cytometry upon fluorescent staining. Tomographic images of single cells were analysed to identify the cell constituents and fluorescence images of single cells were analysed to determine nuclei diameter and mitochondria number. We are now performing computational analyses to define physical features that are peculiar of NB cells and that can be detected by TPM. A comparison between the results obtained by Amnis ImageStream^X Mk II flow cytometer and TPM will be presented.

Summary/Conclusions

High-throughput tomographic imaging of NB single cells in microfluidic platform will define a new diagnostic tool based on morphological parameters as biomarkers to identify NB CTCs. The proposed method can open the way for new label-free diagnostic tool for fast blood analysis and effective advanced NB screening.

Type Basic

Presentation Preference Rapid Fire Poster Presentation

Abstract nr. 430

“A tale of two diseases”: a single-cell approach to neuroblastoma identifies two diseases entities, distinct for developmental origin, site of onset and genomic alteration profile

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Topic Neuroblastoma as developmental disease

Background/Introduction

Neuroblastoma (NB) is an elusive cancer deriving from a transient population of multipotent and highly motile cells during the embryonic development; the neural crest cells (NCC). Depending on their localization along the cranium to sacrum antero-posterior axis the NCC gives rise to a variety of cell types including ganglia, cartilage and bones, the thymus, melanocytes, the adrenal medulla and part of the heart and septum. NB is believed to emerge from the trunk NCC and particularly from the sympathoadrenal precursor (SAP) sublineage to which previously consolidated knowledge attributes the formation of both the sympathetic neurons of the paravertebral ganglia and the ChCs of the adrenal medulla. However, we now know that in mouse and zebrafish 80% of the ChCs of the adrenal medulla originate from a totally different NCC progeny than the SAPs, the Schwann cell precursors (SCPs). These cells can migrate along the visceral motor nerves to the vicinity of the developing adrenal gland, where they detach from the nerves and form 80% of the postsynaptic neuroendocrine ChCs. The other 20% of ChCs is contributed by the conventional SAP lineage. Therefore, according to this 20/80% double origin, the ChCs of the adrenal medulla will be a mix in this proportion of cells of SAP and SCP origin, respectively.

Aims

We aim at revising NB taxonomy on a developmental basis, by exploiting a single-cell biology approach.

Methods/Materials

To define an hypothetical model for NB taxonomy and for the relative segregation of genomic profiles, we combined (a) the available radiogenomics data on NB topology of origin; (b) the data coming from recent work about the spatiotemporal structure of cell fate decisions in murine NCC; (c) the high evolutionary conservation of the NCC developmental patterns along vertebrate evolution, including the alternative SCP route of NCC in zebrafish and later in humans; and (d) the patterns of the partial or total mutual exclusivity of NB mutations based on the classification of the tumour grade. Thanks to this approach, we could define a model for the NB origin which greatly simplifies the taxonomy of this disease. We verified this model by single-cell biology of selected human NB samples.

Results

We provide *topological* (precise site of origin of the tumour), *developmental* (mutually exclusive presence of SAP lineage or SCP lineage markers), and *genetic* (exact segregation of mutations) evidence that single neuroblastoma cells derived from tumours belong to either the SCP or the SAP developmental lineages. We also provide evidence that all the current mouse and zebrafish NB models do not match the human condition, being all artificially “hybrid” versions of the two different human diseases.

Summary/Conclusions

On the basis of the findings, NB can be divided into two distinct diseases: the sympathoadrenal precursor neuroblastoma (SAP-NB) deriving from the sympathoadrenal lineage, and the Schwann cell precursor neuroblastoma (SCP-NB) deriving from the alternative and parallel Schwann cell route of NCC differentiation. These findings will have a profound impact on NB diagnosis, prognosis and therapy.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 431

Self-renewing neuroblastoma cells of the bone marrow share a mesenchymal phenotype which is associated with poor outcome: an NCRI CCL CSG Neuroblastoma Group Study.

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

Neuroblastoma cells in the bone marrow are a hallmark of high-risk disease in children over the age of 1 year, identifying children for more intensive treatment at diagnosis and being a frequent site of relapse. For some children the elimination of these cells is one of the greatest challenges for cure.

Aims

To characterise neuroblastoma cells that are capable of self-renewal from bone marrow aspirates to identify candidate biomarkers of risk and targets for the design of new treatments.

Methods/Materials

Neuroblastoma cells were isolated from bone marrow aspirates collected at diagnosis from children with stage M high-risk disease using immune-magnetic bead selection for the cell surface disialoganglioside GD_2; CCLG biological study 2019 BS05. The number of GD2 positive cells was recorded and cells characterised by immunocytology for adrenergic, neuronal and haemopoietic markers. Single cell self-renewing neuroblastoma cells were identified by seeding 1 cell into each well of 10 Primeria™ 96 well plates (960 single cells; Poisson distribution probability of $\lambda<1=0.9$) and the number of wells containing ≥5 cells recorded after 21 days by light microscopy. RNA was extracted from clones and paired-end sequencing performed on the Illumina® Hiseq3000. Using a bespoke RNA-sequencing pipeline we identified cell-surface proteins that might be good targets for the development of new treatments. Expression candidates was confirmed in bone marrow aspirates and trephines by immunocytology and immunohistochemistry respectively and their prognostic potential explored.

Results

Median infiltration of bone marrow aspirates with GD_2 positive neuroblastoma cells at diagnosis was 7% (range 0 – 60%; n=100). Expression of the neuroblastoma markers was heterogeneous. Neuroblastoma cells did not express the haematopoietic markers CD45 or CD57. At diagnosis high infiltration of bone marrow aspirates with GD2 positive neuroblastoma cells was predictive of a worse event free survival as previously reported, as was infiltration with PHOX2B expressing cells (chi^2 6.75, p = 0.009, n=62). Total RNA sequencing revealed that single cell self-renewing clones shared a mesenchymal signature that included high expression of vimentin, fibronectin 1, paired related homeobox 1 (PPRX1), periostin and YAP1. Protein expression of the mesenchymal markers PRRX1 and periostin was confirmed on cytospins of bone marrow aspirates and bone marrow trephines. High infiltration of bone marrow with periostin expressing cells was associated with a two-fold increased risk of an event; event-free survival hazard ratio 2.2, p=0.008, 95%CI 0.9 – 5.7 and over-all survival hazard ratio 2.7, p=0.05, 95%CI 0.9-7.7.

Summary/Conclusions

Neuroblastoma cells in the bone marrow contain two divergent transcriptional phenotypes consistent with an adrenergic or mesenchymal lineage. Self-renewing drug resistant cells share a mesenchymal signature which may be associated with poor outcome. The prognostic significance of mesenchymal markers should be investigated further in prospective studies.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 432

Single cell RNA sequencing reveals a pro-inflammatory tumor microenvironment in human neuroblastoma

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

Immunotherapy has been emerging as an important modality for metastatic melanoma and other cancers. Pediatric malignancies, however, are often not treatable with immunotherapy because they are known as “cold tumors”. Cold tumors lack tumor antigens and infiltration of immune cells, meaning they are not targetable. For neuroblastoma, anti-GD2 immunotherapy has been approved and has increased the survival rate when combined with cytokines. However, still half of high-risk neuroblastoma patients are currently not cured. Certain neuroblastomas respond to anti-inflammatory treatment and recently subsets of high-risk neuroblastomas were reported to show an inflammatory phenotype with infiltrating inflammatory cells of prognostic significance. However, these cells remain to be further classified to establish novel therapeutic options. Therefore, we aimed to characterize the molecular landscape of immune cells in human neuroblastoma by single cell RNA sequencing analysis.

Aims

Methods/Materials

We used droplet based single-cell RNA-sequencing (10x Chromium Single-Cell 3’ v2 protocol) and profiled 17 samples from 15 children. The cohort consisted of primary and recurrent neuroblastomas, untreated and treated, core needle biopsies and surgical specimen, low,

intermediate and high-risk patients of different ages and genetic subsets. We used *in silico* analysis and various bioinformatic tools for cell annotations and cell functionality scoring curated from published literature.

Results

We profiled ~70,000 single cells from human neuroblastomas, used unbiased clustering and we identified 13 main cell types spanning tumor cells, stromal cells and immune cells. Our data suggest that neuroblastomas consist of high proportions of immune cells including T cells, myeloid cells, NK cells, B cells and mast cells. The highest proportion of immune cells were T cells followed by cells of myeloid origin. Through subclustering *in silico* analysis, we show that the myeloid cell cluster consisted of six subpopulations, from which two subclusters showed high monocyte and inflammatory scores and expressed inflammatory genes such as IL1B and TNF. Further, three subclusters showed high macrophage score with a distinct M2 signature when compared to monocyte subclusters.
The T and NK cells consisted of twelve distinct subpopulations, deconvoluting into two NK cell populations, a population of NKT cells, a population of innate lymphoid cells (ILCs), a population of regulatory T cells and three CD4+ populations including a naïve, an activated and a T helper 17 population of cells. We detected three distinct populations of CD8+ cytotoxic T lymphocytes (CTLs) showing a high cytotoxicity and exhaustion signature score.
Finally, we observed expression of the antigen presenting genes B2M and MHC class I in the *Mesenchymal* tumor cell subpopulation while the *Adrenergic* tumor subpopulation expressed very low levels. Strikingly, the Adrenergic tumor cells expressed NK cell inhibitory ligands suggesting a possible mechanism of NK cell killing evasion.

Summary/Conclusions

In this study, we further analyzed a complex immune cell landscape in human neuroblastomas using single-cell RNA-sequencing. Our results show that the neuroblastoma tumor microenvironment consists of pro-inflammatory cells many of which appear to be fully functional, however, without exerting efficient tumor cell killing. Further studies are required to test functionality of the immune cells and implications for novel immunotherapy.

Single cell RNA sequencing reveals a pro-inflammatory tumor microenvironment in human neuroblastoma

Center for Regenerative Medicine

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Type Basic

Presentation Preference Oral presentation

Abstract nr. 433

Treatment-related toxicities during anti-GD2 immunotherapy in high-risk neuroblastoma patients in the Netherlands

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

The introduction of immunotherapy using a chimeric anti-GD2 monoclonal antibody (ch14.18, dinutuximab) together with immunostimulatory cytokines [interleukin-2 (IL2) and granulocyte-macrophage colony-stimulating factor (GM-CSF)] has significantly improved survival rates for high-risk neuroblastoma patients. However, this improvement in survival is accompanied by a substantial immunotherapy-related toxicity burden.
Since the approval of ch14.18 for the treatment of neuroblastoma in 2016, high-risk neuroblastoma patients received immunotherapy in accordance with the COG ANBL0032 protocol as post-consolidation therapy in the Netherlands.

Aims

The primary objective was to describe treatment-related toxicities during immunotherapy with ch14.18 antibody, cytokines IL2 and GM-CSF, and isotretinoin in high-risk neuroblastoma patients after completing standard induction and consolidation according to the DCOG (Dutch Childhood

Oncology Group) NBL 2009 treatment protocol.

Methods/Materials

Descriptive retrospective review of medical records of patients with high-risk neuroblastoma treated with immunotherapy as post-consolidation therapy between August 2016 and October 2019 in a single-center in the Netherlands. Toxicities were recorded and graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE; version 3.0). Although a patient may have reported a given toxicity multiple times per course, only the highest grade of toxicity per patient per type per course is given. Besides toxicities, results of blood cultures and dose modifications of dinutuximab and IL2 were collected.

Results

Twenty-six patients (11 girls and 15 boys), with a median age at diagnosis of 3.5 years (range 4 months - 18 years) received immunotherapy. In two patients immunotherapy was discontinued after two courses; in one patient due to disease progression and in another patient due to unacceptable toxicities (catheter-related infection with ICU admission in combination with severe sensory and motor neuropathy).

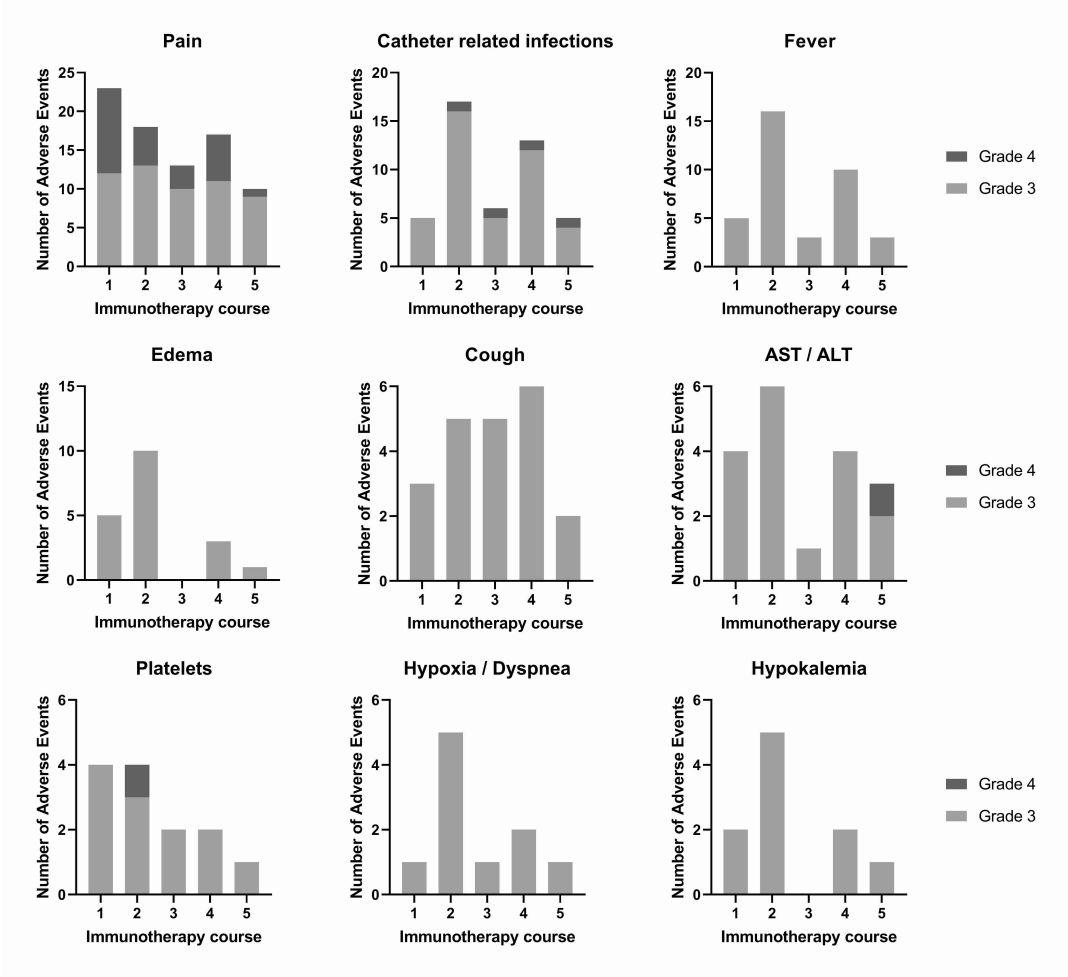
The most common grade ≥ 3 toxicities for courses 1–5, respectively, were pain (88, 69, 54, 70, 41%), catheter-related infections (19, 65, 25, 54, 21%), and fever (19, 62, 13, 42, 13%) (Figure 1). In total, 310 grade ≥ 3 toxicities were recorded in 124 courses of immunotherapy Thirty-three grade 4 (11%) and no grade 5 toxicities (death) were seen. Grade ≥ 3 toxicities were not evenly distributed among the five courses, with most toxicities described in courses with IL2 [course 2 (36%) and course 4 (23%)].

The total dose of dinutuximab was not administered due to toxicity in 0, 27, 0, 21, 13% of patients in course 1-5, respectively.

All 26 patients experienced fever during immunotherapy; 81% suffered from fever >40.0°C (grade 3). Most fever episodes were recorded in the immunotherapy courses with IL2 (course 2 and 4). Catheter-related bloodstream infections were identified in 81% of patients. Four of these episodes were severe (grade 4) and led to ICU admission followed by full recovery.

Summary/Conclusions

Immunotherapy-related toxicity after induction and consolidation according to the DCOG NBL 2009 treatment protocol is considerable but manageable.



The most common grade ≥ 3 toxicities of immunotherapy for courses 1–5.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 434

A Comparative Study of Extracellular Vesicle Encapsulated miRNA from 3D in vitro and in vivo Neuroblastoma Models.

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

The main challenge in treating high risk neuroblastoma is the development of multidrug resistance and metastatic spread, resulting in this rare cancer accounting for 15% of childhood cancer deaths. Neuroblastoma cells communicate with a vast array of neighbouring and distant cells via paracrine and endocrine signalling such direct cell-to-cell contact, cell-to-matrix adhesion and extracellular vesicles (EVs). In our pilot studies, EVs isolated from drug sensitive and resistant neuroblastoma cell lines conferred an increased colony forming ability and drug resistance to sensitive neuroblastoma cells, highlighting the ability of EV cargo containing DNA, RNA and protein to alter the behaviour of recipient cells in an oncogenic manner. Interactions between tumour cells and the extracellular matrix (ECM) also influence disease initiation, progression, and outcome. Therefore, the use of a more physiologically reflective preclinical model which facilitates these 3D ECM interactions enables more reliable testing of therapeutic agents, biomarker identification and basic research. Recently, we described a physiologically relevant porous 3D collagen-nanohydroxyapatite scaffold *in vitro* model for neuroblastoma that demonstrated cell growth and chemosensitivity similar to murine models. We hypothesised that this 3D model could be used to study mechanisms of EV communication in neuroblastoma.

Aims

We aimed to profile the miRNA cargo of EVs derived from drug resistant and sensitive neuroblastoma cells grown in orthotopic mouse models compared to corresponding cells grown in the physiologically relevant 3D porous collagen-nanohydroxyapatite scaffolds.

Methods/Materials

The Kelly (sensitive) and KellyCis83 (resistant) cell lines were grown in 3D *in vitro* scaffold and in murine orthotopic models. Circulating EVs were isolated from murine plasma and from conditioned media surrounding *in vitro* cells by ultracentrifugation, and validated by Western Blot and

nanoparticle-tracking analysis. Profiling of EV miRNA cargo was performed using TaqMan™ OpenArray™ Human MicroRNA Panels.

Results

Cell growth and viability in the 3D *in vitro* model reflected that seen *in vivo* with resistant cells exhibiting higher growth than sensitive. Both cell lines exhibited a considerably higher tolerance to cisplatin in 3D *in vivo* and *in vitro* models at clinically relevant dose (200μM) compared to 2D (20 μM). We identified several miRNAs including miR-17/-92a/-106a and -483-3p which are abundantly expressed in EVs exported from cancer cells grown in the 3D *in vitro*/*in vivo* models and those which are altered in response to chemotherapeutic resistance. High expression of these miRNA in high risk neuroblastoma is significantly associated with poor overall and progression free survival indicating their potential role in mediating response and resistance to therapy.

Summary/Conclusions

Our study demonstrates the ability of EVs derived from drug resistant neuroblastoma cells to reprogram recipient cells, driving tumour progression and resistance. These results also demonstrate the potential of our 3D *in vitro* scaffold based model for the investigation of tumour behaviour, toxicity testing and its utility for the identification of EV encapsulated miRNA biomarkers for further exploration in liquid biopsies.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 435

Trogocytosis: a potential mechanism of resistance in neuroblastoma following anti-GD2 immunotherapy

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

Children with high-risk neuroblastoma (NB) have a poor prognosis with an overall survival of less than 50%. The anti-GD2 monoclonal antibody, dinutuximab, has been shown to improve survival, but many children recur with resistant disease. We recently discovered that treating neuroblastoma cells with activated natural killer cells (aNK) and dinutuximab leads to tumor cell death and the acquisition of the neuroblastoma membrane marker GD2 by the natural killer cells, a phenomenon called trogocytosis.

Aims

To characterize the process of trogocytosis following immunotherapy in neuroblastoma and to determine the efficacy of GD2+ NK cells in neuroblastoma cytotoxicity.

Methods/Materials

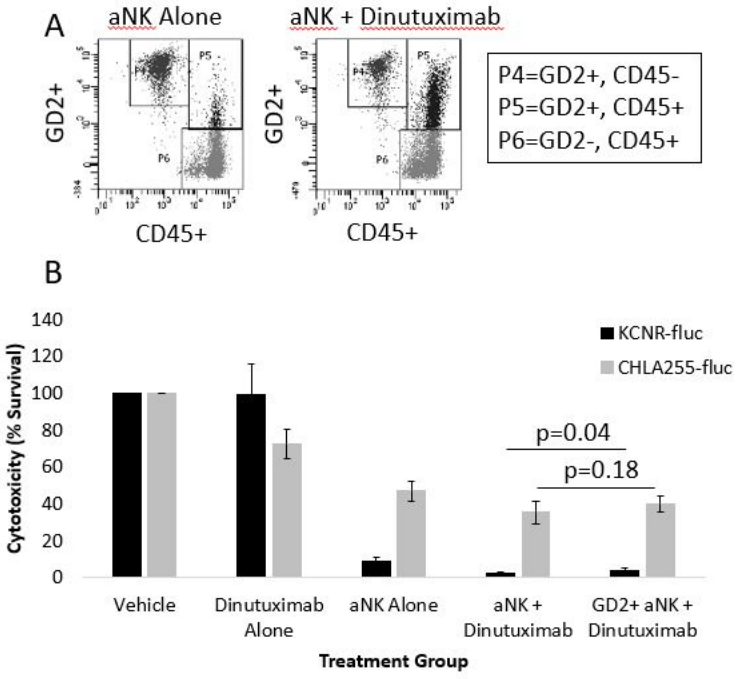
Human NB cells from the CHLA255-fluc and KCNR-fluc cell lines were treated with vehicle control, dinutuximab (100ng/mL) alone, aNK cells alone (E:T 1:1), or aNK cells plus dinutuximab. The treatment groups were then incubated, harvested hourly from 0 minutes to 6 hours, and analyzed by flow cytometry to evaluate expression of GD2 (NB cell marker, APC) and CD45 (NK cell marker, AlexaFluor-488). Live cell microscopy images were obtained at various timepoints. The GD2+ NK cells were then incubated with fresh NB cells for 24 hours to assess cytotoxicity. Luciferase cytotoxicity assays were performed to evaluate the efficacy of the GD2+ NK cells compared to fresh, naïve aNK cells. Student’s t-test was performed; $p<0.05$ was considered significant.

Results

Following treatment with aNK cells and dinutuximab, a novel GD2+/CD45+ population was observed and reached maximal expression at 3 hours (Figure 1A). As tumor cells were killed, there was a notable time-dependent loss of GD2 expression from the NB cell population with a concomitant rise in a GD2+/CD45+ population. Live cell imaging demonstrated the acquisition of NB membrane-bound GD2 by the CD45+ NK cells, resulting in GD2+ NK cells. Lastly, compared to fresh aNK cells, GD2+ NK cells show decreased cytotoxicity to CHLA255 and KCNR neuroblastoma cells alone (p=0.11, p=0.008) and in combination with dinutuximab (p=0.18, p=0.04), respectively (Figure 1B).

Summary/Conclusions

Our study demonstrates the phenomenon of trogocytosis following aNK cell and dinutuximab immunotherapy *in vitro*, which may represent a novel mechanism of resistance in neuroblastoma. Ongoing *in vitro*, *in vivo*, and patient specimen studies are being conducted to further characterize and validate the functional effects of trogocytosis on immunotherapy for neuroblastoma.



Type Basic

Presentation Preference Oral presentation

Abstract nr. 436

The INRG visualization and analytics platform

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Topic Other

Background/Introduction

The INRG Data Commons houses data on over 21,000 children diagnosed with neuroblastoma over the past 25 years. With data on over 30 clinical and phenotypic variables from subjects in North America (COG), Europe (SIOPEN, GPoH) and Japan. Updated outcomes data are periodically added. Current use is restricted to cohort discovery, data requests, and linkage to other data (TARGET, BPC). The purpose of this study is to add visualization and analysis tools to the INRG data commons to provide researchers with a platform for discovery and experimental planning.

Aims

Aim 1 - Allow researchers to select a cohort of interest based selection from the 30+ variables
Aim 2 - Give researchers the ability to build survival curves to examine the event-free (EFS) and overall survival (OS)
Aim 3 - Enable the platform to ingest variables for survival curve analysis from other platforms (e.g., Gabriella Kids First Data Resource).

Methods/Materials

The INRG data commons platform went live in 2015 and is publicly-available at http://www.inrgdb.org for cohort discovery over 21,000 international research subjects and 30+ clinical and phenotypic variables. To create a visualization, the search criteria for a given cohort are passed to the visualization page on which the user provides additional parameters for analysis, including the survival endpoint (OS / EFS), the time window (e.g., 0-10 years), and the variable against which to plot survival (e.g., MYCN status).

The visualization platform makes a call to the API to retrieve a data set consisting of survival time, survival status, and the variable of interest (e.g., MYCN status). These data are then packaged in an R data frame and sent in a call to an R server process running in a web service wrapper. R generates a temporary image file that gets stored on the file system and then displayed in the browser. All of the processing happens server side to prevent the transfer of line-level data to users.

Results

A beta version of the platform was released on January 2020 for testing. Initial results demonstrate a highly-effective platform for generating survival curves for cohorts of patients. Further enhancements will be added to increase functionality and add features. Users will be able to save and download their visualizations as well as use them for formal data requests. Finally, connections will be made to other data sources to allow the consumption of genetic data on the INRG subjects, keyed on the publicly-available Universal Specimen Identifier (USI).

Summary/Conclusions

This represents the first time that a visualization and analysis platform will be available to the research community that facilitates searching over harmonized data from an international cohort of patients. This powerful platform will allow researchers to develop and test hypotheses for further examination. The platform should be useful for a wide variety of researchers, from clinicians with little informatics experience up to bioinformaticians that want an easy cloud-based platform for data visualization and analysis. The platform will be demonstrated during the presentation and a variety of different kinds of analyses will be shown.

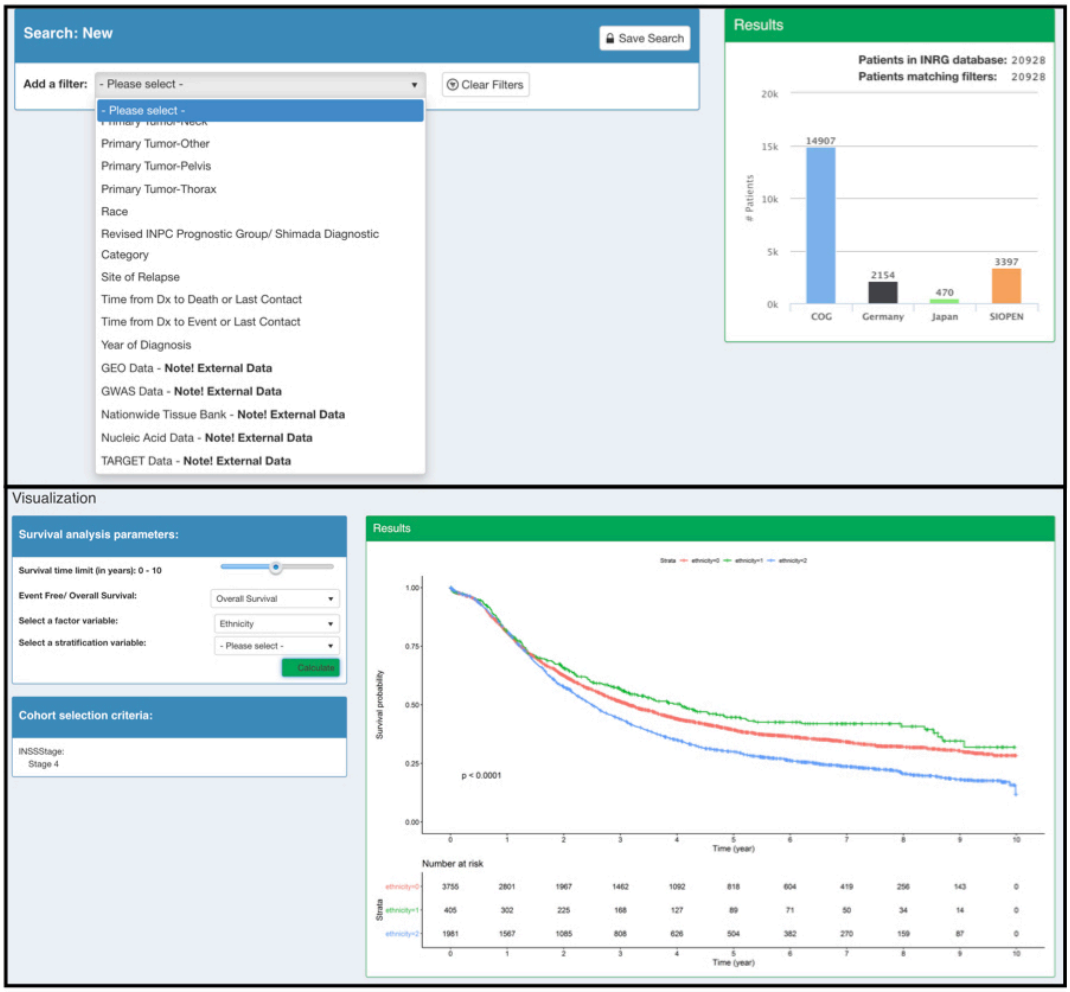

Figure 1 - INRG cohort selection and visualization. Top: Cohort discovery. Bottom: Survival curve generation. Details in the text.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 437

NRAS status determines sensitivity to SHP2 inhibitor-combination therapies targeting the RAS-MAPK pathway in neuroblastoma

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

Survival for high-risk neuroblastoma (NB) remains poor and treatment for relapsed disease rarely leads to long-term cures. Large sequencing studies of NB tumors from diagnosis have not identified common targetable driver mutations other than the 10% of tumors that harbor mutations in the anaplastic lymphoma kinase (*ALK*) gene. However, at NB recurrence more frequent mutations in genes in the RAS-MAPK pathway have been detected. The *PTPN11*-encoded tyrosine phosphatase SHP2 is an activator of the RAS pathway, and we and others have shown that pharmacologic inhibition of SHP2 suppresses the growth of various tumor types harboring KRAS and HRAS mutations such as pancreatic and lung cancers.

Aims

We hypothesized that SHP2 inhibitors might inhibit growth of NB cells and that sensitivity may depend on the genetic status of *RAS, PTPN11*and other components of the RAS-MAPK pathway signaling. Thus, we assessed the sensitivity of multiple SHP2 inhibitors alone and in combination with drugs targeting RAS-MAPK signaling *in vitro* and*in vivo*using cells with and without different RAS pathway activating mutations.

Methods/Materials

The sensitivities ofthree SHP2 inhibitors (NSC-87877, II-B08, and SHP099) were assessed in a panel of human NB cell lines with diverse genetic backgrounds, including mutations in the neuroblastoma *RAS*oncogene (*NRAS)* as well as *NF1* and *ALK*. We also generated isogenic NB cells with endogenous wild-type NRAS (NRAS $^{wt)}$expression that stably overexpressed NRAS ^{wt} or mutant NRAS (NRAS ^{mt}) proteins. Growth inhibition of ranges of doses of SHP2 inhibitors alone and in combination with trametinib, vemurafenib, and ulixertinib were deteremined *in vitro*

using Alamar blue and bromodeoxyuridine incorporation assays, and synergy was determined using excess-over-bliss methods. Downstream RAS-MAPK signaling was assessed by western immunoblot. NB xenografts were generated in NOD-SCID mice and treated with SHP099 alone and in combination with trametinib and tumor measurements and bioluminescence imaging were analyzed.

Results

We observed inhibition of growth and downstream RAS-MAPK signaling in NB cells in response to treatment with the SHP2 inhibitors NSC-87877, II-B08, and SHP099. Cells with RAS pathway alterations, most notably $NRAS^{Q61K}$, were less sensitive to all three SHP2 pharmacologic inhibitors. Furthermore, in contrast to cells with wild-type NRAS, isogenic NB cells engineered to overexpress $NRAS^{Q61K}$ were more resistant to SHP099 and II-B08. Interestingly, dual inhibition of SHP2 and RAS effectors MEK, RAF, or ERK with trametinib, vemurafenib, and ulixertinib, respectively, showed synergistic effects *in vitro* in NB cells harboring RAS-activating mutations and reversed SHP2 inhibitor resistance. Using xenograft models we also demonstrated that, in comparison to vehicle or single agent treatment, combinations of SHP099 with the MEK inhibitor trametinib reduced tumor volume and increased survival *in vivo*. Immunoblots of treated tumor lysates showed increased apoptosis and potent reduction of p-SHP2, p-MEK and p-ERK levels in mice treated with SHP099 and trametinib in comparison to vehicle or either drug alone.

Summary/Conclusions

These results suggest that combination therapies of SHP2 inhibitors with other drugs that target the RAS-MAPK pathway may be effective in relapsed NB including those that acquire RAS-MAPK pathway mutations.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 438

MYCN mediated metabolic processes in childhood neuroblastoma

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Topic Other

Background/Introduction

Neuroblastoma (NB) is one of the most aggressive solid tumors of early childhood. Amplification of the *MYCN* oncogene can be found in around 30% of patients and is associated with rapid tumor progression and poor prognosis. In spite of treatment development, overall and event-free survival for high-risk neuroblastoma patients remains around 40-50%, and survivors often suffer long-term side effects. Alternative approaches, as differentiation-inducing therapies, are especially interesting for high-risk patients in which resistance to conventional chemotherapy arises.
We have shown that a small chemical molecule, 10058-F4, previously identified as a c-MYC inhibitor also targets the MYCN/MAX complex resulting in apoptosis and neuronal differentiation in *MYCN*-amplified NB cells. Importantly, we demonstrated that inhibition of MYC results in metabolic changes including mitochondrial dysfunction leading to accumulation of lipid droplets (Zirath 2013).

We have previously also shown that the *miR-17~92* locus is directly activated by MYCN, highlighting the importance of this cluster in tumorigenesis. We have identified several nuclear hormone receptors including estrogen receptor alpha (ERα) and the glucocorticoid receptor (GR) as targets of *miR-17~92* and have demonstrated their connection to neural differentiation and that overexpression of ERα led to reduced tumor burden (Lovén 2010; Ribeiro 2016; Dzieran 2018).

Aims

To study the impact of MYCN on neuroblastoma cell metabolism and the possible synergy of combined activation of estrogen receptor alpha and the glucocorticoid receptor for neuronal differentiation.

Methods/Materials

Here, we have combined proteomics, gene expression profiling, functional analysis, and metabolic tracing as well as in vitro differentiation analysis and xenograft studies.

Results

Analysis of protein and gene expression highlighted changes in key enzymes involved in glycolysis and β-oxidation in *MYCN*-amplified NB. Metabolic profiling showed that these cells display both enhanced glycolytic and respiratory capacity compared to non-*MYCN* amplified NB cells. In response to high energy demand conditions *MYCN*-amplified cells significantly increased the glycolytic rate and the mitochondrial capacity. In addition, we discovered that the cells showed *de novo* glutamine synthesis. Importantly, we found that high expression of the key enzyme of β-oxidation, CPT1, correlates with reduced survival of NB patients and that inhibition of CPT1 with etomoxir reduced tumor burden in NB xenografts *in vivo* (Oliynyk 2019). We are now further examining the contribution of glutamine and glucose to fatty acids in high versus low MYCN expressing neuroblastoma cells by metabolic tracing followed by mass spectrometry analysis. We are also analysing a second inhibitor of CPT1, teglicar, in neuroblastoma xenograft experiments. We have injected GR-overexpressing neuroblastoma cells into mice and show that high levels of GR reduces tumor growth. We further found that coactivation of ERα and GR in BE(2)-GR/ERα cells resulted in a similar robust neurite outgrowth as with retinoic acid treatment alone. Importantly, the neuronal differentiation markers TH and $p75^{NTR}$ were upregulated by estradiol (E2) but not by dexamethasone (dex). Furthermore, TH and $p75^{NTR}$ expression levels were higher by E2+dex treatment than by E2 treatment alone. Collectively, these data show that co-expression of GR and ERα possess synergistic effects on neuronal differentiation in *MYCN*-amplified NB cells.

Summary/Conclusions

Taken together, our findings provide novel information on MYCN-mediated high-energetic metabolic phenotype, which can be a possible mechanism of cancer cell survival during NB pathogenesis. We also present novel data on induction of neuronal differentiation in neuroblastoma. These new knowledge may form the basis for novel cancer therapies for children with neuroblastoma.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 439

Comprehensive immune monitoring in patients with high-risk neuroblastoma during chemo- and immune therapy

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

Neuroblastoma (NBL) is the most common extracranial solid tumor in childhood. Despite intense treatment, children with high-risk (HR) disease have a poor prognosis. Immunotherapy showed a significant improvement in event-free survival in high-risk neuroblastoma patients receiving chimeric anti-GD2 in combination with cytokines and isotretinoin after myeloablative consolidation therapy. However, response to immunotherapy varies widely, and often therapy is stopped due to severe toxicities. Nassin et al. (*bbmt 2018*) showed that most patients with HR NBL do not have full immune reconstitution at the start of immunotherapy and that immune recovery may correlate with disease-related outcomes in patients with HR disease. Data on the presence and function of immune cells during therapeutic interventions after autologous hematopoietic cell transplantation (aHCT) is largely lacking.

Aims

The aim of the present study is to describe the dynamics of immune cell subsets and their function at diagnosis and during therapy in HR NBL patients.

Methods/Materials

We performed in-depth immune monitoring in peripheral blood of 25 HR NBL patients at diagnosis, during induction chemotherapy, before high dose conditioning and during immunotherapy. Immune subsets (leukocytes, lymphocyte, monocyte, neutrophil, eosinophil and

lymphocyte, including subsets within B-cells, NK-cells and T-cells) were measured in fresh peripheral blood samples using multi-color flow cytometry. To assess proliferation of T-cells, PBMCs were labelled with CTV and cultured for 3 days in presence of different stimuli (anti-CD3, PHA) and analyzed using flow cytometry. Suppression of Tregs, was measured similarly in co-cultures of isolated CD4+CD25highCD127low Tregs and PBMC from patients and healthy controls. Supernatants were measured using multiplex immunoassays (IL-2, sIL-2, IL-10, IL-13, IL-17, INF-g, TNFa).

Results

The dynamics of immune cells during multi-agent chemotherapy varied largely between patients after chemotherapy. Large variation between cell counts, between patients and between treatment cycles was observed. During immune therapy, we found that IL-2 and GM-CSF induced expansion of suppressive Treg and increased circulating eosinophils. In addition, GM-CSF increased neutrophil, lymphocyte and monocyte counts, but had no effect on CD3+ T cell, NK cell or CD19+ B cell populations. IL-2 administration resulted in increase of both CD16+ and CD56+ NK cells, and had no effect on CD3+ T cell numbers, CD19+ B cell and monocyte counts. Patient Treg were functional albeit a slightly reduced ability to suppress T cell proliferation when compared to Treg from healthy controls. In contrast, we found that patient T-cell proliferation was significantly impaired at diagnosis and after aHCT. This was confirmed by impaired cytokine profiles measured *in vitro*.

Summary/Conclusions

Our findings show that the status of the immune system is highly variable between patients post-aHCT. Nevertheless, immune therapy protocols do not take the immune status into account for dose calculation or timing of treatment. Moreover, we show that treating patients with immune therapy does not restore the corrupted effector T cell function, but instead induces an expansion of functional Treg. These new insights are valuable for the design of combination strategies and new therapeutic approaches.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 440

Predicting induction chemotherapy using dynamic modelling of the p53 network

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Topic Gene regulatory networks and epigenetics: from basic science to therapy

Background/Introduction

Neuroblastoma has a diverse clinical presentation and course. Risk classification of Neuroblastoma has refined treatments with reduced therapy for low- and intermediate-risk disease and intensive therapy in high-risk cases. Advances in therapy for these high-risk patients increased 5-year overall survival to 50% and includes intensive induction chemotherapy comprising high dosages of DNA-damaging agents. These agents are highly toxic and have severe short- and long-term side effects such as neutropenia, secondary tumours, infertility, bone necrosis, and irreversible hearing loss. Unfortunately, patient responses vary and about 20% of patients do not respond. Thus, predictive and prognostic makers for refining risk-stratified Neuroblastoma therapies present an urgent clinical need. The p53 signal transduction network (STN) is critical for mediating the cellular DNA damage response. In response to chemotherapeutics, the activation of p53 regulates critical cell fate decisions such as cell-cycle arrest and apoptosis. Hereby, the specific time- and dose-dependent p53 activation patterns are critical for deciding which cell fate is triggered. Because these dynamic activation patterns are difficult to understand intuitively, systems biology utilises mathematical models. Recently, mathematical models have also begun to emerge as a new concept in patient-stratification. In contrast to classic biomarkers that usually consist of snapshot measurements mathematical models describe drug response mechanisms using a set of dynamic mathematical rules. Inherently, these models can predict time-dependent changes using precise computer simulations.

Aims

Our aim was to understand how the p53 STN links genotype to phenotype, and how specific expression profiles of the p53 network components control the dynamic p53 response and influence patient survival.

Methods/Materials

We built a mathematical model of the p53 network and calibrated it on dynamic perturbation data from cell lines. The model contains patient-specific parameters that can be adjusted to measurements from patient tumour samples. Retrospective tumour profiling data from several neuroblastoma cohorts (n=688, n=249, DNA array; n=143 RNA-seq) were used to simulate the patient-specific responses and test whether the p53 response patterns associate with tumour aggressiveness and patient survival. Both, sub-stratification and multivariate Cox regression was

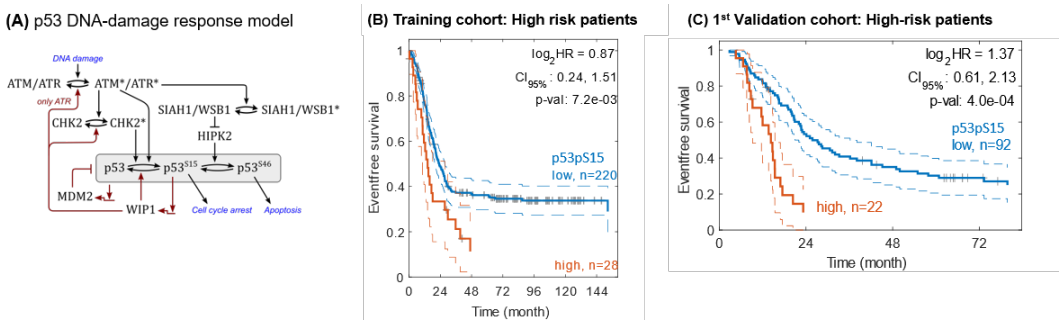
used to correct for potential confounding factors such as MYCN amplification and risk (Children's Oncology Group, COG). Sensitivity analysis was used to identify optimal combination drug-targets for non-responding patients.

Results

We found several associations that link specific p53 STN response patterns to tumour aggressiveness and patient survival. In particular, the simulated p53 phosphorylation response of S15 (pS15) emerged as a robust prognostic maker in neuroblastoma, with increased pS15 responses indicating poor event-free and overall survival. In about 10% of the high-risk patients, the model predicted a highly activated pS15 cell-cycle-arrest signal and extremely poor event-free survival of less than 20%. The optimal drug-combination targets to re-instate the normal p53 activation pattern in these patients differed between patients. CHEK2 was the in optimal target in 69% of patients, SIAH1 in 28% of patients.

Summary/Conclusions

The results show that personalised mathematical modelling can help us to understand the p53 DNA responses of individual patient. Patient-specific simulations might be useful to predict therapy responses, stratify patients, and refine treatments.



Patient stratification based on mathematical modeling of the p53 DNA damage response

Type Basic

Presentation Preference Oral presentation

Abstract nr. 441

VISIOMICS – a docker-based platform supporting the exploration and integrated analysis of multiOMICs data for tumor diagnostics

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

In cancer diagnostics tumor sub-grouping, risk stratification and/or survival prediction typically involve either genomics, gene expression or epigenetic analysis. If combined with bioimaging and clinical data and integrated based on expert knowledge and statistical methods, multi-scalar genomics (multiOMICs) analyses have demonstrated feasibility in terms of time-to-analysis and promise to improve patient outcome. However, there is an urgent need, but lack of platforms supporting a technical and biological data integration.

Aims

The aim was to build a platform allowing integration and visualization of multiOMICs, cell/tissue imaging and clinical datasets as a basis to explore biological data, to unravel yet unidentified causal relations and to finally translate the gained knowledge into certified clinical analysis workflows.

Methods/Materials

We use state-of-the-art technologies to develop a datastore to host raw data files and metadata information as output from multiOMICs data analysis and tissue image analysis. Image-based deep learning frameworks are used to extract marker expression/gene features from interphase fluorescence in situ hybridization (iFISH) and multi-epitope ligand cartography (MELC) images whereas single nucleotide polymorphism array (SNPa)-based deep learning frameworks are developed to support efficient genomic data annotation. Based on statistical methods for feature selection and correlation analysis and combined with innovative concepts for visual analytics, tools allowing an explorative visualisation and analysis of multiOMICs, clinical and imaging datasets are developed.

Results

We established a modular system to address biological research questions for cancer diagnostics consisting of dockerized components, orchestrated by a central workflow manager. Data extraction modules, including bioinformatics pipelines, image processing pipelines and genomics data annotation workflows, extract and store linkable data from all data sources in a central database. The system then allows integration and visual exploration of multiOMICs data using a data integration module and a visual analytics framework embedded in a React-based webclient. All developed modules interact using REpresentational State Transfer (REST) interfaces and are being integrable into certifiable clinical diagnostic workflows.

Summary/Conclusions

In VISIOMICS, experts from biology, bioinformatics, data analysis, bioimage analysis, deep

learning and visual analytics use state-of-the-art methods to develop a platform achieving multiOMICs and histopathology data integration and visualization gaining new knowledge and serving as basic components to develop certifiable clinical diagnostic workflows.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 442

The dual PLK1-/PI3K inhibitor Rigosertib shows antitumoral activity in preclinical neuroblastoma models

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

High-risk neuroblastoma responds poorly to conventional chemotherapy and overall survival of patients with high-risk neuroblastoma is below 50%. New targeted therapeutics promise to be a solution for individual patients, yet recent studies have shown that tumors can rapidly develop resistance to single-target drugs. The multi-kinase inhibitor Rigosertib suppresses phosphoinositide 3-kinase (PI3K) pathway activation as well as polo-like kinase 1 (PLK1) activity. Both, *PLK1* and *PI3K*, are expressed at elevated levels in high-risk neuroblastoma, and high expression correlates with unfavorable disease outcome. Small molecule inhibitors of either PI3K or PLK1 have previously been shown to have specific antitumoral efficacy in neuroblastoma. These earlier studies report that single inhibition of either target reduced cell viability, proliferation, caused cell cycle arrest, and massively induced apoptosis.

Aims

We have investigated the response of preclinical models of high-risk neuroblastoma to Rigosertib,

a dual pathway inhibitor.

Methods/Materials

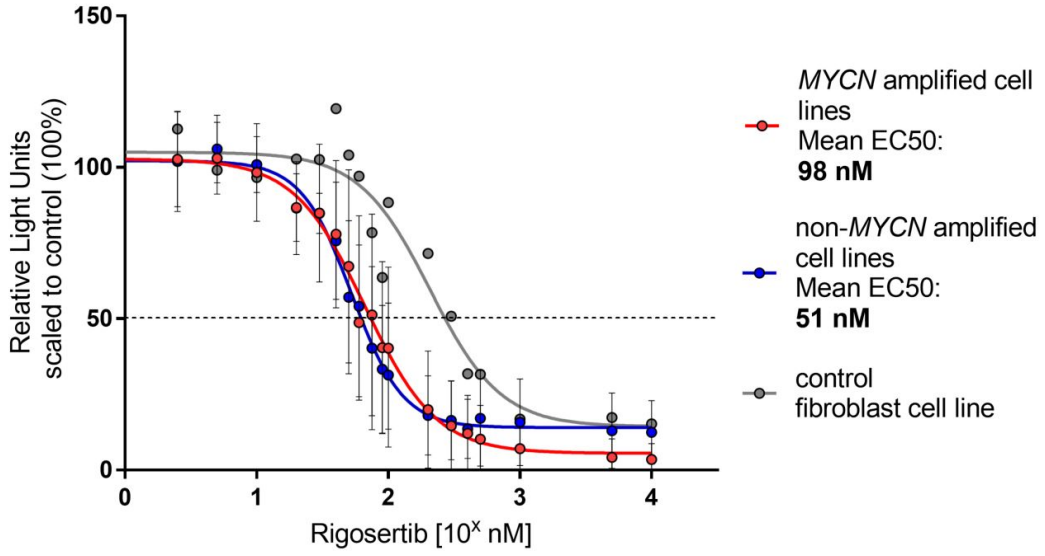
Rigosertib was applied to neuroblastoma cell lines at various concentrations. Its effect on cell viability was measured applying a luminescence assay. Wound healing and clonogenic assays were used to investigate the effect of Rigosertib on migration and cell-cell interactions. Using qPCR and Western blot experiments, we investigated the effect of Rigosertib on PI3K-PLK1 pathway proteins. Furthermore, we functionally characterize the cellular effects of dual PI3K-PLK1 pathway interference in neuroblastoma cells, focusing on proliferation, apoptosis and differentiation.

Results

Treatment of a variety of neuroblastoma cell lines with Rigosertib exhibited potent antiproliferative effects at nanomolar concentrations (IC50: 30nM – 238nM). Wound healing and colony formation are strongly impeded by application of Rigosertib. Analyses of PLK1, PIK3 and corresponding pathway components on transcript as well as protein level validated the target-specific mode of action in response to the Rigosertib treatment.

Summary/Conclusions

Our results indicate that Rigosertib is a highly effective multikinase inhibitor *in-vitro*, which warrants further preclinical evaluation in *in-vivo* neuroblastoma models. Combinations of Rigosertib and classical chemotherapy as well other small molecule inhibitors or targeted CAR-T-cell therapy may be a promising approach for treating high-risk neuroblastoma.



Three days after treatment with Rigosertib, Neuroblastoma cells show reduced cell viability at nanomolar concentrations.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 443

Defining the effects of polyamine synthesis inhibition on the neuroblastoma immune microenvironment

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

MYCN remains the primary oncogenic driver, and MYC signaling is often deregulated in high-risk neuroblastoma (NB). Although direct *MYC* antagonism has not been achievable, *MYC* regulates several downstream cellular modules (including polyamine (PA) metabolism), that are druggable. Indeed, the FDA-approved drug difluoromethylornithine (DFMO) significantly decreases PA synthesis by irreversibly inactivating the enzyme ornithine decarboxylase. In a preclinical model of *MYCN*-driven high-risk NB (*TH-MYCN*$^{+/+}$ transgenic mice), we find that DFMO significantly extends survival. We hypothesized that in addition to its tumor-intrinsic effects, DFMO may be altering the tumor microenvironment (TME) to enhance antitumor immunity.

Aims

To gain a better understanding of the ways in which DFMO alters the NB TME, we set out to characterize the frequencies and functional capacities of the hematopoietic cells that infiltrate the tumors of *TH-MYCN*$^{+/+}$ transgenic mice.

Methods/Materials

Tumors from *TH-MYCN*$^{+/+}$ transgenic mice were excised and subjected to mechanical and enzymatic dissociation. Single cell suspensions were subjected to multiparameter flow cytometry using a variety of panels aimed at determining the frequencies of live CD45+ cells and their expression of markers of functional capacity. Cytokine levels were assayed using quantitative PCR and ELISA. The microbiota of DFMO-treated and untreated mice were assayed using 16S rRNA deep sequencing.

Results

We find that DFMO administration results in multiple alterations in the frequency and phenotype of tumor-infiltrating immune cells. Given DFMO's known anti-protozoal activity, we examined the microbiota of the respiratory and intestinal tracts of treated and untreated mice; these analyses revealed no significant changes in the microbiota of DFMO-treated mice when compared to age-matched untreated mice, suggesting that the observed TME effects were not driven by microbiome alterations. Most notable amongst the changes induced by DFMO in the tumor-infiltrating hematopoietic cells, was a dose-dependent increase in the frequency of natural killer (NK) cells. Additionally, while NK cells in untreated mice displayed significantly decreased expression of the stimulatory receptor NKG2D, DFMO partially restored this loss. Concomitantly, tumor cells from DFMO-treated mice expressed higher levels of Rae1-γ, a known ligand for NKG2D. Decreases in NKG2D expression had previously been described in human NK cells exposed to transforming growth factor beta 1 (TGFβ1), a multifunctional cytokine that is produced in several solid tumors including NB. TGFβ1 is synthesized as a pro-peptide that undergoes further processing for generation of its free (and active) form. Our preliminary analyses indicate that DFMO-treated and untreated tumors express similar levels of TGFβ1 transcripts, yet treated tumors contain lower concentrations of free TGFb1 protein. Studies are now ongoing to determine how DFMO treatment alters the processing of pro-TGFβ1 to its active form.

Summary/Conclusions

These findings collectively demonstrate that DFMO increases intra-tumoral NK cell frequencies and protects them from loss of anti-tumor activity. Understanding the mechanisms by which interference with PA synthesis results in reduction in intra-tumoral NK cell immunosuppression bears significant clinical relevance, as dinutuximab is believed to exert its effects via NK cell-mediated antibody-dependent cellular cytotoxicity. Future studies will be aimed at determining if inhibition of TGFβ1 activity could synergize with PA interference to enhance NK cell-mediated antitumor immunity in NB.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 444

BET inhibition as monotherapy and combination therapy in MYC-driven neuroblastoma models

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Topic Gene regulatory networks and epigenetics: from basic science to therapy

Background/Introduction

MYC signaling is a predominant driver of high-risk neuroblastoma, caused either by amplification of *MYCN* or by activation of cMYC. The Bromodomain and Extra-terminal (BET) domain-containing protein BRD4 was reported to cooperate with MYCN in the epigenetic regulation of super-enhancer driven genes in neuroblastoma. Inhibition of BRD4 by small molecules such as JQ1 and OTX015 was shown to repress BET/MYCN-mediated transcriptional control and to exert antitumoural efficacy in MYCN-driven neuroblastoma. The BET inhibitor TEN-010, a derivative of JQ1, is currently in clinical trials for various MYC-driven adult tumours but its efficacy against neuroblastoma is yet to be established.

Aims

Our objective was to compare the antitumoural efficacy of TEN-010 to the previously published BET inhibitors and to evaluate its potential to be implemented in the clinical treatment regimen of high-risk neuroblastoma.

Methods/Materials

In a preclinical setup, we investigated the antitumoural activity of TEN-010 (n=18), OTX015 (n=12) and JQ1 (n=12) in a panel of neuroblastoma cell lines and fibroblasts. We employed an ATP detection assay to assess cell viability 72h after treatment. Western Blot and qPCR were

used to measure changes in mRNA and protein expression. Effects on proliferation and cell death were investigated using colorimetric BrdU ELISA and Annexin V/7-AAD FACS analysis. To mimic the simultaneous administration of TEN-010 with conventional drugs of neuroblastoma study treatment, we subsequently turned towards combination therapies. We assessed combinations of TEN-010 with conventional chemotherapeutics (cisplatin, doxorubicin, etoposide, vincristine) in neuroblastoma cell lines IMR-5/75, CHP-134, NBL-S and SK-N-AS and fibroblast cell line BJ hTERT. Combinatorial effects were analysed using the Bliss Independence Model.

Results

In general, cell lines with high MYCN/MYC expression, according to RNA sequencing data, showed higher sensitivity to TEN-010 monotherapy than cell lines with low or moderate expression of these genes. Nine cell lines were highly sensitive to TEN-010 with IC50 values ranging 79nM to 632nM. Overall the efficacy of TEN-010 was comparable to the documented efficacies of JQ1 and OTX015. Treatment with TEN-010 exerted inhibition of proliferation and induction of cell death, which was confirmed by BrdU ELISA and Annexin V/7-AAD FACS analysis. The efficacy of TEN-010 monotherapy was further enhanced when cells were treated for longer durations up to seven days. Western Blot and qPCR confirmed on-target activity of TEN-010 by upregulation of the bona-fide response marker HEXIM1. Investigating combinatorial treatment approaches, we observed synergistic interactions of TEN-010 and cisplatin or vincristine in MYCN-driven neuroblastoma cell lines, while combinations with etoposide or doxorubicin indicate additive effects. Conversely, all combinations except for etoposide showed antagonistic or additive interactions in fibroblasts, suggesting a potential benefit on toxic side effects.

Summary/Conclusions

Our results support the notion that BET proteins have crucial functions in MYC/MYCN-driven neuroblastoma and promote BET inhibitors, such as TEN-010, as an effective, beneficial addition to current standard therapy regimen for high-risk neuroblastoma.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 445

Biomarker Analysis for checkpointkinase 1 inhibitor prexasertib in in vitro high-risk neuroblastoma models

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

Checkpoint kinase 1 (chk1) is a serine/threonine protein kinase that is essential for DNA damage repair and cell cycle checkpoint regulation. Ultimately chk1 activation plays a role in genomic integrity by helping to prevent propagation of cells with DNA damage. Prexasertib (LY2606368)—a small molecule chk1 inhibitor in clinical development for adult and pediatric solid tumors—has been shown to potentiate DNA damage and induce cytotoxic effects. Additionally, mRNA analysis of high-risk neuroblastoma (HR-NB) tumors indicates that low chk1 expression gives an overall survival advantage for NB patients. Taken altogether, chk1 inhibition by prexasertib is a novel therapeutic approach that demands further in vitro and in vivo investigation for use in the treatment of HR-NB.

Aims

The aims of the project are to a) further validate prexasertib as a therapeutic approach for NB, b) identify synergistic drug combinations with prexasertib and c) establish a reliable clinical biomarker for prexasertib combination therapy to ensure the development of high-quality clinical trials.

Methods/Materials

High-throughput screening of 23 NB classical cell lines and 10 NB organoids using a drug library containing 170 compounds that are clinically approved or currently in clinical development was completed to identify new therapeutic approaches. Western blot and flow cytometry were used to further validate prexasertib treatment in NB cells and reporter cell lines were developed to aid in the identification of potential biomarkers. Additional high-throughput screens of 10 compounds combined with prexasertib were completed to identify synergistic combinations.

Results

High-throughput screening of NB classical cell lines and NB organoids demonstrates high in vitro sensitivity to prexasertib treatment. The observed IC50 values for prexasertib are well within the clinically achievable range that has been reported in literature. Further validation of prexasertib demonstrates that NB sensitivity to chk1 inhibition is related to baseline levels of DNA damage and that DNA repair defects/replication stress can potentiate these effects. As such, players within the DNA repair pathways are investigated as potential therapeutic biomarkers and we included DNA damaging agents as well as small molecule inhibitors targeting DNA repair pathways in synergy screens.

Summary/Conclusions

Our study supports further pre-clinical development of prexasertib as a therapeutic approach for the treatment of NB. Additionally, we show that existing or induced DNA damage can act synergistically with chk1 inhibition and that this DNA damage could potentially be used as a clinical biomarker.

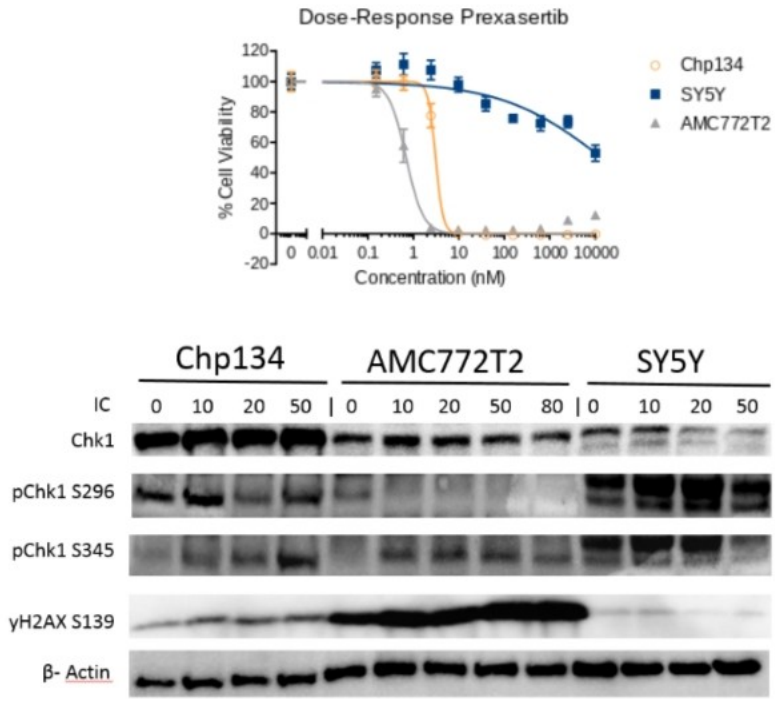

Figure 1- Prexasertib dose-response curves and western blot analysis of relevant proteins

Type Translational

Presentation Preference Traditional poster

Abstract nr. 446

MYC transcription activation mediated by OCT4 as a mechanism of resistance to 13-cisRA-induced differentiation in neuroblastoma

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

MYCN genomic amplification is one of the risk factors in neuroblastoma. 13-*cis* retinoic acid (13-*cis*RA), a differentiating agent, down-regulates MYCN expression and is part of neuroblastoma maintenance therapy. Despite the improvement in clinical outcome from maintenance therapy of neuroblastoma given in first response with 13-*cis*RA and anti-GD2 monoclonal antibody plus cytokine, ~40% of high-risk neuroblastoma patients still die of recurrent disease. Although *MYC* genomic amplification is rare in neuroblastoma (~1%), 11% of neuroblastoma primary tumors collected at diagnosis have high c-MYC protein suggesting that *MYC* transcriptional activation rather than genomic amplification drives such tumors.

Aims

We sought to investigate the role of *MYC* oncogene in 13-*cis*RA resistance and to molecularly characterize mechanisms of *MYC* expression in neuroblastoma.

Methods/Materials

All neuroblastoma models were obtained from the COG repository for cell lines and xenografts (www.CCcells.org). Transcription factors associated with increased expression of c-MYC in 13-*cis* RA-resistant neuroblastoma cell lines were identified using protein/DNA hybridization arrays. Binding of OCT4 to the MYC enhancer/promoter region was identified and confirmed by EMSA and ChIP assay. The binding kinases to OCT4, MAPKAPK2 (MK2) and DNA-PK, and the phosphorylation sites of OCT4 were identified by mass spectrometry. RNAi-mediated gene silencing was used to validate the MK2-OCT4-MYC pathway.

Results

Neuroblastoma cell lines selected for resistance to 13-*cis*RA showed low MYCN and high c-MYC expression. Transcriptional activation of *MYC* mediated by OCT4 (encoded by *POU5F1*) functionally replaced MYCN in 13-*cis*RA-resistant cell lines. In neuroblastoma patient-derived cell lines (PCLs),19 established at diagnosis (Dx) and 16 at progressive disease (PD), and patient-derived xenografts (PDXs) (8 Dx and 9 PD), we confirmed that c-MYC expression levels were higher in PD relative to Dx lines ($P = 0.0005$). We identified OCT4 and TCF3 as transcription factors highly expressed in neuroblastoma cells with high c-MYC expression. We then identified two novel OCT4-binding sites (including OBS1 and OBS2) located in the *MYC* promoter/enhancer region: -1209 to -1140 and found that OCT4 NH_2-terminal domain (NTD) and POU specific domain (POUs) are critical for *MYC* transcriptional activation. To identify kinases that are associated with OCT4-induced c-MYC activation, we used mass spectrometry and PhosphoMotif Finder® and identified MK2 as one of the upstream kinases that can bind to and directly regulate the OCT4 biological function by phosphorylation at its amino acid Ser^{111} residue to transcriptionally activate *MYC* expression. RNA expression in 175 *MYCN* non-amplified high-risk primary tumors (TARGET database) showed that *MAPKAPK2* positively correlated with *MYC* expression ($P < 0.001$) and overall survival was lower ($P < 0.001$) for patients with high *MAPKAPK2*. Also, OCT4, MK2, and c-MYC were higher in PD relative to Dx neuroblastoma PCLs and PDXs. Functional studies by gene knockdown of the *POU5F1* or *MAPKAPK2* using shRNAs showed decreased c-MYC expression, inhibition of cell proliferation, and restoring growth arrest and neurite outgrowth in response to 13-*cis*RA.

Summary/Conclusions

High c-MYC expression, independent of genomic amplification, is associated with disease progression and resistance to 13-*cis*RA in neuroblastoma. The MK2-mediated OCT4 transcriptional activation is a novel mechanism for *MYC* activation in PD neuroblastoma and provides a potential therapeutic target.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 447

Combining immunomodulating chemotherapy with checkpoint blockade antibody provides effective therapy in the spontaneous TH-MYC-N transgenic neuroblastoma model

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

Neuroblastoma typically has a low mutational burden, with few neoantigens and an immunosuppressive tumour microenvironment. Not surprisingly, immune checkpoint blockade antibodies (e.g. anti-PD-1), which have achieved remarkable success in adult malignancies, have not shown clinical activity as single agents in neuroblastoma, or indeed many paediatric tumours. However, tumour specific T cells are found in patients with neuroblastoma and, where present, these correlate with favourable outcome. Although chemotherapy is generally considered immunosuppressive, there is increasing recognition that many chemotherapy agents may have favourable effects on the anti-tumour immune response, such as deletion of regulatory T cell and induction of immunogenic cell death.

Aims

Here we aimed to investigate the immunomodulating effects of cyclophosphamide and doxorubicin, and explore if these chemotherapy agents can be combined with checkpoint blockade antibodies to provide effective therapy in preclinical models of neuroblastoma.

Methods/Materials

The ability of cyclophosphamide and doxorubicin to induce expression of recognised markers of immunogenic cell death in a range of murine neuroblastoma cell lines was assessed by flow cytometry and immunohistochemistry. Syngeneic and spontaneous murine neuroblastoma models were used to examine the effects of different doses of the chemotherapy agents on tumour infiltrating lymphocyte populations, and also to investigate the therapeutic effects of combining chemotherapy with the immune checkpoint blockade (anti-PD-1 monoclonal antibody). An

apoptosis resistant neuroblastoma cell line and mouse strain (vav-BCL-2) were used to investigate the mechanism of action of therapeutic synergy between these agents.

Results

In vitro exposure of neuroblastoma cell lines to either cyclophosphamide or doxorubicin was found to result in induction of ecto-CRT, Hsp-70 and HMGB1, suggesting induction of immunogenic cell death. In vivo, low dose cyclophosphamide was found to selectively deplete and reduce the suppressive activity of tumour infiltrating regulatory T cell (Treg) in both NXS2 and 9464D neuroblastoma models, whilst maintaining and activating CD8+ and CD4+ T cells. The combination of low dose cyclophosphamide with anti-PD-1 was demonstrated to improve survival, as compared to either agent alone, in 3 different immunocompetent neuroblastoma models, including the TH-MYC-N transgenic spontaneous tumour model. A metronomic, weekly dosing strategy was found to achieve long term survival in the spontaneous model. The therapeutic effects were lost when wild type neuroblastoma cell lines were inoculated into apoptosis resistant vav-BCL-2 mice, suggesting that host mechanisms (e.g. Treg depletion) are essential in addition to the direct cytotoxic effects of the chemotherapy on tumour cells.

Summary/Conclusions

Low dose cyclophosphamide results in depletion of intratumoral Treg cells and synergises with anti-PD-1 antibody to achieve effective long term therapy in the TH-MYC-N spontaneous tumour model. This combinational therapy warrants further investigation and is potentially easily translatable into clinical trial.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 448

Natural history of ganglioneuroma (GN) and intermixed ganglioneuroblastoma (iGNB): An International Neuroblastoma Risk Group (INRG) project

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Topic Other

Background/Introduction

GN and iGNB represent the benign extremes of the neuroblastic tumour spectrum. Historically surgical resection was recommended, and in some cases chemotherapy. Recent reports suggest that a more conservative approach, including watchful observation, could be adequate and avoid surgical morbidity. However, large studies are lacking.

Aims

To describe the natural history of a large cohort of GN and iGNB, with a focus on presentation, treatment, outcomes and patterns of relapse.

Methods/Materials

Eligible (diagnosis of GN and iGNB, age 0-24 years, diagnosed 1990-2016) patients were identified in the INRG Data Commons and descriptively analysed. Kaplan-Meier curves of event-free survival (EFS) and overall survival (OS), were generated, and the outcome of subgroups was compared using a log rank test. Treatment data reported to the INRG Data Commons are the initial assigned treatment, and not the actual treatment administered.

Results

945 eligible cases were identified, 198 (21%) GN and 747 (79%) iGNB. 323 (39%) were males. Median age was 4.5 years (range: 0-20.5 years), with 893 (94.5%) ≥18 months at diagnosis. The primary tumour was thoracic in 346 (39%) cases, abdominal in 261 (29%) cases, and adrenal in 184 (21%) cases. The majority (526 cases, 56%) were INSS stage 1, 241 (26%) were stage 2, 122 (13%) were stage 3, 2 (0.2%) were stage 4S, and 49 (5%) were stage 4. Only 51/945 cases (21%) presented with metastases (INSS stage 4 or 4S): 16 (31%) in bone marrow, 20 (39%) in bone, 18 (35%) in distant lymph nodes, 11 (22%) in other sites and 21 patients with unknown site of metastasis. *MYCN* was studied in 847 cases (90%) and was amplified in 17 (2%). None of the cases had other molecular investigations. The initial assigned treatment was: observation (16, 6%), surgery and observation (174, 61%), conventional-dose chemotherapy (2-8 cycles) plus surgery (66, 23%), and only 29 (10%) were treated with intensive chemotherapy. Of these 29, 17 were reported to have received stem cell transplant, and 4 received immunotherapy. The 5-year EFS survival was 94±0.9%, and 5-year OS was 96±0.8% (n=945).

Summary/Conclusions

Patients with GN and iGNB have excellent survival outcomes. The majority of patients underwent an attempt at surgical resection. Further studies focusing on long term morbidity of the disease and/or treatment will be important, in order to inform more accurate ‘balance of risk’ for future treatment recommendations.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 449

A comparison between 123I-MIBG imaging, trephine biopsy histology, GD2-immunocytology, and RT-qPCR to assess bone marrow involvement in high-risk neuroblastoma

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

Bone marrow (BM) is the most common site of dissemination in patients with metastatic neuroblastoma. Currently, histology of BM trephine biopsies, GD2-immunocytology, and ^{123}I-MIBG (meta-iodobenzylguanidine) scintigraphy + SPECT-CT are used to assess BM infiltration. We have previously demonstrated that minimal residual disease detected by RT-qPCR (reverse transcriptase quantitative PCR) using neuroblastoma-specific target genes is a sensitive method to detect BM disease involvement. In this study, we compared the various diagnostic modalities to assess BM involvement.

Aims

To perform matched comparisons of RT-qPCR, trephine biopsy histology, GD2-immunocytology, and ^{123}I-MIBG scintigraphy + SPECT-CT assessing BM infiltration in high-risk neuroblastoma patients. Moreover, the additional value of bilateral versus unilateral sampling was investigated.

Methods/Materials

After matching bilateral BM aspirates and biopsies to ^{123}I-MIBG imaging (performed within one week), 73 BM assessments from 23 high-risk neuroblastoma patients were identified from a single center (22/23 INRG Stage M >1 year; 1/23 INRG Stage M <1 year with MYCN amplification). RT-qPCR was performed as previously described (Stutterheim 2009; van Wezel 2016). GD2-immunocytology was done as previously described using pooled bilateral samples (Swerts 2005). Imaging results from ^{123}I-MIBG scintigraphy and SPECT-CT were quantitatively evaluated using Curie scores by two nuclear medicine physicians. Histology of bone marrow trephine biopsies was assessed by a pathologist using immunohistochemical markers (CD56, chromogranin A, synaptophysin).

Results

Of the 73 matched BM assessments, 17 samples were taken at diagnosis, 35 during induction chemotherapy, 10 at the end of induction chemotherapy, and 11 after consolidation therapy. All 17 diagnostic samples were RT-qPCR positive (15/17 bilateral, 2/17 unilateral). BM involvement could be confirmed by trephine biopsy and/or whole-body MIBG imaging in 14/17 cases. In the other 3/17 cases, stage M disease was diagnosed on the basis of soft tissue metastatic disease. In 2/3 cases, GD2-immunocytology was positive.

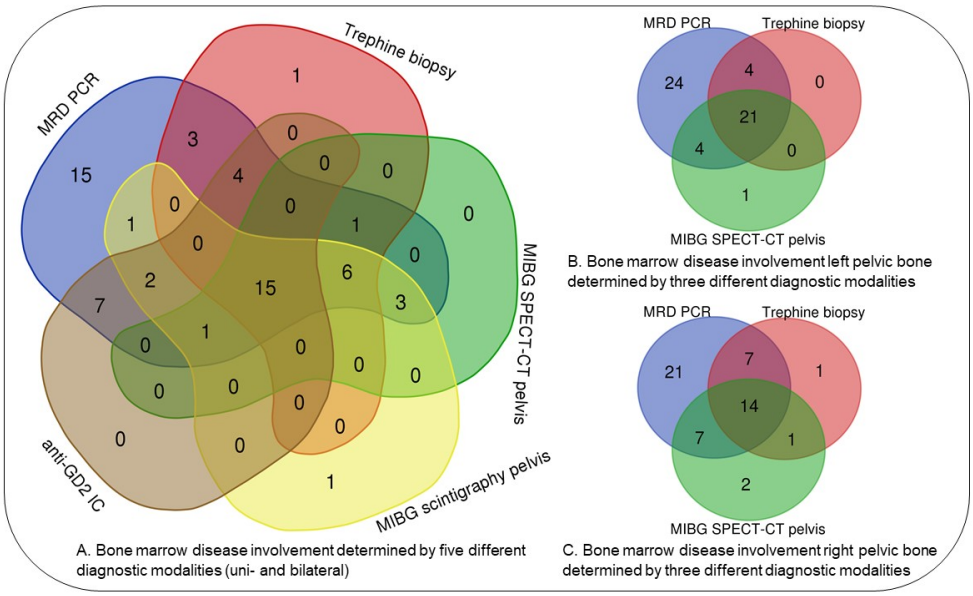
RT-qPCR was positive for pelvic BM infiltration in 79% compared to 41% for trephine biopsy histology, 43 % for GD2-immunocytology, 40% for MIBG scintigraphy, and 36% for SPECT-CT (Figure 1A). Overall, whole-body MIBG scintigraphy detected bone marrow dissemination in 64%. Of the 15/73 bilateral negative RT-qPCR samples, 5/15 showed BM involvement on whole-body MIBG imaging, and one sample had a unilateral positive trephine biopsy. These six samples were taken during and after induction chemotherapy and after consolidation therapy (respectively, n=4; 1; 1).

Eleven of 30 positive trephine-biopsies were unilaterally positive only. In 9/11 samples other diagnostic modalities (RT-qPCR and/or MIBG) identified BM disease dissemination. In 2/11 BM involvement could have been missed if unilateral sampling had prevailed.

Fourteen samples were unilaterally RT-qPCR-positive. In 4/14 samples, the other diagnostic modalities were unable to detect BM involvement, and thus are at the risk of being missed if unilateral samples would have been taken.

Summary/Conclusions

For the detection of BM involvement, application of complementary diagnostic modalities is superior to one single technique. RT-qPCR seems to be the most sensitive technique to detect BM disease involvement. Bilateral pelvic BM sampling identified more BM involvement than unilateral sampling. These results needs to be validated in a prospective analysis. The correlation of RT-qPCR positivity with outcome warrants further investigation in a prospective study.



Bone marrow disease involvement determined by different diagnostic modalities

Type Translational

Presentation Preference Oral presentation

Abstract nr. 450

In vitro and in vivo validation of combination strategies with Venetoclax for neuroblastoma

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

The majority of neuroblastoma patients overexpress the anti-apoptotic protein B cell lymphoma/leukemia 2 (BCL-2). We previously showed that the BCL-2 inhibitor Venetoclax causes programmed cell death in BCL-2-dependent neuroblastoma, which contributed to the initiation of a phase I trial to study the safety and pharmacokinetics of Venetoclax in children with relapsed or refractory neuroblastoma. Unfortunately, Venetoclax monotherapy only led to tumor growth stabilization and not to complete tumor regression. This was in line with previous *in vitro* and *in vivo* studies showing resistance development after Venetoclax treatment.

Aims

In the current study, we aim at the identification and validation of more effective targeted combination strategies to prevent or overcome neuroblastoma resistance to Venetoclax.

Methods/Materials

Two strategies were followed to identify effective Venetoclax combination options for neuroblastoma patients. First, we focused on finding more personalized combination therapies for subgroups of BCL-2-dependent neuroblastoma patients with additional ALK mutations or RAS-MAPK pathway aberrations. Neuroblastoma model systems harboring these genomic backgrounds were exposed to a library of targeted compounds in combination with a series of Venetoclax concentrations to determine synergistic interactions with Venetoclax. Secondly, approaches to overcome Venetoclax resistance were studied by directly targeting the MCL-1-driven resistance mechanism and by performing an additional high-throughput drug screen. In the latter study, non-resistant and Venetoclax-resistant neuroblastoma cells were exposed to a drug library to identify compounds that were more effective in Venetoclax-resistant cells. Taken together, these strategies revealed promising combination therapies with Venetoclax that were further validated *in vitro* and *in vivo* using BCL-2-dependent neuroblastoma xenografts or patient-derived xenografts.

Results

High-throughput drug screens on BCL-2-dependent neuroblastoma cell lines harboring additional ALK mutations surprisingly did not yield ALK inhibitors as most effective or synergistic drugs in combination with Venetoclax. Instead, CHK1 inhibitor Prexasertib, PARP inhibitor Talazoparib and dual PI3K/HDAC inhibitor CUDC-907 were the most promising hits for combination therapy with Venetoclax. In RAS-MAPK-mutated neuroblastoma cells, the FDA-approved MEK inhibitor Trametinib showed synergistic responses with Venetoclax. These promising effects were confirmed *in vivo* and were found to be related to MEK-mediated phosphorylation and consequently inactivation of the pro-apoptotic protein Bim.
In previous preclinical studies, we demonstrated that Venetoclax resistance occurs due to upregulation of the anti-apoptotic protein MCL-1 and sequestration of Bim released from BCL-2 by MCL-1. *In vitro* combination studies confirmed strong synergism between Venetoclax and MCL-1 inhibitors (i.e. S63845, AZD-5991 and AMG-176) in a panel of BCL-2- and MCL-1-dependent neuroblastoma model systems. In addition, high-throughput screening of non-resistant and Venetoclax-resistant neuroblastoma cells identified the MDM2 inhibitor Idasanutlin as a re-sensitizing drug for Venetoclax-resistant neuroblastoma cells. Idasanutlin caused BAX-mediated apoptosis in Venetoclax-resistant neuroblastoma cells in the presence of Venetoclax, while it caused p21-mediated growth arrest in non-resistant cells and Venetoclax-resistant cells in the absence of Venetoclax. *In vivo* combination therapy showed tumor regression and superior efficacy over single-agent treatments in different high BCL-2-dependent neuroblastoma mouse models. Less BCL-2-dependent mouse models were not sensitive to Venetoclax-Idasanutlin combination therapy.

Summary/Conclusions

We identified various targeted combination strategies with Venetoclax that will guide the clinical implementation of BCL-2 inhibition for the treatment of children with BCL-2-dependent neuroblastoma.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 451

New dose strategy for omega-3 fatty acids for neuroblastoma maintenance trials using body surface area and establishment of a novel omega-3 index, the EDD-index.

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Topic Other

Background/Introduction

Children surviving cancers, including high-risk neuroblastoma receiving multi-modal therapy, often develop chronic health conditions including late-effects on cognition and metabolism. We have recently shown that the long-chain omega-3 fatty acids, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), have significant therapeutic impact on neuroblastoma in vitro and in vivo including inhibition of tumor development suggesting a role against minimal residual disease. Omega-3 may have a role as adjuvant therapy showing synergism with several established modalities. In particular, oral DHA and EPA for long-term maintenance after concluded neuroblastoma therapy has been suggested to both limit risk of relapse and modulate risk of late adverse effects. However, the doses needed in clinical trials have not been established.

Aims

To establish a dose for future phase 2 trials we performed a dose-finding phase 1 study, the OmegaChild study (EPN 2014/120-31/1, NCT02134600).

Methods/Materials

A single blind, dose-escalating study of n-3 fatty acids supplementation was performed in 40 pediatric patients (21 girls) with median age 9.5 years (range 3-16 years). All children were in clinical remission (3-36 months) after concluded cancer therapy. The omega-3 dose was prescribed per body surface area (BSA) for 90 days. Participants were allocated to one of five

daily doses: 400, 800, 1600, 2400 or 3000 mg/m^2 of EPA and DHA (40:60) administered as a daily dose in fruit juice. Fatty acid concentration in plasma phospholipids and red blood cells (RBC) were determined by GC and given as mol%. Omega-3 index (ΣEPA, DHA)), as well as our newly suggested EDD-index which also includes DPA were calculated at day 0, 45 and 90.

Results

Thirty-three participants completed the study with excellent compliance and no significant side effects. Median adherence was 96%. The omega-3 supplementation correlated with dose dependent increase of EPA and DPA, but less of DHA, indicating that DPA should be included in a novel n-3 index (EDD) ($P<0.0001$, rho=0.82 CI (0.655 – 0.909)). The highest doses influenced both omega-6 and monounsaturated fatty acids, suggesting an optimal dose of about 1500 mg/m^2 BSA. However, doses up to 3448 mg/m^2 were tolerable and without severe adverse events.

Summary/Conclusions

Adjusting the dose of omega-3 supplementation to BSA resulted in a highly significant dose-dependent increase of the omega-3 fatty acids. We suggest a daily omega-3 dose of about 1500 mg/m^2 BSA for future phase-2 studies in children including long-term maintenance to evaluate impact on clinical outcome of high-risk neuroblastoma. The marked increase of DPA suggests its inclusion in evaluation of the clinical effects, using our suggested novel EDD-index (**E**PA, **D**PA, **D** HA).

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 452

PPM1D is a neuroblastoma oncogene and therapeutic target in childhood neural tumors.

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Majority of cancers harbor alterations of the tumor suppressor *TP53*. However, childhood cancers, including unfavorable neuroblastoma, often lack *TP53* mutations despite frequent loss of p53 function, suggesting alternative p53 inactivating mechanisms.
Chromosome 17q-gain are common in poor-risk neuroblastoma and found in other cancers including childhood medulloblastoma. Wip1, encoded by the 17q23.2 gene *PPM1D* is a serine/threonine phosphatase inhibiting p53 and other key proteins in cell-cycle control, DNA-repair and apoptosis, involved in immunity/inflammation with activating mutations in cancers and developmental disorders.

Aims

We used multiomic approaches, in conjunction with clinical data, to characterize a neuroblastoma oncogene commonly activated by gain of chromosome 17q.

Methods/Materials

We investigated the genetic landscape of human neuroblastoma, in particular *PPM1D* and corresponding WIP1 expression and function in clinical tumors and a range of in vitro and in vivo models including a novel transgenic model to understand oncogenic induction of neuroblastoma and other cancers.

Results

We show that neuroblastomas harbor frequent *PPM1D* copy number gain and gene fusions, in addition to the detection of activating truncating germ-line and somatic mutations.
Analysis of the genetic landscape in a national neuroblastoma material showed 17q+ to be the

most common genetic aberration. Detailed analysis of 17q-gain and corresponding break-points identified PPM1D, present in extra copies in all 17q+ neuroblastomas, as the most suitable oncogene candidate. Multiple independent clinical cohorts showed that *PPM1D* expression correlates with adverse prognostic factors and poor clinical outcome. Unbalanced translocations leading to 17q+ was detected to be the single first event in development of unfavorable human neuroblastoma and analyzing clonal evolution trajectories revealed segmental 17q-gain being further accumulated during clonal evolution.
Pharmacological and genetic manipulation established WIP1 as a druggable target in neuroblastoma. Genome-scale CRISPR-Cas9 screening demonstrated *PPM1D* genetic dependency in *TP53* wild-type neuroblastoma cell lines, and shRNA *PPM1D* knockdown significantly delayed in vivo tumor formation. WIP1-inhibition with the specific phosphatase inhibitor SL-176 was highly potent in cytotoxic/cytostatic effect in neuroblastoma cells. This non-toxic WIP1-inhibitor significantly inhibited neuroblastoma growth in nude mice.
Establishing a transgenic mouse model overexpressing *PPM1D* showed that these mice develop cancers phenotypically and genetically similar to tumors arising in mice with dysfunctional p53 when subjected to low-dose sublethal (4.5Gy) irradiation. Tumors included T-cell lymphomas harboring *Notch1*-mutations, *Pten*-deletions and p53-accumulation, as well as adenocarcinomas and *PHOX2B*-expressing neural crest-derived neuroblastomas.
Hence, 17q-gain is not only the most common genetic aberration always including PPM1D extra copies, but also shown to be the single first event in neuroblastoma development, and together with neuroblastoma development linked to an oncogenic truncating germline PPM1D-mutation, and neuroblastomas induced in mice overexpressing PPM1D as their only aberration provide evidence that PPM1D is a neuroblastoma-inducing oncogene.

Summary/Conclusions

Our data establish *PPM1D* as a *bona fide* oncogene in wtTP53 cancer and more specifically in childhood neuroblastoma. Pharmacological inhibition of WIP1 suppressed the growth of neural tumors in nude mice proposing WIP1 as a therapeutic target in neural childhood tumors.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 453

Characterization of the anti-tumor effects of Diphenyleneiodonium Chloride in MYCN-amplified neuroblastoma

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Neuroblastoma is one of the deadliest childhood cancers. Despite extensive research, the prognosis for children with high-risk neuroblastoma remains as low as 50%. Amplification of MYCN drives half of the high-risk cases. Previously, we reported that Diphenyleneiodonium Chloride (DPI) induces apoptosis and differentiation of MYCN-amplified neuroblastoma cells and inhibits NB growth both *in vitro* and *in vivo* in zebrafish.

Aims

In this project, our aim was to shed light on the mechanisms of action of DPI in MYCN-amplified neuroblastoma cells.

Methods/Materials

We applied mass spectrometry-based label-free quantitative proteomics and phosphoproteomics to study the DPI induced molecular changes in MYCN amplified neuroblastoma cells.

Results

We found that DPI interferes with cell cycle progression and reduces MYCN levels in addition to the induction of mitochondrial superoxide-mediated apoptosis in MYCN-amplified neuroblastoma cells.

Summary/Conclusions

Diphenyleneiodonium Chloride is a potential novel treatment strategy for the therapy of MYCN-amplified neuroblastoma.

Type Basic

Presentation Preference Traditional poster

Abstract nr. 454

RELAPSED/REFRACTORY PEDIATRIC NEUROBLASTOMA: EXCELLENT TOLERABILITY AND SUSTAINED RESPONSES WITH TEMOZOLOMIDE-BASED REGIMENS.

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Topic Other

Background/Introduction

Refractory/relapsed neuroblastoma (rrNBL) remains a significant clinical challenge. Temozolomide (TMZ) is active and well tolerated in this context as shown by small phase I/II trials with limited follow-up.

Aims

To describe the clinical characteristics and outcomes of a real-world cohort of children with rrNBL treated with TMZ.

Methods/Materials

A descriptive, retrospective, international (1 Spanish, 3 French centres) study, of children with rrNBL treated with single-agent or combined TMZ, diagnosed between 1/1/04–31/12/17, follow-up to 31/10/19.

Results

163 children were included, of whom 9(5.5%) had initially localised disease; the remainder had

metastases. Median age at diagnosis was 3.7 years (range 0.3–17.8y).
Refractory NBL: Sixty-three children received TMZ-based chemotherapy for induction-refractory disease. Formal response evaluation (imaging+/-bone marrow) was available for 52/63. Overall best response rate was 48% (3CR/22PR). 32/63 (50.8%) responded sufficiently to proceed to intensification with high dose chemotherapy (HDC). At last follow-up, 22/32 were alive (19CR/PR, 3PD) compared with 7/31 (5CR/PR, 2PD) who did not receive HDC. Overall, for children with induction-refractory NBL, 2-year PFS and OS were 34.8% (95%CI 22.7–46.9%) and 60.7% (48.1–73.3%) respectively.
Relapsed NBL: 100 children received TMZ-based chemotherapy for relapsed neuroblastoma; 85(85%) for a first relapse, 9(9%) for a 2^{nd}, and 6(6%) for 3^{rd} or subsequent relapse.
Formal best response evaluation was available for 70/100. Overall best response rate was 47% (11CR/22PR). Twenty (29%) had stable disease; 17(24%) progression. Best response was seen after a median 5.5 (range 3-19) cycles. Best clinical evaluation was available for 93/100 children; 29(31%) never had clinical improvement or stability. PFS and OS at 2 years were 13.8% (95%CI 6.3–21.3%) and 23.2% (13.8–32.5%) respectively.
Prolonged treatment: Twenty-two of 163 children received at least 12 sequential cycles of TMZ-based chemotherapy. Fifteen are alive (9 CR/PR, 6 with disease), with median follow-up since starting TMZ of 5.3years (range 1.6–10y). Two remain on TMZ (20 and 66 cycles to date). Treating clinicians electively stopped TMZ-based chemotherapy for sustained remission in 12/22 children. One patient died of NBL 7 months after stopping, 5 have progressive disease. Six remain relapse-free on no treatment with a median follow-up since TMZ-withdrawal of 2.5years (range 0.5–5.1y). Patients remaining relapse-free received a median 31 cycles of TMZ-based chemotherapy (range 12-37), compared with 16 (range 12-31) for those who progressed or died.
Tolerability: Patients received a median of 3 continuous cycles (range 1-29) for refractory and 3.5 (1-66) for relapsed disease. TMZ-based chemotherapy was well tolerated; 16% of all cycles were delayed overall, only 9% of all cycles for toxicity. TMZ combined with irinotecan (TEMIRI) was the least well tolerated, with more cycles delayed and the only in which a patient withdrew for toxicity. Performance scales were optimal (≥80%) after 6 months therapy with TMZ.
None of 163 patients developed secondary malignancy or myelodysplastic disease, in a cohort with an overall median follow-up of 1.2years (range 0-11.3y); at least 2 years for 49 patients.

Summary/Conclusions

TMZbased regimens can gain and maintain disease control in rrNBL, enabling consolidation for refractory disease and prolonged disease control for relapsed patients.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 455

The landscape and evolution of somatic mutations captured in the NEPENTHE precision medicine clinical trial for relapsed high-risk neuroblastoma

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Patients with relapsed high-risk neuroblastoma (HR NBL) remain largely incurable despite a dramatic increase in our knowledge of the genetic basis of the disease. Genomic profiling of paired diagnostic and relapsed tumor samples demonstrates a paucity of targetable mutations at diagnosis but significantly higher rates of clonal mutation in relapsed disease, suggesting that the emergence of resistant subclones drives relapse. Understanding the mechanisms of this tumor evolution is necessary to develop superior therapeutic options that prevent resistance and target driver mutations.

Aims

NEPENTHE (Next Generation Personalized Neuroblastoma Therapy) is a pediatric precision medicine trial that matches genomic aberrations in NBL cells at the time of relapse to rationally designed combinations of molecularly targeted agents. In its therapeutic arm, the trial assesses the safety, toxicity, and anti-tumor efficacy of 3 combinations of targeted therapies. For the correlative studies comprising the molecular arm of the trial, we aim to understand the evolutionary genomic landscape of HR NBL from diagnosis through treatment and relapse, and establish

patient derived xenografts (PDXs) from these patients for preclinical testing of rational drug therapies.

Methods/Materials

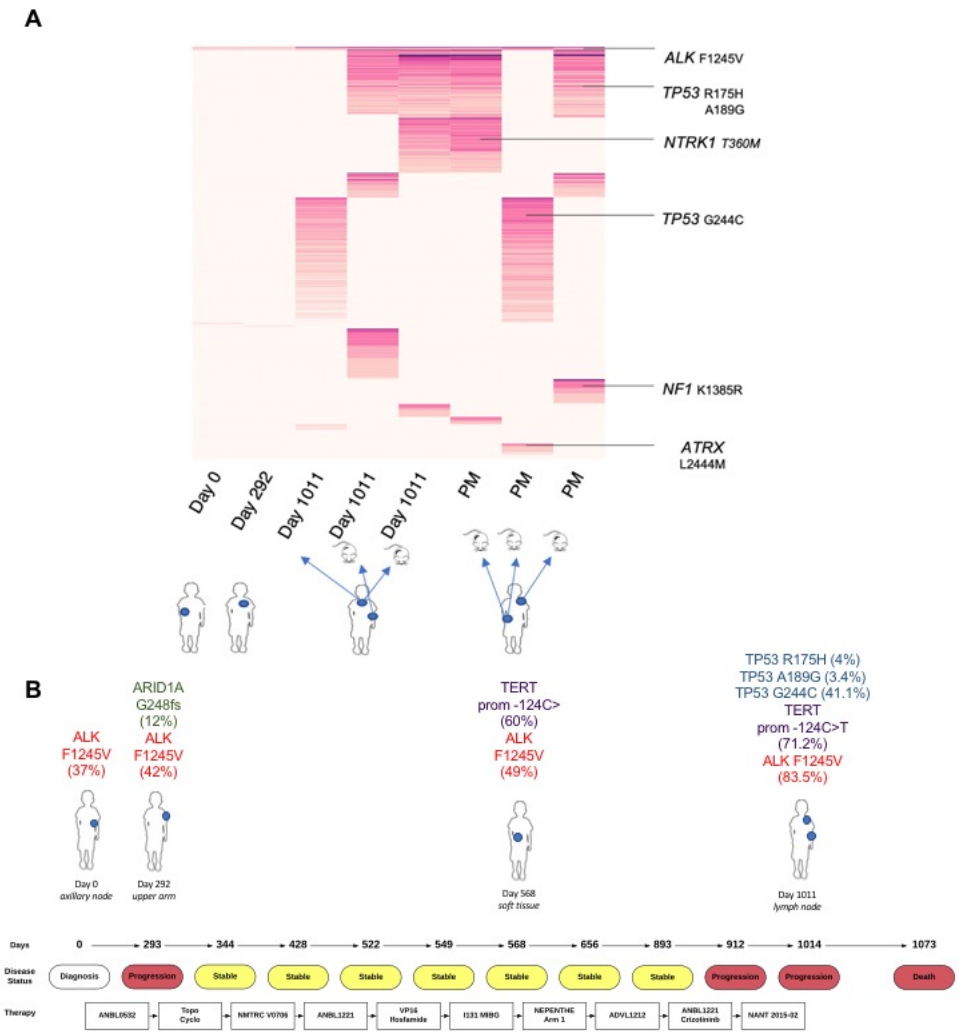
Patients with relapsed HR NBL undergo biopsy at the time of trial enrollment with real-time gene panel molecular profiling of the tumor tissue for therapy assignment with the FoundationOne platform. Residual tissue as well as retrospective tumor and normal samples are obtained and subjected to whole exome sequencing (WES) and RNA sequencing. For PDX establishment, viable tumor tissue is injected with Matrigel into the flanks of NSG mice and propagated with serial passaging.

Results

Fifty-one patients have enrolled on NEPENTHE to date. The molecular profiling data currently available from 49 patients confirms higher rates of clonal mutation in relapsed patients, with 22% harboring the R1275, F1174, or F1245 mutations or amplifications in *ALK*. An additional 10% of patients possess mutations in the RAS-MAPK pathway (*BRAF* or *NF1*), 8% harbor copy number aberrations (CNAs) in *CDKN2A* or *CDK4/6*, 7% have *TERT* promoter mutations, 5% have variants or CNAs in *ATRX*, and 4% harbor *TP53* mutations. In parallel, we have performed WES and RNA sequencing on a total of 167 samples from 23 enrolled patients, including tissues from 12 established PDX models. Preliminary analysis of these data shows that many of these clonal variants are relapse specific and acquired throughout the course of therapy. Moreover, lesions from serial timepoints sampled from differing metastatic sites show divergent evolutionary trajectories with corresponding transcriptional changes, as well as chemotherapy signatures of treatment.

Summary/Conclusions

Serial exome and RNA sequencing from patients with relapsed HR NBL demonstrate patterns of tumor evolution leading to resistant and relapsed disease. Our data underscore the complexity of temporal and spatial tumor evolution, as well as the limitations of single site biopsy and the need for development of further assays, such as circulating tumor DNA, to more efficiently capture patient tumor heterogeneity at diagnosis and throughout treatment.



Tissue Profiling in NEPENTHE Patient. Panel A shows a heatmap with the variant allele frequencies (VAFs) of single nucleotide variants from whole exome sequencing from serially obtained samples in one study participant. Each column portrays a different sample with tissue origin shown on the x-axis. Notable likely pathogenic variants are indicated on the right. Panel B demonstrates the patient's clinical course and therapy, with notable mutations detected from the respective timepoint and corresponding VAFs indicated. Increasing *ALK* and *TERT* mutant VAF, as well as the emergence of newly detected *TP53* mutations, accompany disease progression.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 456

In vivo identification of active drugs and drug combinations for high-risk neuroblastoma using zebrafish models

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

Neuroblastoma is a high-risk pediatric solid tumor that accounts for 15% of childhood cancer deaths. Most children with high-risk neuroblastoma respond initially to chemotherapy, but a large proportion will experience therapy-resistant relapse. In addition, due to intensified therapies, survivors of high-risk neuroblastoma often face long-term toxicities such as skeletal dysplasia, cardiac dysfunction and premature mortality.

Aims

We aim to identify drugs and drug combinations with selectively activity against high-risk neuroblastoma cells in vivo, without undue toxicity to normal tissues.

Methods/Materials

We capitalize on the advantages of zebrafish as a model organism to address pressing questions relevant to the generation of effective and selective therapies for high-risk neuroblastoma. We have optimized zebrafish growth and feeding conditions to obtain consistent and reproducible neuroblastoma tumor onset, tumor penetrance, drug response and fish survival across multiple fish lines and batches. The zebrafish neuroblastoma tumor cells, expressing a dbh:GFP fluorescent reporter, are visualized in living fish using fluorescent stereomicroscope, and the drug effects are quantified by comparing the fluorescent tumors before and after the treatment.

Results

We have developed zebrafish models of high-risk neuroblastoma driven by overexpression of MYCN or MYC oncogene, as well as zebrafish models of refractory neuroblastoma harboring either hyperactivation of RAS-MAPK pathway or inactivation of the p53 pathway. These zebrafish develop aggressive neuroblastoma with up to 80% tumor penetrance by 3 weeks of age, ideal for

the rapid analysis of small-molecule drugs by adding the drugs to fish water in 12-well plates. Using these faithful preclinical models of refractory neuroblastoma, we are analyzing FDA-approved drugs and drugs currently in clinical trials by simultaneously assessing drug toxicity to normal tissues and activity against primary neuroblastoma tumors in juvenile zebrafish. Post-treatment fish can be followed for tumor relapse, normal development and long-term toxicities. In addition, we also developed assays to analyze drug effects on the progression of very early tumor lesions after tumor initiation in vivo, as well as the awakening of dormant disseminated neuroblastoma cells. Using these zebrafish models, we screened a panel of kinase inhibitors, and identified entrectinib (a RTK inhibitor that is active against NTRK1/2/3, ALK and ROS1) as the most active candidate drug. We validated its anti-neuroblastoma activity in human neuroblastoma cell lines. Our data showed that entrectinib is more active than the ALK inhibitor lorlatinib or the NTRK inhibitor larotrectinib, and less toxic than the multi-RTK inhibitor crizotinib. Importantly, we found that the activity of entrectinib is independent of MYC/MYCN, ALK, NF1, P53 mutation status, suggesting entrectinib as a potential anti-neuroblastoma drug.

Summary/Conclusions

We have established zebrafish neuroblastoma models for preclinical basic science studies, as multiple dose levels of each drug alone and in combination with other drugs can be readily tested at low cost and in high-throughput. The effective and safe drugs and drug combinations identified in our zebrafish platforms will inform follow-up testing in murine neuroblastoma primary patient-derived xenografts, before moving forward in clinical trials for neuroblastoma patients.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 457

Multi-omics network inference identifies novel drug targets in high risk neuroblastoma

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Topic Gene regulatory networks and epigenetics: from basic science to therapy

Background/Introduction

Neuroblastoma, an aggressive embryonal tumor of the sympathetic nervous system, is dominated by DNA copy number alterations. Tumors with segmental chromosomal aberrations are associated with poor survival. Although some oncogenic driver genes e.g. *MYCN* have been identified in neuroblastoma, many drivers remain to be found on the major recurrent DNA copy number alterations such as 2p and 17q.
Regulatory networks provide an in depth, systems-level understanding of complex biological processes and their perturbed regulation upon disease. Network inference, the prediction of regulatory networks from omics data and a set of potential regulators (e.g. driver genes), is known to benefit from different algorithms and data types.
Since cancer driving DNA copy number alterations are thought to target genes in a relatively small number of signaling and regulatory pathways, regulatory networks can provide a framework to study the convergence of these alterations (i.e. driver genes) on specific network modules (i.e. driver pathways).

Aims

We hypothesized that one or more dosage sensitive genes on the gained copy number regions act as cooperative drivers during neuroblastoma development. Through integrative computational analysis and network inference, we aim to identify these causal drivers and the regulatory networks in which they operate, since these may open up new avenues for targeted therapy.

Methods/Materials

On available matching transcriptome, copy number alteration and clinical data for > 200 primary tumor samples, we applied several multi-omics data integration strategies, including the multi-omics module network inference algorithm Lemon-Tree and different data in the regulator assignment such as expression data for genes on the recurrently amplified 2p and 17q regions and for all transcription factors. Using ensemble methodologies, we clustered genes in consensus co-expression modules and predicted a prioritized list of drivers and regulators for each of them.

We characterized modules and drivers in the regulatory network through network analysis, functional enrichment analysis, clinical data correspondence and further integration with other data.

Results

Module network inference resulted in over 150 tight co-expression modules containing over 8000 genes. Over 90% of the modules showed significant functional enrichment, often related to the hallmarks of cancer. Retaining the 1% top predictions, we identified novel drivers on chromosomes 2p and 17q as well as additional transcriptional regulators. Several predicted drivers are known to participate in biological pathways overrepresented in the co-expression modules. Amongst others, we detected *BRIP1* and *RRM2* as neuroblastoma driver genes, for which we have gathered preclinical evidence already (see abstracts Vanhauwaert et al. & Nunes et al.). Correlation to neuroblastoma specific transcriptomic signatures and survival data provided further validation of the predicted modules and drivers. We discuss the obtained hypotheses in light of known experimental findings in neuroblastoma. We are currently integrating patient single cell transcriptome data with this reference regulatory network for patient-specific drug target identification.

Summary/Conclusions

In conclusion, we report on the relevance of multi-omics network inference for identifying novel key driver genes and regulators of neuroblastoma. We provide a neuroblastoma reference regulatory network, from which novel drug targets and perturbed biological pathways for specific patient subtypes are identified.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 458

Novel metabolic vulnerabilities: Inhibition of cysteine uptake as a therapeutic concept for MYCN/MYC-driven tumors

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Aberrant *MYCN* oncogene expression determines a subset of highly aggressive neuroblastomas (NB) with poor clinical outcome, mainly due to resistance to conventional treatment. Recent findings have established a novel functional link between oncogenic *MYCN* and ferroptosis – a regulated, iron-dependent form of cell death, characterized by accumulation of ROS-mediated lipid hydroperoxides dependent on the availability of the amino acid rate-limiting for glutathione synthesis, cysteine.

Aims

Our aim is to develop a precise mechanistic model of cysteine usage in ferroptosis sensitive/resistant cells and to validate this novel metabolic vulnerability in various preclinical models. We focused on understanding the impact of cyst(e)ine uptake, cysteine synthesis via methionine-to-cysteine conversion (transsulfuration) and cysteine catabolism on the glutathione metabolism, particularly glutathione peroxidase 4 (GPX4)-detoxifying activity controlling lipid hydroperoxide formation.

Methods/Materials

To understand the drivers of cysteine addiction of NB cells we performed cystine titration experiments that allowed us to define a panel of ferroptosis sensitive and resistant neuroblastoma cells lines. These models are currently tested in flux experiments using stable isotope labelled cysteine/cystine, glutamine, and methionine (13C, 34S) to quantify the distribution and flux into

different synthetic processes (glutathione, protein and CoA cofactor synthesis). Moreover, multi-omics profiling of NB cell lines and primary NBs were used to define genetic markers that predict sensitivity of NB cells to ferroptosis indcution. NB xenograft mouse models were used to validate this metabolic vulnerability *in vivo.*

Results

Titration experiment show that *MYCN*-amplified NB cells are strongly addicted to cyst(e)ine supply, whereas cells lacking *MYCN* or *MYC* expression are largely resistant to limiting cystine supply. More importantly, downregulation of selected ferroptosis-related proteins increased sensitivity of *MYCN*-amplified NB cells to cystine deprivation as well as ferroptosis inducers. Flux experiments using stable isotope labelled amino acids revealed striking differences in distribution and flux into different metabolic processes when comparing both cell types. Of note, activation of the transsulfuration pathway due to epigenetic remodeling of genes controlling this process is a novel oncogenic function of *MYCN*, which provides a critical additional cysteine source in a subset of high-risk NBs.

Summary/Conclusions

In summary, our data show that ferroptosis execution emerges as a novel tumor suppressor function that needs to be inactivated during tumor formation and malignant progression. Conversely, *MYCN*-amplified tumors acquire metabolic activities to overcome ferroptosis sensitivity. Nevertheless, *MYCN*-amplified cells are highly sensitive to ferroptosis induction, disclosing a yet unknown metabolic vulnerability that can be exploited therapeutically.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 459

Intratumor heterogeneity and single cell changes induced by traditional chemotherapy

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

With the advent of bulk DNA and RNA sequencing, our knowledge of the genomic and transcriptomic landscapes of neuroblastoma (NB) has increased drastically. This has helped create a stronger fundamental understanding of the disease; however, it has not provided great insights into new therapeutic approaches. This can be partially attributed to shortcomings in bulk sequencing techniques. Using bulk techniques, we remain unable to answer crucial questions about tumor heterogeneity and the tumor evolution in response to treatment. Ultimately this limits our understanding of neuroblastoma and thus our ability to develop of novel, more effective therapies for neuroblastoma.

Aims

In this study, we will use single cell RNA sequencing of tumor samples to gain a greater insight into intratumor heterogeneity and intratumor transcriptomic changes over the course of standardly given chemotherapy. With a greater understanding of tumor evolution in response to therapy, we aim to identify and validate more effective therapeutic approaches for NB.

Methods/Materials

Tumor biopsies from patients at diagnosis and after chemotherapy are collected from surgery and immediately processed. Following mechanical digestion, samples are enzymatically dissolved to achieve a cell suspension. Cells are then stained and FACS sorted for single cell sequencing

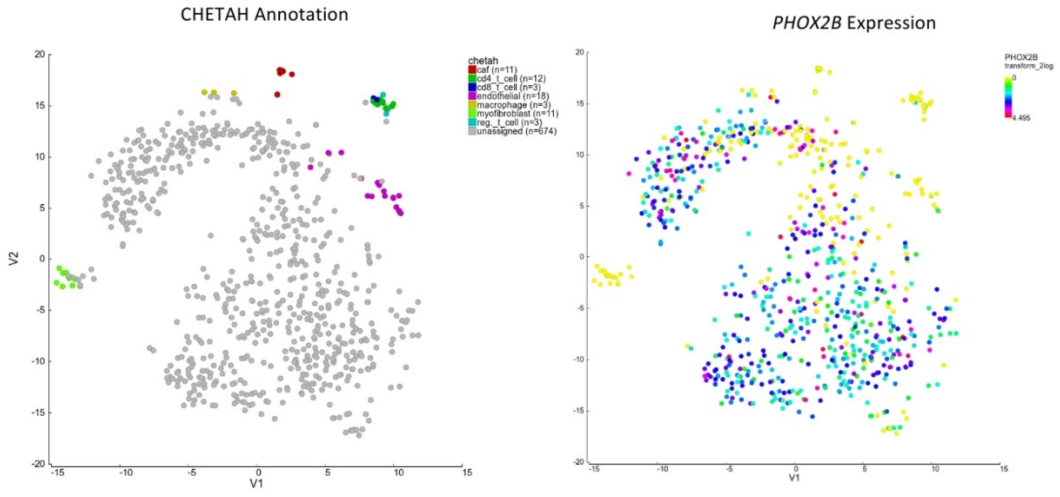
using the CELL-Seq2 protocol. After data processing and parsing, results are visualized using R2 (genomics analysis and visualization platform).

Results

Due to the clinical procedure of collecting diagnostic (or primary) NB samples, samples received are typically needle biopsies, offering very limited material for analysis. Initial FACS strategies for single-cell sorting resulted in poor sorting quality due to the overall low number of cells. However, incorporation of a nuclear stain with a viability stain allowed for elimination of debris and more efficient sorting of tumor material for single-cell RNA sequencing. Using the CHETAH (Characterization of cEll Types Aided by Hierarchical clustering) algorithm, preliminary results of a tumor sample reveal that diagnostic NB samples are mainly composed of tumor cells (verified via PHOX2B expression) with small populations of fibroblasts, immune cells, macrophages and endothelial cells. Tumor cells cluster based on expression of apoptosis-associated genes which could be intrinsic to the tumor, however, more tumor samples need to be evaluated to rule out processing artifacts. Upon collection of more single-cell sequencing data, matched untreated and treated tumor samples will be compared to elucidate therapeutic targets.

Summary/Conclusions

Single cell sequencing of limited diagnostic material offers a high-resolution overview to investigate intratumor heterogeneity. In order to further investigate tumor changes induced by chemotherapy, matched patient samples need to be collected and sequenced.



Single cell RNA sequencing data of primary, untreated NB.

Type Translational

Presentation Preference Traditional poster

Abstract nr. 460

The synthetic lethality landscape of key drivers and drug targets in Neuroblastoma

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

Neuroblastomas (NB) are tumors originating from cells of the neural crest and are driven by few genetic alterations with a limited number of druggable target genes.

Aims

Here we apply a novel data-driven approach to identify synthetic lethal (SL) partners of key driver genes and drug targets in NB. These provide a new approach for discovering novel tumor vulnerabilities in NB.

Methods/Materials

SL interactions denote the relationship between two genes whose combined inactivation is lethal to the cell, while their individual inactivation is not (and we also considered synthetic dosage lethal (SDL) relations, which denote an analogous functional relation between the activation of one gene and the inactivation of another). We focused on a list of 25 key known driver genes and drug targets of NB patients. To identify their SL and SDL partners in NB, we applied the previously published and validated ISLE computational pipeline (Lee et al., 20018, Nature Communications) to analyze 54 pediatric and 17 neuroblastoma cell lines from the Cancer Dependency Map (*depmap,* Ghandi et al., 2019, Nature) and 523 patients' NB primary tumors omics data (n=142 patients from TARGET, n=190 from the SEQC/MAQC-III Consortium, n=191 from Ackerman et al., 2018). Briefly, ISLE is applied in three steps: First, it identifies putative SL gene pairs from *depmap.* Second, it selects those gene pairs whose co-inactivation is under-represented in primary NB tumors. Thirdly, it prioritizes candidate SL pairs whose co-inactivation is associated with better patient prognosis.

Results

Performing this ISLE analysis in NB, we identified 496 significant SL interactions for 18 of the 25 driver or drug target genes. First, we tested, whether the predicted SL interactions can predict

gene essentiality by analyzing the results of published experimental *in vitro* CRISPR screens. Reassuringly, we find that for several driver genes/drug targets, one can successfully predict their essentiality in the CRISPR screen and drug validations based on their SL pairs, and that, as expected, the SL inferred are more predictive of gene essentiality and drug response in the NB cell lines than in general pediatric cell lines.
Second, our analysis predicts many novel SL partners of key drivers in NB such as MYCN, ALK and CHD5. For MYCN, the SL interactions inferred include MYCN-ATRX and MYCN-BAZ1A (both ATRX and BAZ1A are involved in chromatin remodeling) and MYCN-CDCA4 (cell cycle regulation). Notably, 13 of these MYCN SL interactions involve druggable SL partners, providing novel selective putative drug target candidates. Six MYCN SL interactions showed evidence of physical interactions with MYCN. REACTOME pathway analysis of MYCN SL interactions shows high enrichment of pathways involved in cell cycle (eg. M phase, Cell Cycle Checkpoints, Mitotic Prometaphase) and organic cation/anion transport (FDR <0.2). Interestingly, the ISLE workflow identified MYCN as a synthetic lethal partner of the tumor suppressor CHD5. Indeed, the SL partners of CHD5 and MYCN share many common enriched pathways such as those of cell cycle regulation (Cell Cycle Checkpoints, G2/M DNA damage checkpoint, Mitotic Prometaphase). Other enriched pathways for CHD5 involve DNA repair pathways (Homology Directed Repair, DNA Double-Strand Break Repair, DNA Repair) (FDR <0.1).

Summary/Conclusions

Taken together, these results motivate further experimental investigations exploring the feasibility of a new paradigm for whole exome SL-based precision treatments in NB but also for other pediatric tumors.

The synthetic lethality landscape of key drivers and drug targets in Neuroblastoma

Type Translational

Presentation Preference Oral presentation

Abstract nr. 461

Late-effects following high risk neuroblastoma treatment

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Topic Other

Background/Introduction

Survival from high risk neuroblastoma (HRNB) used to be very poor resulting in escalation of treatment intensity to include radiotherapy, high dose chemotherapy with stem cell rescue and more recently immunotherapy. While survival has improved and is currently reported to be about 50% at 5 years, this improvement comes at a cost. There is emerging data highlighting the physical burden of intense treatment. In survivors of all stages of neuroblastoma the 20yr cumulative incidence of chronic disease has been reported at 41%[1]. This is four times that of siblings with the main systems affected being musculo-skeletal, neurological, endocrine and second malignancy. The ENSG 5 study of high risk patients showed that 77% of patients had one late effect with deafness and endocrine disturbance being the most frequent[2]. Only one study assessed psychological outcomes in survivors of HRNB and used siblings as controls. They demonstrated increased anxiety / depression, reduced attention, increased antisocial behaviour and increased social withdrawal[3].

Aims

To identify the number of patients entering the long term follow up phase following high risk treatment for neuroblastoma in 2 UK principal treatment centres.
To assess the incidence and pattern of late effects and the relationship to changing treatment intensity.

Methods/Materials

Using local cancer registries survivors more than 5 years from treatment for HRNB were identified in 2 CCLG principal treatment centres (Newcastle & Aberdeen). A retrospective review of case notes and electronic records was performed and data collected on age at diagnosis, location of tumour, length of follow up, subsequent diagnoses and need for medical intervention and details about late mortality. Late effects were graded as per CTCAE 4.03.

Results

A total of 31 patients were identified with 27 patients from Newcastle and 4 from Aberdeen. They

were diagnosed between 1987 and 2013 and were treated with a variety of different treatment regimens. All received high dose chemotherapy with stem cell rescue, 60% radiotherapy and 26% anti GD_2 immunotherapy. Mean age at diagnosis was 3.6 years (range 0.16-20.9 years). Length of follow up ranged from 5-25.5 years with mean 13.5yrs. There were 3 late relapses with one death from disease. All survivors had at least one late effect with a median of 2 per person with a maximum of 10. Hearing loss and endocrine disturbance / fertility problems were the most common late effects. One patient is awaiting a lung transplant after developing lung disease secondary to therapy. In those with information available about dental health over 50% had dental issues. All 3 patients to develop scoliosis had paravertebral disease with radiotherapy to spine.

Summary/Conclusions

Adult survivors of high risk neuroblastoma exist with a life-long burden of treatment. It is important to ascertain the variation in late effects in relation to treatment and the impact the late effects have on daily functioning and quality of life.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 462

ABCC3 drug transporter in Neuroblastoma cell invasion

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Topic Other

Background/Introduction

Neuroblastoma, a neural crest derived tumor, is one of the most common extra cranial solid cancer in childhood. The amplification of MYCN oncogene is the most important marker of aggressive neuroblastomas. This subgroup of tumors often shows a multiple drug resistant phenotype and although many high-risk neuroblastoma tumors initially respond to the first cycles of intensive chemotherapy, they frequently become refractory to treatment as the disease progresses. Chemoresistance can be due to several cellular mechanisms, including those related to altered activity of specific ATP-binding cassette (ABC) drug transporters. ABC transporters shuttle hydrophobic lipophilic compounds across cell membranes in an ATP-dependent manner. ABCC3 is one of the most de-regulated ABC transporter gene whose expression is strongly down-regulated in case of high MYCN levels.

Aims

To test if the low ABCC3 expressing neuroblastoma cells SK-N-BE 2c could change their invasivity potential, we've measured invasiveness by transwell-matrigel invasion assay of neuroblastoma cells over-expressing both ABCC3 wt and the ATP binding site mutant. Transcriptomic analysis has been performed by a Nimblegen oligonucleotide microarray and QRT-PCR assay has been used to validate microarray data. Bioinformatic analyses were performed to determine which specific pathways are significantly de-regulated in case of ABCC3 WT over-expression when compared with both control and ABCC3 ATP binding site mutant cells.

Methods/Materials

We've measured invasiveness of cells over-expressing ABCC3 by transwell-matrigel invasion assay. Transcriptomic analysis has been performed by a Nimblegen oligonucleotide microarray. QRT-PCR and luciferase gene reporter assays have been used to validate microarray data. We've

also analysed the potential perturbation of key Transcription Factor activity by using the VIPER algorithm (Virtual Inference of Protein-activity by Enriched Regulon analysis). Dichlorofluorescein-diacetate assay has been employed to measure intracellular ROS level.

Results

MYCN amplified neuroblastoma cell clones stably over-expressing ABCC3_WT coding sequence were found to be less invasive in-vitro if compared to control cells. Gene ontology analysis of microarray data have revealed a strong de-regulation of genes involved in cell migration/invasion pathways. Among them, MET is the most down-regulated gene. Met encodes for the Hepatocyte Growth Factor Receptor (HGFR) whose expression have been largely demonstrated to be strictly correlated to cell invasiveness and advance stage of many types of tumors. Transcription factor network analysis (VIPER) has shown multiple de-regulated TF-networks including key cancer related factors such as HAND2, GATA3 and TWIST1.

Summary/Conclusions

High level of MYCN expression in tumors may trigger an altered ABC transporter expression profile, hence contributing to the control of drug and physiological substrate efflux in cancer cells. Further investigations on MYCN-ABCC3 axis could reveal key druggable pathways correlated with metastatic behavior of neuroblastoma cells. Our results suggest that MYCN amplification and the resulting ABCC3 repression lead to an aggressive cell phenotype due in part to the inhibition of the transporter function of ABCC3. High level of MYCN expression in neuroblastoma tumours may trigger an altered ABC transporter expression profile, hence contributing to the control of drug and physiological substrate efflux in cancer cells. Further investigations on MYCN-ABCC3 axis could reveal key druggable pathways correlated with metastatic behaviour of neuroblastoma cells.

Type Basic

Presentation Preference Rapid Fire Poster Presentation

Abstract nr. 463

Extracellular domain shedding of the ALK receptor mediates neuroblastoma cell migration

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

Mutations of the anaplastic lymphoma kinase (ALK) cell surface receptor tyrosine kinase are the most common directly targetable genetic aberrations in neuroblastoma, affecting approximately 10% of these tumors. However, the wild-type receptor is also overexpressed in the vast majority of neuroblastoma tumors, where it undergoes proteolytic shedding of the extracellular domain (ECD), the functional significance of which is unclear. Here we mapped the ALK cleavage site and show that ECD shedding is important for neuroblastoma cell invasion and migration. CRISPR-based editing of the ECD cleavage site of the wild-type ALK receptor in neuroblastoma cells resulted in their decreased migration and invasion, a phenotype that was associated with downregulation of

an epithelial-to-mesenchymal signature, decreased nuclear localization of β-catenin and reversal upon reintroduction of wild-type ALK. Importantly, inhibition of ECD cleavage led to significantly decreased metastasis and longer survival in mouse models of neuroblastoma. Retention of the ECD also potentiated retinoic acid-induced differentiation of neuroblastoma cells. We further show that the ALK ECD is cleaved by matrix metalloproteinase 9, whose inactivation leads to cleavage inhibition and loss of neuroblastoma cell migration. Together, our results indicate pivotal roles for ECD cleavage of wild-type ALK in neuroblastoma metastasis and differentiation, and suggest approaches to harnessing this process for therapeutic gain.

Aims

Methods/Materials

Results

Summary/Conclusions

Type Basic

Presentation Preference Oral presentation

Abstract nr. 464

Genomic Analysis and precision therapy for high risk neuroblastoma at diagnosis and relapse

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Despite recent advances, high risk neuroblastoma (NB) accounts for 15% of all pediatric cancer deaths. Many patients will attain remission but approximately half of patients relapse within five years of diagnosis. Those who relapse often transiently respond to additional therapy but have a high rate of subsequent relapse. Further genomic understanding and novel therapies are needed.

Aims

Compare genomic analysis of high-risk NB at diagnosis with those at relapse. Characterize differences in DNA and transcriptomes and evaluation of novel subgroups.

Methods/Materials

Genomic Analysis was performed on patients enrolled on either Molecular Guided Therapy NMTRC008/009 study “Feasibility Trial Using Molecular Guided Therapy for the Treatment of Patients with Relapse and Refractory Childhood Cancer” or “PEDS-PLAN – Pediatric Precision Laboratory Advanced Neuroblastoma Therapy”. Whole-exome sequencing (WES) and RNA-Seq were performed at The Translational Genomics Research Institute (TGen) on the Illumina HiSeq. The RNA expression levels were compared to a normal whole-body reference composed of 22 normal tissues as well as a pediatric cancer reference. Differential expression data was interpreted in the context of systems biology annotation for the purpose of identifying activated cellular processes targetable by drugs.

Results

Tumor WES was performed on 116 patients (69 diagnosis, 47 relapse) and RNA-Seq was performed on 127 patients (69 diagnosis, 58 relapse). DNA analysis exhibited fewer mutations in primary diagnosis compared to relapse (median 53[26-78] coding mutations per subject to 164[28-

5000]). Most common mutations or copy number alterations (diagnosis, relapse) are seen in MYCN (29%, 34%), ATRX (9%,30%), ALK (17%,11%), CDKN2A (0%,13%), PPM1D (0%,15%). RNA expression analysis identified subsets of patients with high/low expression of SSX1, SSX2 or HOXD13 genes. SSX1 and SSX2 genes were expressed moderate to high in relapse and mutually exclusive when compared to diagnosis patients. High SSX1, SSX2 expression are seen in 20%,14% of patients at diagnosis as opposed to 43%, 34% at relapse. We also identified two expression subtypes within MYCN amplified tumors. The first was characterized by low SSX1, SSX2/high MYCN expression (SSX1/SSX2 is low in 95% of MYCN amplified tumors vs 56% of MYCN negative tumors). The second was characterized by high HOXD13/high MYCN expression (HOXD13 is overexpressed in 65% of MYCN amplified tumors vs 28% MYCN non-amplified tumors).

Summary/Conclusions

Genomic sequencing for precision therapy was feasible both at diagnosis and relapse. RNA analysis points towards identification of new subtypes which could be used to stratify patients and develop further understanding of relapse. Validation of this approach in preclinical models may lead to novel therapeutic strategies. In order to improve outcome and decrease long term toxicities of current chemotherapy, further study of genomic guided treatments at diagnosis is warranted.

Elizabeth

Genomic Analysis and precision therapy for high risk neuroblastoma at diagnosis and relapse

Karl Dykema

Elizabeth

Type Translational

Presentation Preference Traditional poster

Abstract nr. 465

De-novo germline ALK mutation associated with congenital metastatic (Ms) neuroblastoma, dysmorphism and developmental delay.

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

Anaplastic Lymphoma Kinase (ALK) is a transmembrane receptor tyrosine kinase that plays an important role in nervous system development. Germline *ALK* mutations predispose to neuroblastic tumours and are the most common cause of hereditary neuroblastoma. Within the *ALK* gene are a number of mutational hotspots. F1174 is the 2nd commonest site of somatic mutation with only one previously reported case in the germline, an F1174V mutation in a child with congenital neuroblastoma and fatal encephalopathy / brain stem abnormality.

Aims

To report, to our knowledge, the first case of a child now 3 years old, with congenital neuroblastoma and developmental delay with a germline F1174L *ALK* mutation.

Methods/Materials

We report the clinical and genetic features of a child (now 3 years old) with congenital neuroblastoma and developmental delay who was found to have a germline F1174L *ALK* mutation by Sanger DNA sequencing. We hypothesised this mutation was responsible for the child's phenotype and the parents consented to enter the child into the Genomics England 100,000 genomes project http://www.genomicsengland.co.uk. Whole genome sequencing (WGS) was performed on germline DNA from the child and both parents together with tumoural DNA. Samples were prepared using an Illumina TruSeq DNA PCR-Free library preparation kit and then sequenced on a HiSeq X generating 150 bp paired-end reads. Germline samples were sequenced to produce at least 85 Gb of sequences with sequencing quality of at least 30. Written informed

consent was given by the child's family to report this case.

Results

A 3 year old boy presented at birth with "blueberry muffin spots" and was found to have congenital Ms neuroblastoma with a right adrenal primary tumour and skin/subcutaneous tissue and liver metastases. Biopsy confirmed International Neuroblastoma Pathology Classification favourable histology neuroblastoma with no evidence of *MYCN* amplification or segmental chromosomal abnormalities. After an initial period of observation the patient required 2 cycles of chemotherapy due to disease progression and poor feeding. Since then his tumour has slowly and continuously regressed. Genetic testing revealed an ALK c.3520T>C p.(Phe1174Leu) *ALK* mutation in the tumour which was subsequently found to be a *de-novo* germline mutation. The child had other health problems since birth including feeding difficulty, poor growth, gross motor developmental delay, hypertonia and convergent squint. Fragile X testing was normal. Whole genome germline DNA sequencing from the child and parents showed no other genetic cause for the child's phenotype and cancer whole genome sequencing revealed 8 variants at low allele frequency of unknown clinical significance.

Summary/Conclusions

Germline *ALK* mutations predispose to early onset neuroblastoma. To our knowledge this is the first report of a germline F1174L mutation in a child which is likely to be responsible for his phenotype of poor feeding, small stature and developmental delay. Future reports of germline F1174L mutations will confirm or refute this finding and may lead to identification of a new genetic syndrome which could be termed an "ALKopathy".

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 466

Surviving High-Risk Neuroblastoma: A Preliminary Descriptive Report from Project LEAHRN (Late Effects After High-Risk Neuroblastoma) Lisa Diller, Wendy B. London, Jenna Bardwell, Paige Kao, Anna Gilmore, Paul C. Nathan, Wendy Landier, Sogol Mostoufi-Moab, Tara Brinkman and Tara O. Henderson

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Topic Other

Background/Introduction

The Late Effects After High-Risk Neuroblastoma (LEAHRN) trial [Children's Oncology Group (COG) ALTE15N2] was designed to study the long-term outcomes in this emerging group of childhood cancer survivors and to analyze the impact of immunotherapy and other new agents on outcomes.

Aims

To describe clinical outcomes in a large cohort of high-risk neuroblastoma survivors.

Methods/Materials

Eligible patients were at least 5 years from a new diagnosis of high-risk neuroblastoma and had been previously enrolled anytime after January 1, 2000 on the COG Neuroblastoma Biology study; they were age 5-50 years, not currently receiving cytotoxic therapy and at least 5 years from original neuroblastoma diagnosis. Eighty-eight COG institutions elected to participate in this study. Subjects participated in a clinical evaluation at a single time point and cross-sectional analyses to determine the proportion of survivors with severe toxicity, the impact of toxicity on quality-of-life, and patient and treatment-related risk factors for adverse outcomes are planned. Preliminary descriptive data for patients enrolled during the first 2.5 years of the study are presented herein.

Results

As of October 1 2019, 268 patients were enrolled from 53 institutions with median age at enrollment 12 years (range: 5-24). The median age at neuroblastoma diagnosis was 2.5 years (range: 0 - 15.8) and the median time from diagnosis to enrollment was 9.8 years (range: 0.4-17.9). Among 250 patients with known disease status, 89.6% were neuroblastoma disease-free

after primary therapy with no history of relapse, 6% were neuroblastoma-free after completion of treatment for primary and relapsed disease; and 3.6% reported stable disease after primary therapy or after completion of relapse therapy.
In 240 patients with data regarding prior treatment, all patients had received chemotherapy in combination with other therapies. Other therapy included single transplant (n=180); tandem transplant (n=53); immunotherapy (n=139); radiation therapy (n=228); isotretinoin (n=220); and MIBG-therapy (n=12).
Severe ototoxicity (requiring hearing aids) was observed in 140/222 (63%). Major organ system toxicities include cardiac toxicity in 35/246 (14%), pulmonary toxicity in 60/245 (24%) and non-reproductive endocrine toxicity in 105/247 (63%). Specific toxicity counts included hypothyroidism (n=31), pulmonary hypertension (2), congestive heart failure (7), restrictive lung disease (16), abnormal pulmonary function tests (13), hypertension (12) and stroke (1). Height Z- scores were less than -1.7 in 57/142 (40%)
Eleven of 246 (4.5%) patients have developed secondary malignant neoplasms (SMN), including malignancies of bone (1), brain (1), kidney (1) and thyroid (1) as well as mucoepidermoid carcinoma (2) spindle cell carcinoma (1), lymphoma (1), and leukemia/MDS (3). Only 4/11 SMN were reported to have occurred within the radiation field.

Summary/Conclusions

A growing number of high-risk neuroblastoma survivors face a range of health challenges. Future analyses, after completion of enrollment (n=400), will include risk factor analyses, quality-of-life outcome assessment and impact of specific exposures, including immunotherapy and MIBG, on long-term toxicity. These results will be important in the design of future studies and in the long-term follow-up care of survivors.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 467

Issues on a rare case of ganglioneuroblastoma

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

Ganglioneuroblastoma is a very rare histological subgroup of peripheral neuroblastic tumors (pNTs) in the pediatric population; its clinical behavior is extremely heterogeneous.

Aims

Methods/Materials

Case Presentation: Patient G.L., male, aged 3 years, was admitted to our Department with a left supraclavicular adenopathy that was noted from the parents about 3-4 weeks earlier. He had no history of pain, fever, weight loss or other comorbidities. His family and personal pathological history was insignificant.

At admission, physical examination revealed good general status except for a 2 cm left supraclavicular adenopathy. Laboratory tests did not detect anemia or thrombocytopenia and the vanylmandelic acid was at upper reference values (12.23 mg/gr urinary creatinine). Ultrasound of the abdomen was normal. A chest X-ray was performed, which showed a solid nodular mass and calcifications in the posterior superior mediastinum. Thoracic computed tomography (CT) scan showed a left apical thoracic tumor mass (6.4 x 4.7 x 6.7 cm) involving the left costo-vertebral groove (D1-D2) and displacing the subclavian vessels that appeared partially stenotic. A CT-guided biopsy of the tumor without complications was performed. The first pathological assessment was ganglioneuroblastoma, intermixed type, Schwannian stroma-rich, MYCN non-amplified and presence of segmental chromosomal aberrations.

A whole body MRI showed an hyperintense signal area in D8 vertebra on STIR and DWIBS sequences, without signs of bone erosion. The same abnormalities were found at MIBG scintigraphy. Bone marrow aspirate from bilateral iliac crest was negative.

A multidisciplinary discussion to perform a resection of tumor was done and surgery was planned and approached using left thoracotomy under general anesthesia with the complete excision of the tumor and the supraclavicular adenopathy. Histopathologic exam of the surgical samples were more consistent with composite neuroblastoma (intermixed-nodular ganglioneuroblastoma), MYCN non-amplified; this histological pattern was confirmed in a more careful evaluation of the previous bioptic samples.
The patient underwent 4 cycles of chemotherapy (SIOPEN NB HR); a good response was evidenced in the next radiological exams by minimal abnormal signal in the vertebral lesion on MRI DWIBS without MIBG uptake on SPECT.

Results

Summary/Conclusions

Ganglioneuroblastoma is a rare subtype of pNTs; its histopathological diagnosis sometimes has some issues but a proper diagnosis is of utmost importance because of the clinical, therapeutic and prognostic significance among the different subgroups. Although if the multimodal imaging could guide the diagnostic approach, a correct assessment of the histologic samples is the cornerstone; the identification of nodular lesions in the second samples changed the prognosis and the therapeutic approach.
Clinicians and pathologists should be aware of the various clinical presentations of pNTs, and the appropriate use of multimodal imaging and pathologic investigations is crucial for the diagnosis and therapy.

Type Clinical

Presentation Preference Traditional poster

Abstract nr. 468

A novel cre-conditional MYCN-driven neuroblastoma mouse model

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Topic Other

Background/Introduction

Genetically engineered mouse models for neuroblastoma enable fine-tuned mechanistic investigation of this neural crest-derived malignancy and provide a suitable *in vivo* platform for preclinical drug testing. Our group previously developed a conditional MYCN-driven mouse model for neuroblastoma (*Dbh-iCre;LSL-MYCN-luc*) in which Cre recombinase expression is restricted to dopamine β-hydroxylase-expressing cells hence enabling and confining high-level expression of human *MYCN* to those cells and their progeny. This model closely recapitulated human disease and overcame several limitations of previous mouse models for neuroblastoma. Neuroblastic tumors initially arose at a high incidence (75%) regardless of strain background. Tumors displayed immature, undifferentiated histology and were predominantly derived from adrenal glands and superior cervical ganglia. However, genetic drift and the BAC-transgenic design of the Cre donor strain used resulted in vastly diminished tumor incidence in later generations.

Aims

We sought to establish a novel Cre recombinase conditional mouse model for neuroblastoma employing and evaluating alternative Cre recombinase donor strains.

Methods/Materials

We acquired four transgenic models as alternative Cre recombinase donors, with Cre expression under the control of murine *Wnt1*, *Sox10*, *Phox2b* or the *Th* promoter activity. These models were cross-bred with the previously generated *LSL-MYCN-luc* mice to investigate potential tumor

incidence, latency, localization, and histopathology in the double-transgenic offspring and compare these properties to those in the *Dbh-iCre;LSL-MYCN-luc* mouse initially.

Results

Cross-bred *Cre donor, LSL-MYCN* double-transgenic offspring were monitored for 40 weeks. *Sox10-cre x LSL-MYCN* crosses yielded far fewer double-transgenic progeny than expected based on Mendelian ratios, indicating embryonic lethality. No tumor development was observed in 10 *Wnt1-Cre;LSL-MYCN*, 10 *Phox2b-IRES-Cre;LSL-MYCN* and the surviving three *Sox10-Cre;LSL-MYCN* mice. On the other hand, 100% of the 10 *Th-IRES-Cre;LSL-MYCN* mice died prematurely at an average age of 220 days due to tumor development. Tumor tissue was recovered from 70% of these mice, and tumors were often localized to the mesentery, and less frequently, the liver or adrenal, indicating neoplasms derive predominantly from the celiac ganglia in this model. Tumor histopathology, evaluated by an expert pathologist, confirmed a neuroblastic phenotype of a mixed differentiation grade. We immunohistochemically detected neuronal and neuroblastoma marker proteins, markers of active proliferation and human MYCN protein in tumor sections. Molecular tumor characterization by transcriptome and exome sequencing is currently ongoing and will also assess secondary genetic alterations acquired during oncogenesis.

Summary/Conclusions

Considering four neural crest-specific Cre donor models, we established *Th-IRES-Cre*;*LSL-MYCN* as a new mouse model yielding neuroblastic tumors at an extremely high incidence. Histologically, tumors resemble human neuroblastomas with a mixed degree of differentiation, in contrast to our previous *Dbh*-based MYCN-driven model, in which tumors predominantly presented with an undifferentiated histology. This model may prove especially suitable to investigate mechanisms of high-risk neuroblastoma biology, such as research focusing on MYCN co-drivers and their impact on tumor onset, differentiation and drug response.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 469

The neuroblastoma surfaceome is regulated by adrenergic or mesenchymal subtype-specific transcriptional regulatory networks.

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Topic Gene regulatory networks and epigenetics: from basic science to therapy

Background/Introduction

While the implementation of GD2 immunotherapy has improved high-risk neuroblastoma outcomes, many patients experience significant acute toxicities and relapse. Thus, identifying new cell-surface targets that are linked to crucial gene regulatory networks could improve patient responses, limit toxic side-effects and mitigate immune evasion via downregulation of the target.

Aims

To develop an integrated epigenomics and RNA-sequencing-based approach to identify neuroblastoma-specific surface molecules in adrenergic and/or mesenchymal subtypes that may be amenable to synthetic immunotherapies.

Methods/Materials

We utilized singscore (ssGSEA) with published adrenergic and mesenchymal gene signatures to subset high-risk neuroblastoma patient samples (TARGET RNA-seq, adrenergic=130, mesenchymal=11). We used limma voom to determine differentially expressed genes in each neuroblastoma subtype compared to 1641 normal tissue samples from 25 organs (GTEx). We processed ChIP-seq data of transcription factors in the adrenergic core regulatory circuit (CRC) in two adrenergic neuroblastoma cell lines (KELLY and SKNBE2C) and applied the bedtools and HOMER toolkits to identify CRC-bound differentially expressed genes. Finally, the differentially expressed genes in each neuroblastoma subtype were further analyzed for cell surface localization using the COMPARTMENTS database or referencing the neuroblastoma ligandome via coimmunoprecipitation of MHC Class I and II coupled to mass-spectrometry (see Yarmarkovich, ANR 2020).

Results

For the adrenergic subtype, we first showed that genes bound by 6+ CRC transcription factors were significantly more likely to be differentially expressed compared to GTEx normal samples

(3.6-fold enrichment; zscore=12.67). Of the 116 adrenergic differentially expressed genes, 41 (35%) were bound by 6+ CRC transcription factors, and 26 of these 41 genes (63%) were either resident cell surface proteins or detected as MHC-bound peptides. These included several proteins currently in clinical development (ALK, L1CAM, and SLC6A2 [NET]). As expected, the mRNA expression of many adrenergic-specific targets was much lower or completely absent in the mesenchymal subtype. We identified 2 mesenchymal-specific genes, and several genes present in both subtypes, that are differentially expressed compared to GTEx normal samples. Notably, the peripherin gene (PRPH) had similar mRNA expression in both adrenergic and mesenchymal patient samples and showed broad H3K27Ac signal in cell lines of each neuroblastoma subtype. Finally, we showed that 8 peptides derived from PRPH were detected in the neuroblastoma ligandome and 4/8 primary neuroblastoma samples presented at least one peptide. Moreover, 3/8 neuroblastoma samples presented a common PRPH peptide that was predicted to bind to HLA-A0201 with an affinity of 9.7 nM (NetMHC4.0). Overall, many candidate immunotherapeutic targets are adrenergic-specific, the exceptions being L1CAM and several MHC-presented peptides derived from PHOX2A, PHOX2B, or PRPH that are present in both adrenergic and mesenchymal neuroblastoma subtypes.

Summary/Conclusions

Identification of CRC-bound genes encoding cell surface proteins or MHC-presented peptides can help prioritize immunotherapeutic targets for clinical development. Targeting proteins expressed in both the adrenergic and mesenchymal subtypes could abrogate classical mechanisms of immune escape such as selection of subclones with low target expression.

Type Basic

Presentation Preference Traditional poster

Abstract nr. 470

Synergistic antitumor effects of combining selective CDK7 and BRD4 inhibition in neuroblastoma

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

Cyclin-dependent kinase 7 (CDK7) with roles in transcription initiation and cell cycle progression has been recognized as a therapeutic target in cancer. We have previously shown that inhibition of CDK7 with THZ1, leads to striking cytotoxicity in mouse models of high-risk neuroblastoma (NB) driven by downregulation of super-enhancer-associated with oncogenic drivers. However, potent off-target activity of THZ1 on CDK12/13 also contributed to this phenotype.

Aims

Investigate the therapeutic potential of a novel, selective CDK7 inhibitor, YKL-5-124 in pre-clinical models of NB.

Methods/Materials

Established cell line and patient-derived xenograft (PDX) models of human NB.

Results

Using a novel, highly selective covalent inhibitor of CDK7, YKL-5-124, we demonstrate that selective inhibition of CDK7 has no effect on C-terminal domain (CTD) phosphorylation of RNA Pol II. Rather, we observe that YKL-5-124 significantly inhibits CDK1 and CDK2 T-loop phosphorylation in NB cells. Consistent with these findings, CDK7 inhibition with YKL-5-124 leads to aberrant cell cycle progression and DNA replication stress without the induction of extensive apoptosis. We next identified that combining YKL-5-124 with inhibitors of the bromodomain-containing protein 4 (BRD4), further potentiates the effect of YKL-5-124 on DNA synthesis and induces apoptosis in NB cells. This translates to significant tumor regression in both established cell line and patient-derived xenograft mouse models (PDX) of NB with tolerable toxicity. We

determined that cytotoxicity of combination treatment was associated with inhibition of RNA Pol II transcription. RNA-sequencing analysis identified effects on genes involved in DNA replication and the TP53 pathway.

Summary/Conclusions

Taken together, our findings suggest that combined inhibition of CDK7 and BRD4 provide a novel therapeutic option for NB. Ongoing work is focused on understanding the mechanism underlying such synergistic cytotoxicity.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 471

Targeting Survivin in neuroblastoma at protein level with the Survivin inhibitor S12

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

The oncogene *BIRC5* (encoding the protein Survivin) is located on chromosome arm 17q, upregulated by MYCN, and was found to be strongly overexpressed in high-risk neuroblastoma (NB). Survivin is involved in suppression of apoptosis, mitosis, cell cycle, metabolism, invasion and several other key cellular functions. Knock-down of *BIRC5* in neuroblastoma cell lines has been shown to result in cell death. Treatment of neuroblastoma cell lines and mice with neuroblastoma xenografts with the Survivin inhibitor YM155 resulted in cell death and tumor regression. However, more recent studies have revealed that YM155 treatment leads to unspecific DNA damage and other unspecific side effects rather than specific inhibition of Survivin.

Aims

Our aim is to explore strategies for targeting Survivin on protein level and test the novel Survivin inhibitor S12, which binds to Survivin and prevents it from homodimerization and binding to other proteins.

Methods/Materials

For a panel of 20 NB cell lines, mRNA expression data, epigenetic data and exome sequencing data were obtained, and the cell lines were treated with S12 or YM155. The cell lines were stratified according to sensitivity to the inhibitors, and machine learning was applied to generate a predictive signature for sensitivity to S12. The cellular effects of S12 on neuroblastoma cell lines

were further analysed using cell-based assays for cell viability, proliferation, apoptosis and differentiation. Survivin expression before and during treatment with S12 was analysed using western blot analysis.

Results

Treating 20 NB cell lines with S12 and YM155 at a wide range of concentrations, we found that the sensitivity to S12 treatment did not correlate well with the sensitivity to YM155 treatment. The mean IC50 of S12 was approx. 10μM in cell lines stratified as sensitive to S12 treatment. Using a machine-learning algorithm, a molecular predictor based on gene expression and epigenetic features could be established and was validated using a leave-one-out cross-validation strategy. As expected, treatment of neuroblastoma cells with did not alter survivin protein levels. S12 treatment significantly reduced cell viability, which was driven both by reduced cell proliferation and the induction of apoptosis. S12 treatment also induced cellular differentiation, as indicated by cell shape, the outgrowth of neurite-like processes and the upregulation of markers of neuronal differentiation.

Summary/Conclusions

Inhibition of Survivin by interfering with Survivin homodimerization and protein-protein interactions using the small-molecule inhibitor S12 outlines a new strategy for targeting Survivin in neuroblastoma. For translation of this strategy into clinical application we propose further development and improvement of S12-based inhibitors that achieve efficacies at nanomolar concentrations.

Type Translational

Presentation Preference Oral presentation

Abstract nr. 472

Long term follow-up of children with Neuroblastoma receiving radiotherapy to metastatic lesions within the German Neuroblastoma Trials NB97 and NB 2004

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

Neuroblastoma (NB) is the most common extracranial solid malignancy in children. Over 50% of patients present with metastatic lesions, and despite a multimodal treatment approach the prognosis of these patients is not satisfactory

Although radiotherapy has become an integral part for treatment of the primary tumor, the role of radiotherapy for osteomedullary lesions is not well defined. When compared to a cohort of children who did not receive RT to metastatic lesions, previous studies did not presented any significant benefit of adding focal irradiation to metastatic sites (Polishchuk et al., 2014; Kandula et al., 2015). However, small series have reported promising results (Casey et al., 2018; Mazloom et al., 2014).

Aims

We performed a retrospective analysis to assess the impact of RT to osteomedullary lesions in children with NB.

Methods/Materials

Clinical records of patients who received irradiation for osteomedullary metastases during first-line treatment included in the prospective, national, multicenter neuroblastoma trialsNB97 (EU - 20661) or NB2004-HR (NCT 00410631) were retrospectively analyzed

Results

Within the national trials NB97 and NB2004, 18 (57% male, 43% female) patients with residual osteomedullary uptake at the end of induction therapy were irradiated and eligible for this analysis. Seventeen patients received irradiation to MIBG active metastatic lesions at the time of RT,

whereas one patient received RT to one metastasis after complete metabolic response due to previous therapy. At the time of RT two or more lesions (Median 3 (2-5)) were present in 12 children. Among 53 lesions detected before starting RT, 23 received RT.
A medium (range) dose of 36Gy (20Gy-45Gy) to one or more (n=1-3) osteomedullary metastases was applied. The irradiated metastatic sites were located either in the skull (29.1%), extremity (41.6%), spine (20.8%) or rips (8.3%) respectively. The medium follow-up time was 149 months (55-220) in survivors. At the time of last follow up 4 patients were free of relapse or progression. 11 patients had deceased, either due to tumor progression (n=9) or due to complications of the systemic therapy (n=2). An isolated local failure at an irradiated metastatic site was recorded in four patients. In five patients, progressions in the region of the irradiated site together with other progressive osteomedullary sites were reported. Furthermore, 4 patients progressed osteomedullary, but not at the irradiated sites. The global local control rate was 9 of 18 patients.

Summary/Conclusions

Due to the limited number of patients and the retrospective nature of this analysis, the impact of RT to residual metastatic sites cannot be proved. In order to provide evidence, a prospective study would be desirable.
In the absence of convincing evidence, the indication for radiotherapy to metastatic lesions has to be reserved to multidisciplinary protocols or applied only after thoughtful individual multidisciplinary tumor board decisions.

Type Clinical

Presentation Preference Rapid Fire Poster Presentation

Abstract nr. 473

IL-15-expanded Vd1 T cells demonstrate prolonged proliferation and anti-tumour activity as a potential platform for immune therapy against neuroblastoma.

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

Chimeric antigen receptor (CAR)-based immunotherapies developed in αβ T cells have shown wide success against a number of haematological malignancies. However, their efficacy against neuroblastoma (NB) has been limited; specifically, they have been hindered by on-target, off-tumour toxicity as well as CAR T cell exhaustion and persistence within the NB tumour microenvironment. Vδ1+ cells, a subset γδ T lymphocytes, present a promising cellular platform by which to obviate these drawbacks, as they have distinctive cellular properties that suggest they can protect non-tumour cells from lysis and resist exhaustion in a tissue context. Unfortunately, Vδ1+ cell expansion and differentiation are not well understood. γδ T cells only comprise 1 – 5% of circulating T cells, with 10 – 30% of that fraction being Vδ1+ cells that have previously been difficult to expand.

Aims

To study these cells as a potential cell for therapeutic use, we aimed to optimise an Vδ1+ expansion protocol that included TCR stimulation with anti-CD3 [OKT-3] and IL-15 supplementation to produce cytotoxic Vδ1+ cells.

Methods/Materials

We conducted studies with flow cytometric analysis to examine fold change in Vδ1+ cell number and shifts in their phenotypic marker expression, as well as their proliferation in response to tumour targets. We also investigated expanded Vδ1+ cell cytokine production using immunosorbent assays.

Results

Here, we show that our expansion technique is sufficient to produce a large number of Vδ1+ cells that can proliferate for up to 28 days following stimulation. While these cells express phenotypic markers found in naïve and central memory T cells, they also delay expression of exhaustion

markers. Finally, these expanded Vδ1+ cells demonstrate antitumour activity against NB-specific cell lines through surface marker expression and cytokine release when co-cultured *in vitro*. [data in progress]

Summary/Conclusions

Thus, we propose that Vδ1+ cells can be expanded *ex vivo* for immunotherapeutic development targeting neuroblastoma.

IL-15-expanded Vδ1+ T cells demonstrate prolonged proliferation and anti-tumour activity as a potential platform for immune therapy against neuroblastoma.

Type Basic

Presentation Preference Traditional poster

Abstract nr. 474

Glycoprotein GD2 presentation on the surface of neuroblastoma cells grown on 3D bio-engineered scaffolds

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

Neuroblastoma is an aggressive paediatric cancer of the sympathetic nervous system. Current therapies are not effective in the long term for almost 80% of patients with the clinically aggressive disease (Olga Piskareva and Raymond L. Stallings, 2015). GD2 is a glycoprotein that is abundantly expressed on NB cells. Due to its high expression on NB cells and low expression on other cells of the body it is a useful target for immunotherapy in NB patients. Immunotherapy using anti-GD2 combination therapy has been implemented as a treatment for minimal residual of disease. Yet 1/3 of individuals have a poor outcome due to relapse (Yu et al., 2010). A major hurdle in the drug development pipeline for cancers is the discrepancy between *in vitro* and *in vivo* testing results, leading to only 1 in 10 drugs entering clinical trials and approval by the FDA. Recently, we have characterised the growth of numerous NB cell lines on differing collagen scaffolds with the ability proliferate, infiltrate, deposit extracellular matrix and respond to stimuli similar to *in vivo* biological systems (Curtin *et al.*, 2018).

Aims

The aim of this study was to characterize the growth pattern of four well characterized neuroblastoma cell lines on collagen-nanohydroxyapatite (coll-nHA) scaffolds and subsequently evaluate GD2 presentation on the surface of four neuroblastoma cell lines grown in scaffolds using immunohistochemistry.

Methods/Materials

Multiple NB cells were characterised for DNA content using the pico green dsDNA assay when grown on coll-nHA scaffolds. Cell growth and distribution on the scaffolds was examined with H&E. Immunohistochemical analysis was performed for the surface marker GD2.

Results

Upon analysis of cell numbers per scaffold using the Pico green Assay, results showed cell growth is cell line dependant. This may possibly be in relation to cell line doubling time and invasive nature of each cell line in the scaffolds. Cellular growth peaked at 14 or 21 days before decreasing. This may be due to inability of the cells to infiltrate the scaffold correctly and nutrient availability becoming depleted. We have now optimised immunodetection of GD2 both in 2D and 3D *in vitro*. GD2 is uniformly expressed across all NB cell lines grown in 2D. This data is in agreement with previous studies. At the next step, we investigatied GD2 expression in 3D *in vitro* scaffolds to assess the cellular distribution. Results showed differing growth patterns for cell lines, ranging from tight colony clusters to dispersed cell growth.

Summary/Conclusions

To conclude, 3D bio-engineered models have great potential to better act as a new platform for testing antibody based immunotherapies that more accurately reflect NB and the ECM. Future studies will assess the ability of the GD2 antibody to induce immune cell-mediated killing in a co-culture of immune cells and NB cells on these new emerging 3D scaffolds.

Type Basic

Presentation Preference Traditional poster

Abstract nr. 475

Kalirin-RAC controls nucleokinetic migration in ADRN-type neuroblastoma

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Topic Neuroblastoma as developmental disease

Background/Introduction

Neuroblastoma (NB) is thought to be a result of impaired differentiation of neural crest-derived progenitor cells, which promotes the expansion of a population of cells susceptible to the secondary transforming events (Pei et al., 2013). NB has been recently resolved as a biphasic malignancy with primary tumors containing the cells of ADRN (noradrenergic) type and MES (mesenchymal) type (Boeva et al., 2017; van Groningen et al., 2017). Both ADRN and MES lineages produce aggressive metastatic tumors, yet ADRN identity could be reprogrammed towards MES "toolkit". The migrational propensity of NB is affected by the identity, but the mechanisms behind the divergence remain unknown.

Differentiation failure in ADRN NB has been traced at the epigenetic, genetic and transcriptional level and manifests as the down-regulation of the genes involved in maintaining neuronal morphology (Henrich et al., 2016). Yet, many neuritogenesis genes are essential for cell migration and failure in their regulation might be involved in NB invasion program. The question remains open as to how differentiation block and migratory propensity are balanced in the ADRN lineage's tumors and whether ADRN cells are a subject to EMT process. From a point of clinical relevance, the closest compartment that may reflect migration and dormancy, are disseminated cancer cells detected as part of minimal residual disease (MRD) (Raimondi et al., 2010). Previous studies in NB identified doublecortin (*DCX)* mRNA encoding the core component of neuronal motility, as a MRD marker associated with poor survival in NB patients (Hartomo et al., 2013; Viprey et al., 2014).

Aims

Based on these facts, we reasoned that understanding the mechanisms implicated in migration of DCX-positive NB can shed light on initial steps of the metastatic process in NB.

Methods/Materials

Results

Our data show that *DCX* expression is associated with ADRN identity. Further, ADRN-type cells exhibit nucleokinetic (NUC) migration, resembling motility observed in neurons. Time-lapse movie analysis with ADRN- and MES-type cell lines expressing a nuclear marker allows us to distinguish between mesenchymal (MES) and NUC mode of migration. Using RNAi along with above-described approach, we show that NUC migration relies on integral components of neuronal migration, including the above-mentioned DCX and lissencephaly1 (LIS1). We propose that parallelism between migration modes in neurons and ADRN NB involves transcriptional control and suggest several candidate TFs including the SOXC factor, SOX11, which regulates *DCX* expression in ADRN-type cells. We demonstrate that nucleokinesis is RAC1- and kalirin-dependent in ADRN NB, the latter mediating NUC activity of RAC1.

Summary/Conclusions

Taken together, these data provide a rationale for further investigation of vulnerabilities associated with NUC migration in ADRN-type cells.

Type Basic

Presentation Preference Traditional poster

Abstract nr. 476

MYCMI-7 - a small MYC-binding compound that inhibits MYC:MAX interaction and tumor cell growth in a MYC-dependent manner

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

Deregulated expression of the MYC family oncogenes/transcription factors MYC and MYCN occurs in many types of adult and childhood tumors including neuroblastoma, and is in the latter case strongly associated with aggressive tumor development, resistance to therapy and poor prognosis. In mouse tumor models, inactivation of MYC often leads to tumor regression with well-tolerated side effects, suggesting that MYC is a potential and suitable target for anti-cancer therapy. Unfortunately, no specific MYC-targeting drugs are available clinic today. Thus, developing inhibitors the cripple MYC activity is an unmet need in cancer therapy. MYC/MYCN is dependent on interactions with its partner protein MAX to assert its function. Using a cell-based MYC:MAX interaction screen we recently identified several potent small molecule MYC:MAX inhibitors including MYCMI-6 and MYCMI-7 (Castell et al., 2018).

Aims

Our long-term goal is to develop compounds that specifically and selectively inhibit MYC:MAX interactions in vivo and that can be of clinical use for cancer therapy for patients with MYC-dependent tumors such as neuroblastoma. The specific aim for this project is to characterize and validate the MYC:MAX inhibitor MYCMI-7 with respect to potency and selectivity in vitro, in cells and in vivo.

Methods/Materials

To assay MYC:MAX and MYCN:MAX interactions, Gaussia Luciferase (GLuc) protein fragment complementation assay (PCA), in situ proximity ligation assay (isPLA), co-immunoprecipitation, microscale thermophoresis (MST), FRET and surface plasmon resonance (SPR) assays were used. MST and SPR was also used to study direct binding of MYCMI-7 to recombinant MYC. The effect of MYCMI-7 on MYC target gene expression was measured by RT-PCR, microarray, RNA-seq and chromatin immunoprecipitation (ChIP). Cell proliferation/viability was measured by WST-1, resazurin, colony assays, TUNEL and annexin V staining, flow cytometry and other assays. Expression of proteins involved in these processes were studied by immunoblot analysis. The efficacy of MYCMI-7 as anti-tumor agents was further evaluated using a mouse xenograft model of MYCN-amplified neuroblastoma. Tumor volume, tumor cell proliferation (Ki67), apoptosis (TUNEL), MYC:MAX interaction (isPLA) and other markers were measured in tumor tissue.

Results

Based on our previous identification of MYCMI-7 (Castell et al., 2018), we here characterized MYCMI-7 in more detail. We show that MYCMI-7 binds to recombinant MYC and efficiently inhibits MYC:MAX and MYCN:MAX protein interactions both in cells and in vitro. In addition, MYCMI-7 treatment results in downregulation of the steady state levels of the MYC and MYCN proteins. Further, MYCMI-7 interfered MYC:MAX interaction with its target genes and efficiently downregulated expression of these genes. We show that MYCMI-7 is a potent inducer of apoptosis, affecting growth of a variety of different tumor cell lines, including neuroblastoma, glioblastoma, Burkitt's lymphoma, acute lymphoblastic leukemia, breast and lung cancer and a number of other epithelial tumors in a MYC-dependent manner, all at low micromolar concentrations. Importantly, normal cells become G_1 arrested without signs of apoptosis upon MYCMI-7 treatment. Finally, treatment with MYCMI-7 in vivo using a mouse model of MYCN-amplified neuroblastoma inhibited tumor growth and prolonged survival of the mice.

Summary/Conclusions

MYCMI-7 can potentially contribute to development of anti-MYC drugs for treatment of cancer.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 477

Germline cancer predisposition gene mutations in sporadic neuroblastoma patients are inherited

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Only 1-2% of neuroblastoma cases present with a family history of the disease. The vast majority of neuroblastomas appear to arise sporadically. Through a genome-wide association study (GWAS) of sporadic neuroblastoma, we have identified over two dozen loci with common variants associated with susceptibility and tumor aggressiveness. More recently, sequencing studies by us and others have estimated 8-10% of sporadic neuroblastoma cases harbor pathogenic (P) or likely pathogenic (LP) germline variants in known cancer predisposition genes (CPGs). However, the heritability and clinical significance of P/LP variants remains unknown due to lack of parental DNA.

Aims

(1) Identify and assess heritability of rare pathogenic germline variants in sporadic neuroblastoma triads and dyads; and (2) Perform integrative tumor-normal analyses to elucidate functional relevance of genetic risk factors.

Methods/Materials

Through the Gabriella Miller Kids First (GMKF) program, we performed whole genome sequencing (WGS) at 50x on robustly annotated patient-parent triads (n=493) and dyads (n=100) along with matched tumor DNA (n=366). In addition, total RNA-sequencing (n=228) and exome sequencing (100x) on matched tumor DNA (n=366) was generated to assess relevance to tumorigenesis. Rare variants (allele frequency < 0.01% in ExAC excluding TCGA) in a predefined set of CPGs (n=198) were extensively annotated and pathogenicity was assessed using ClinVar, the American College of Medical Genetics (ACMG) guidelines as implemented in InterVar, and

manual review. Heritability was assessed using family-based variant calling in n=493 trios. Aggregate gene and pathway-based enrichment testing of P/LP variants was performed using Fisher's Exact test against control cohorts (1000 Genomes, gnomAD, and others). Tumor DNA sequencing was analyzed for somatic SNV, indels, structural variants (SVs), copy number, and mutational signatures to assess "second hits" and functional relevance of germline variants. RNA sequencing data was used to assess preferential allelic imbalance/mono-allelic expression, expression quantitative trait loci (eQTL), and gene fusions.

Results

We observed 66 P/LP germline variants in CPGs involving 65 out of 653 (11.5%) neuroblastoma probands. Trio data was available for 61 of these probands, and in all cases confirmed the P/LP variants were inherited (53% paternal, 47% maternal). All P/LP variants were also observed in the matched tumor DNA. We observed one canonical *ALK* mutation (R1275Q), but no *PHOX2B* mutations. Three *BARD1* loss of function variants were detected. Multiple CPGs showed enrichment of P/LP variants, including *ERCC2* ($p=2.0x10^{-4}$; odds ratio: 50.19, 95% C.I. 2.77-909.11), *ATM* ($1.1x10^{-3}$, odds ratio: 40.99, 95% C.I. 2.20-762.55), *FANCA* ($1.1x10^{-3}$; odds ratio: 40.99, 95% C.I. 2.20-762.55), *GBA* (p=0.006; odds ratio: 31.83, 95% CI. 1.64-617.08), *COL7A1* (p=0.006; odds ratio: 31.83, 95% CI. 1.64-617.08) and *BARD1* (p=0.006; odds ratio: 31.83, 95% CI. 1.64-617.08).

Summary/Conclusions

Approximately 11.5% of sporadic neuroblastoma probands harbor P/LP germline variants in CPGs. In this case series, all P/LP variants were inherited. Work is ongoing to further incorporate tumor sequencing data, extend these efforts beyond CPGs, and investigate the non-coding genome.

Chapel Hill

Rare pathogenic variants in cancer predisposition genes detected in the germline of sporadic neuroblastoma cases are inherited

Type Basic

Presentation Preference Oral presentation

Abstract nr. 478

MYCMI-7 - a small MYC-binding compound that inhibits MYC:MAX interaction and tumor cell growth in a MYC-dependent manner

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Topic Targeted therapy: ALK and other targets in personalized medicine

Background/Introduction

Deregulated expression of the MYC family oncogenes/transcription factors MYC and MYCN occurs in many types of adult and childhood tumors including neuroblastoma, and is in the latter case strongly associated with aggressive tumor development, resistance to therapy and poor prognosis. In mouse tumor models, inactivation of MYC often leads to tumor regression with well-tolerated side effects, suggesting that MYC is a potential and suitable target for anti-cancer therapy. Unfortunately, no specific MYC-targeting drugs are available clinic today. Thus, developing inhibitors the cripple MYC activity is an unmet need in cancer therapy. MYC/MYCN is dependent on interactions with its partner protein MAX to assert its function. Using a cell-based MYC:MAX interaction screen we recently identified several potent small molecule MYC:MAX inhibitors including MYCMI-6 and MYCMI-7 (Castell et al., 2018).

Aims

Our long-term goal is to develop compounds that specifically and selectively inhibit MYC:MAX interactions in vivo and that can be of clinical use for cancer therapy for patients with MYC-dependent tumors such as neuroblastoma. The specific aim for this project is to characterize and validate the MYC:MAX inhibitor MYCMI-7 with respect to potency and selectivity in vitro, in cells and in vivo.

Methods/Materials

To assay MYC:MAX and MYCN:MAX interactions, Gaussia Luciferase (GLuc) protein fragment

complementation assay (PCA), in situ proximity ligation assay (isPLA), co-immunoprecipitation, microscale thermophoresis (MST), FRET and surface plasmon resonance (SPR) assays were used. MST and SPR was also used to study direct binding of MYCMI-7 to recombinant MYC. The effect of MYCMI-7 on MYC target gene expression was measured by RT-PCR, microarray, RNA-seq and chromatin immunoprecipitation (ChIP). Cell proliferation/viability was measured by WST-1, resazurin, colony assays, TUNEL and annexin V staining, flow cytometry and other assays. Expression of proteins involved in these processes were studied by immunoblot analysis. The efficacy of MYCMI-7 as anti-tumor agents was further evaluated using a mouse xenograft model of MYCN-amplified neuroblastoma. Tumor volume, tumor cell proliferation (Ki67), apoptosis (TUNEL), MYC:MAX interaction (isPLA) and other markers were measured in tumor tissue.

Results

Based on our previous identification of MYCMI-7 (Castell et al., 2018), we here characterized MYCMI-7 in more detail. We show that MYCMI-7 binds to recombinant MYC and efficiently inhibits MYC:MAX and MYCN:MAX protein interactions both in cells and in vitro. In addition, MYCMI-7 treatment results in downregulation of the steady state levels of the MYC and MYCN proteins. Further, MYCMI-7 interfered MYC:MAX interaction with its target genes and efficiently downregulated expression of these genes. We show that MYCMI-7 is a potent inducer of apoptosis, affecting growth of a variety of different tumor cell lines, including neuroblastoma, glioblastoma, Burkitt’s lymphoma, acute lymphoblastic leukemia, breast and lung cancer and a number of other epithelial tumors in a MYC-dependent manner, all at low micromolar concentrations. Importantly, normal cells become G1 arrested without signs of apoptosis upon MYCMI-7 treatment. Finally, treatment with MYCMI-7 in vivo using a mouse model of MYCN-amplified neuroblastoma inhibited tumor growth and prolonged survival of the mice.

Summary/Conclusions

MYCMI-7 can potentially contribute to development of anti-MYC drugs for treatment of cancer.

Type Basic

Presentation Preference Traditional poster

Abstract nr. 479

Outcomes of children with ganglioneuroblastoma and ganglioneuroma, a single centre case series

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

Peripheral neuroblastic tumors are the most common extracranial solid tumors in childhood. These tumors are a heterogeneous group with varying degrees of differentiation and malignant potential. They are divided into four broad categories; neuroblastoma (NBL), ganglioneuroma (GN), ganglioneuroblastoma intermixed (GNBi) and ganglioneuroblastoma nodular (GNBn). Historically, GN and GNBi are more benign, GNBn can be divided into favourable and unfavourable based on characteristics such as grade of differentiation, mitosis-karyorrhexis index (MKI), diploidy and age of child, while NBL is considered malignant. Genomic characterisation with *MYC-N* amplification and segmental chromosomal alterations (SCA) (1p loss, 11q loss, 17q gain) has been added to risk stratification in infant NBL and GNBn but is not widely used for GN or GNBi.

Aims

We report a retrospective case review, which highlights patients whose clinical course does not correlate with the historically good prognosis associated with GN, GNBi and GNBn. We also describe the genomic alterations and treatment received by this group of patients.

Methods/Materials

The database of histopathology reports from 2000 to 2018 issued at Great Ormond Street Hospital were searched for the terms ganglioneuroma and ganglioneuroblastoma. A total of 314 histopathology reports were reviewed. 26 reports containing other final diagnoses were excluded. The remaining 288 reports from 153 patients were included in the analysis.

Results

From the 153 patients reviewed, the final treatment diagnosis was GN for 25% (n=38), GNBi for 31% (n=47), GNBn for 3% (n=5) and NBL with evidence of differentiation for 41% (n=63). Clinical

information was available for 72 patients with ganglioneuroma and ganglioneuroblastoma. Of these 72 patients, all except 3 patients had localised disease, 2 with GNBi and 1 with GNBn. 45 patients had genomic information available for analysis. MYC-N amplification was present in 1 case only, a child with GNBn. SCA were found in 2 patients with GN, 11 patients with GNBi and 2 with GNBn. Only 1 patient with GN received chemotherapy and all were well and alive at last clinical follow-up. All 4 children with GNBn with clinical information available received chemotherapy. The remaining 3 children with GNBn are well at time of last review. 11 patients with GNBi were treated systemically, including single agent treatment with isotretinoin. 6 children with GNBn showed evidence of disease progression during their treatment or follow-up period, 5 of who had SCA present. There were a total of 3 deaths in our case series, 2 related to disease progression. 1 child treated for Stage M GBNn with no MYC-N amplification and unknown SCA, had slowly progressive disease, was treated with several lines of therapy but eventually died from disease progression seven years after initial diagnosis. 1 child had localised progressive GNBi with 1p loss.

Summary/Conclusions

Overall, children with ganglioneuroma and ganglioneuroblastoma have a favourable outcome. However children diagnosed with GNBi who have SCA can show disease progression and we describe one child who died as a result of their progressive disease. We also describe a single case of a child with GNBn with slowly progressive disease over seven years, unresponsive to several treatments.

Type Clinical

Presentation Preference Oral presentation

Abstract nr. 480

Modelling the First Step of Metastasis; 3D Culture Assays of Neuroblastoma Invasion.

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

Neuroblastoma (NB) is a highly heterogenous paediatric malignancy that accounts for approximately 7% of all childhood cancers in the United States. However, the same disease is responsible for about 15% of paediatric oncology deaths (1). This disparity is due to the fact that half of the patients have metastatic spread at the time of diagnosis, which represents the most aggressive form of the disease. For this group of high-risk patients, treatment options remain poor resulting in a dismal 5-year survival rate of approximately 50% (2). Therefore, elucidating the molecular basis of the metastatic process may guide novel strategies for drug discovery and therapy.

Aims

The aims of this study were to 1.) model the first step of metastasis by imaging neuroblastoma cell lines and organoids in real-time as they invade 3D hydrogels representing the *in vivo* extracellular matrix 2.) to characterise the different migration strategies employed by NB cells in response to local ECM composition.

Methods/Materials

NB cell lines as well as organoids isolated from patient-derived xenografts (*MYCN*and non-*MYCN* amplified) were cultured in hydrogels of three different compositions. Collagen I was used to model the stromal ECM and Matrigel was used to model the basement membrane. We also cultured in a hydrogel composed of 50% collagen I and 50% Matrigel. We used 3D invasion assays, as described by our collaborator Professor Ewald (3), coupled with DIC time-lapse microscopy and confocal imaging to directly observe the migration strategies employed by NB during invasion of the local microenvironment.

Results

When cultured in 3D hydrogels the migration strategies employed by NB cell lines is not influenced by the local microenvironment. In contrast, NB organoids isolated from patient-derived xenografts (PDXs) employ distinct migration strategies that are dependent on the ECM. In collagen I hydrogels, NB organoids predominantly form multicellular spheroid colonies that do not invade the ECM (C1-C4). However, in hydrogels containing Matrigel, organoids invade the ECM using four distinct migration strategies that we have termed elongated (B1, B4), collective mesenchymal (A2, B2), protrusive (A1), and neuronal (A3). We have also identified a non-invasive bi-layered structure containing a lumen, termed a cyst (A4).

Summary/Conclusions

Our results show that, *in vitro*, NB organoids isolated from PDXs maintain the heterogenous characteristics of native neuroblastomas, regardless of their *MYCN*status. We have demonstrated that the molecular properties of the ECM microenvironment dictate invasion strategies in NB organoids but not cell lines. Furthermore, we have validated the pairing of 3D hydrogel culture and invasion assay as a useful tool for the study of NB migration, invasion and dissemination.

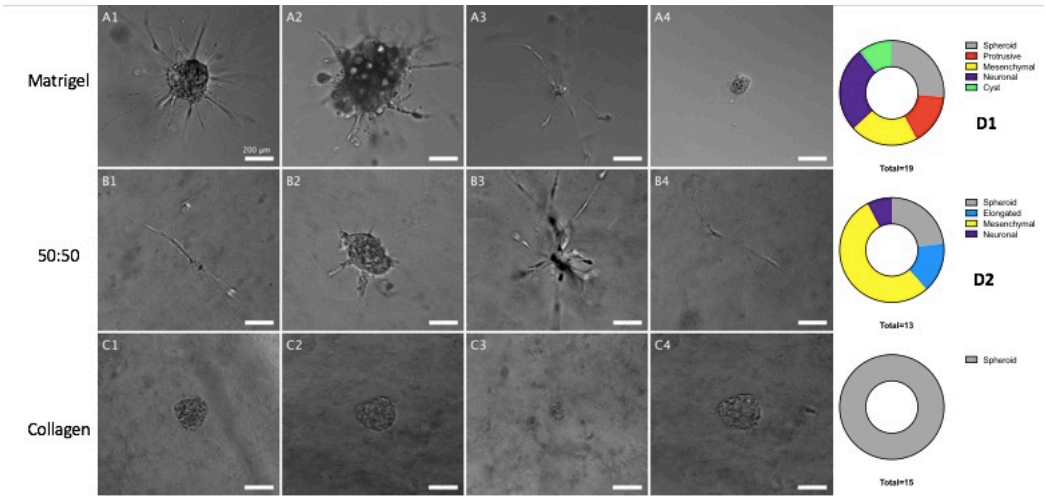

Figure.1 Local microenvironment dictates invasion strategies of NB organoids. Representative images of organoids isolated from Felix-PDX in Matrigel (A1-A4), 50:50 (B1-B4) and collagen I (C1-C4) hydrogels. D1-D3 Indicates proportion of organoids employing specific invasion strategies in respective matrices.

Type Basic

Presentation Preference Traditional poster

Abstract nr. 481

Novel compounds target calcium pathways in drug-resistant Neuroblastoma

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Topic Other

Background/Introduction

Neuroblastoma (NB) is the most common pediatric extra-cranial solid tumor in infants. Up to 60% of high-risk NB patients are either refractory to treatment or experience relapse after treatment, and the remaining tumor cells are resistant to therapy. This is in part due to specific Ca^{2+} defects in NB that inhibit apoptosis. The biggest challenge to treating relapsed high-risk NB is the propensity of NB cells to alter signaling pathways to promote survival in response to cytotoxic stimuli, which results in relapse. Unfortunately, there are currently no effective treatments for relapsed high-risk NB. Our overall goal is to manipulate key pathways to effectively treat high-risk NB.

Aims

The main goals of this project were to identify novel compounds that reduce the viability of drug-resistant NB, and determine the mechanism of action of these compounds.

Methods/Materials

The sulforhodamine B assay was used to screen a library of novel compounds for anti-cancer effects in drug-resistant NB cell lines. Western blot analysis was used to determine if the compounds that exhibited anti-cancer effects inhibited cell cycle progression and/or induced apoptosis. Laser scanning confocal microscopy was used in combination with genetically encoded calcium indicators targeted to the cytoplasm, ER or mitochondria to identify calcium signaling pathways that were affected by the compounds. Electrophysiological patch-clamp measurements were recorded in the whole-cell configuration to determine changes in ion channel activity.

Results

Two novel compounds were identified, compound 248 and compound 249, that exhibited potent anti-cancer effects in drug-resistant NB cells. The compounds inhibited cell cycle progression and induced apoptosis through a mechanism that involved activation of ER-mitochondrial calcium signaling, mitochondrial calcium overload, opening of the mitochondrial permeability transition

pore, and calcium-dependent apoptosis. In addition to regulating calcium stores, the compounds also activated TRP-like channels.

Summary/Conclusions

This study identified two novel compounds that may be effective for treating refractory and relapsed NB. In addition, the study identified a potential treatment strategy that may be targeted for the treatment of refractory and relapsed NB. The knowledge gained from this study may be used to develop more effective treatments for NB.

Type Basic

Presentation Preference Traditional poster

Abstract nr. 482

Difluoromethylornithine Modulates Sphingolipid Metabolism in Human Neuroblastoma Cells

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Topic Other

Background/Introduction

Ornithine decarboxylase (ODC) is the rate limiting enzyme in biosynthesis of polyamines. Polyamines have important role in cell growth, differentiation and apoptosis. Difluoromethylornithine (DFMO) is an irreversible inhibitor of ODC and is current trials for neuroblastoma. Sphingolipids have a crucial role in determining cell fate. It is well known that many chemotherapeutic agents exert their cytotoxic effects via altering the activity of t enzymes in SPL metabolism. Ceramide is the central building block in sphingolipid metabolism and it is well known for its role in cell growth arrest and apoptosis.

Aims

In this study we investigated the effects of DFMO on endogenous sphingolipid and sphingolipid enzymes in SMS-KCNR human neuroblastoma cells.

Methods/Materials

SMS-KCNR cells were treated with 50mM DFMO for 48 Hours. Measurement of endogenous sphingolipids was performed by LC/MS. Sphingolipid pathway enzyme activities were also determined utilizing *in vitro* assays

Results

Ceramide levels were increased ~9.2 fold and dihdyroceramide levels were increased ~5.9 fold. Analysis of individual ceramide species showed increases in all species, with the greatest increases in C24:1 ceramides. All dihydroceramide species showed increases in all species, with the greatest increases in dhC16 ceramides. Since ceramide can be generated by several pathways, including the *de novo* pathway, salvage pathway, or by sphingomyelin (SM) hydrolysis, each enzyme in sphingolipid metabolism was individually investigated. Our assays found that for ceramide generation, Neutral sphingomyelinase 2 (NSMase 2) activity was increased 2.5 fold; while Neutral ceramidase activity was inhibited by 40%. In terms of dihydroceramide metabolism,

DFMO inhibited the activity of dihydroceramide desaturase (DES1).

Summary/Conclusions

These data show for the first time new insight into mechanism of DFMO-induced cytotoxicity in neuroblastoma which may be modulated by alteration in SPL metabolism. Further investigation is required in order to determine the downstream targets of ceramide and the role of increased dihydroceramide in DFMO treatment.

Type Basic

Presentation Preference Traditional poster

Abstract nr. 483

In vitro cytotoxicity and in vivo evaluation of localization and immune sensitivity of human neuroblastoma in xenografts

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

Neuroblastoma (NB) is the most commonly occurring extra-cranial solid tumor in children and accounts for ~10% of all childhood cancers. Half of NB cases occur in children under two years of age. NB is thought to originate from neural crest precursor cells of the developing sympathetic nervous system, with primary tumors localizing in the abdomen, adrenal gland or lumbar ganglia; however its metastatic spread includes such diverse tissues as the lymph nodes, bone, bone marrow, liver and skin. The high-risk metastatic form that accounts for ~50% of NB has survival outcomes of less than 30% with conventional therapy. Due to the heterogenous genetic landscape inherent to NB, a major focus for therapy development is aimed at harnessing the anti-tumour capabilities of the immune system, in particular that mediated by natural killer (NK) cells.

Aims

Approaches to evaluate killing of target cells in vitro include flow-based and chromium-based assays. Here we describe a simple and reproducible system that facilitates both in vitro and in vivo monitoring of NB cell killing by NK cells based on dual color click beetle red (CBR) and green (CBG) luciferase bioluminescence for separate labeling of target and effector cells. In vitro, the system is convenient for longitudinal monitoring and optimization of target cell killing, while in vivo there is the potential for tracking of activities within specific target tissues.

Methods/Materials

A panel of NB cell lines (NB1643, NB1691, NB EBC1, NB SD, SH-SY5Y, SK-N-AS, CHLA136, CHLA79, CHLA90, and IMR32) and NK92 were tagged with CBG and/or CBR using lentivirus. The NK target K562 cell line was also tagged to provide a benchmark evaluation of NB killing. NK92-mediated killing was evaluated in vitro and the cell lines were engrafted intravenously (IV) into NSG mice to create an in vivo model of NB homing and metastasis for evaluating immune-based therapy.

Results

Our results indicate that liver is the most popular homing location when NB cells injected IV; tumors for all NB cell lines formed in the liver. Tumors formed by NB16, CHLA79 and NB1691 initiated both in the liver and BM, while IMR32, NBEBc1, and NBSD BM tumors appeared after full establishment of liver tumors. We never observed CHLA90, SK-N-AS and SH-SY5Y tumors develop or metastasis to BM. Tumor development to humane end point varies among different NB cell lines. SK-N-AS and CHLA136 reach humane endpoint at day 25 and 153 respectably. Bioluminescent and flow cytometry based and cytotoxicity assay results correlate.

Summary/Conclusions

This approach could reveal the impact of modulation of patient NK cells on targeting tumor niches and increasing cytolytic activity. This will establish a more stringent pre-clinical testing platform for the development of immune therapies for NB. The lucifrase-tagged NB cell line panel allows for collective evaluation of NB tracking and tracking in real-time both in vitro and in vivo. This also offers a robust platform to evaluate immune cell killing of localized NB cells in vivo and could accelerate pre-clinical immunotherapy development for this disease.

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Type Basic

Presentation Preference Traditional poster

Abstract nr. 484

Low dose induction-differentiation therapy for Neuroblastoma in children

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Topic Neuroblastoma as developmental disease

Background/Introduction

Neuroblastoma (NB) is the most common pediatric solid tumor that originates in the neural crestthe international therapeutic schedule for neuroblastoma is mainly the maximum tolerance dose chemotherapy + surgery + stem cell transplantation + immunotherapy and other methods, but the maximum tolerance dose chemotherapy makes the hemogram of children worse, the immune system became collapse, and ultimately leads to irreversible consequences. However, low-dose chemotherapy, high-frequency, long-term chemotherapy can not only kill tumor cells, but also effectively protect the immune system, activate the immune response of the body itself, effectively inhibit the growth of tumor. On the other hand, it can also induce tumor differentiation to a benign direction. Finally, these children received surgical resection, or for those patients could not receive a surgery, they showed a balance between the body and tumor, and coexistence with it.

Aims

The aim of this study is to report the efficacy of low dose induction-differentiation chemotherapy in the treatment for neuroblastoma (NB).

Methods/Materials

From 1992 to 2018, 232 NB patients enrolled into this study. Patients were separated into two groups, the initial treatment group: never received the chemotherapy; treated group: relapse patients or received chemotherapy in other hospitals. All the 232 cases were treated by low dose induction-differentiation therapy and traditional Chinese medicine, or low dose chemo+1st initial surgery (mass only partially resected) +low dose chemo continuously+2nd surgery.

Results

Among the 232 NB patients, 75 patients were completely release (CR), 52 patients were partially release (PR), 36 patients were stable disease (SD), 8 patients were progressive disease (PD), 52 patients were dead, and 9 patients loss of follow-up. K-M survival analysis showed that the 5-year survival rate of the initial treatment group was higher than the treated group (68.4% *vs* 43.5%, p=0.025). And 13 patients showed amazing therapeutic outcome, there were 10 patients who were treated by low dose induction-differentiation chemotherapy and traditional Chinese medicine only, the tumor disappeared completely. In these 10 cases, five were Stage IVs, four were Stage IV and one was Stage III. And the three stage III NB patients were treated with low dose induction-differentiation chemotherapy + surgery (only partial resection) after surgery low dose chemotherapy continued, finally, a second surgery was performed, and the mass was completely removed, each tumor mass was transformed into benign GN confirmed by pathology.

Summary/Conclusions

The low dose induction-differentiation therapy is an efficient method for the treatment of NB in children, and it can improve prognosis of the NB children.

Type Clinical

Presentation Preference Traditional poster

Abstract nr. 485

Single cell analysis for circulating neuroblastoma cells

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Topic A deeper look into neuroblastoma tumor heterogeneity and clinical behaviour

Background/Introduction

Circulating tumor cell(CTC) has improved the study of cancer disease as it represents a noninvasive biopsy that can be used as prognostic and prediction biomarkers as well as for future molecular diagnosis as an accurate selection of molecular targets and effective drugs.

Aims

As tumor heterogeneity and defference of malignant grade exist in neuroblastoma (NBL), we aimed to evaluate genetic and phenotypic profiles, as well as lipido-metabolomic molecular profile at the single cell level of CTC for better understanding the heterogeneity and progression of NBL.

Methods/Materials

For attempt of CTC analysis in NBL patients, we have isolated single-CTCs and then analyzed genomic aberrations and expressions using next-generation sequencing after whole genome/transcriptome amplifications (WGA/WTA). And we have tried direct trapping of a single cell within a nanospray tip followed by super-sonication after the addition of ionization solvent for metabolomics analysis (Fig. 1 and 2).

Results

In the NBL-CTCs, the average amount of amplified DNA was 19.0 μg, and the percentage of reads mapped to any targeted region relative to all reads mapped to the reference was 66.5-98.9%. The concordance rates of CTC and tumor mutations were 49-80%. The genomic expression analysis in CTC showed the activation of cancer metastatic, neurogenic, stem cell signaling pathway genes. Metabolomic analysis detected catechol amine metabolites, which are specific to NBL, and drugs included in the patient's course of therapy in addition to vital molecules such as amino acids.

Summary/Conclusions

This "direct single-cell analysis method" seems to be useful for direct and wide range genomic and molecular marker detection in NBL-CTC, for evaluating heterogeneity of NBL tumor at diagnosis as well as during treatments. This method will be applicable for selection of effective drugs and evaluation of treatment efficacy in future.

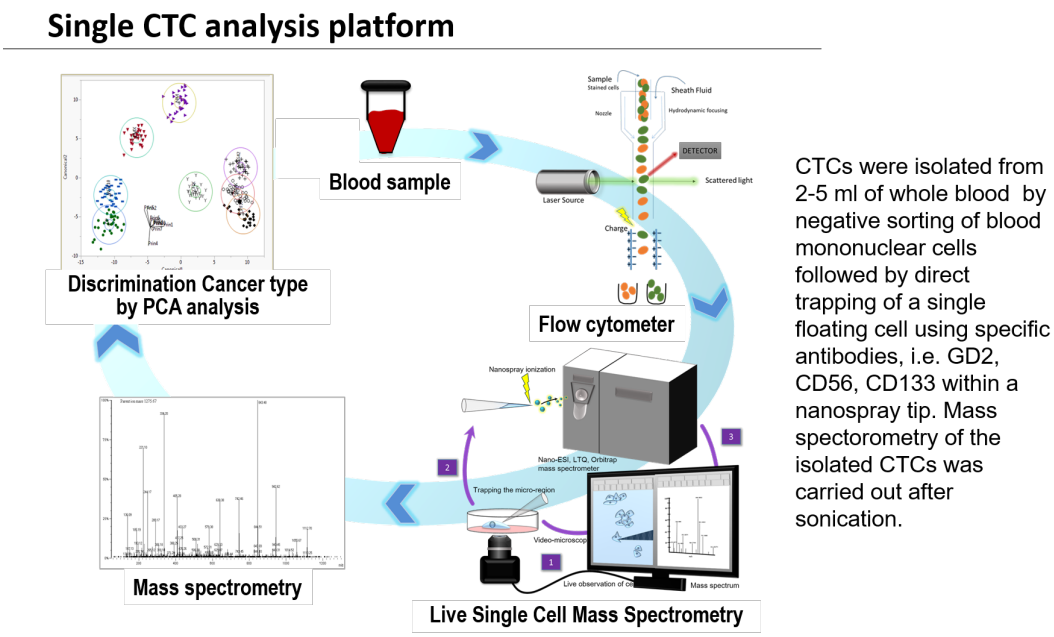

Fig. 1. Single CTC analysis system

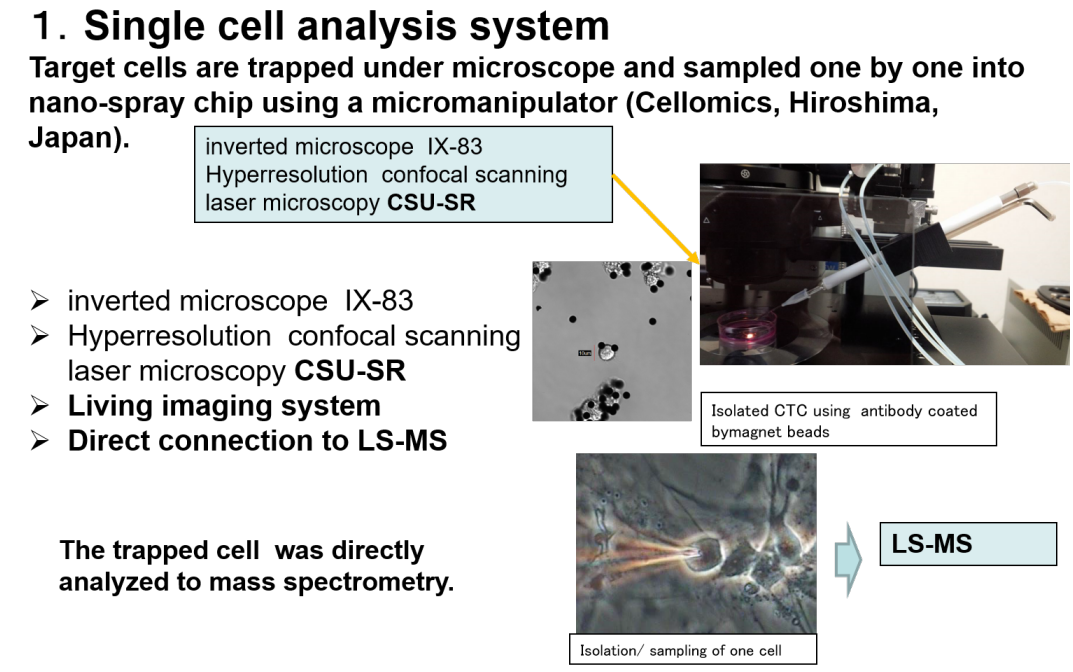

Fig. 2. Single CTC analysis platform

Single CTC analysis platform

Type Translational

Presentation Preference Oral presentation

Abstract nr. 486

Anti-GD2 antibody internalization by neuroblastoma as a mechanism of resistance to immunotherapy

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Topic Progress and hurdles in immune therapy of neuroblastoma

Background/Introduction

Neuroblastoma, a sympathetic nervous system cancer, accounts for a disproportionate number of deaths among childhood malignancies despite intensive multimodal therapy including the relatively recent introduction of antibody targeting disialoganglioside (GD2), a common neuroblastoma antigen. The mechanisms underlying therapeutic resistance in some patients, specifically to antibody-based therapy, remains poorly understood.

Aims

We hypothesized that differential internalization of anti-GD2 antibody by tumor cells upon binding to surface GD2 may act as a mechanism of escape by decreasing tumor sensitivity to antibody-dependent cellular cytotoxicity (ADCC). We aimed to measure antibody internalization differences across 22 human neuroblastoma cell lines and investigate how it relates to GD2 expression and ADCC sensitivity.

Methods/Materials

Antibody internalization was measured using IncucyteS3 live cell imaging and anti-GD2 antibody (14G2a) conjugated to a pH-sensitive red fluorescent dye (pHrodo). GD2 surface expression was quantified by flow cytometry and BD QuantiBrite Beads. Neutrophil-mediated ADCC was measured *in vitro* via one of two methods - DIMSCAN and calcein-labeled tumor cells or IncucyteS3 and GFP-expressing tumor cells. Agents targeting endocytosis (chlorpromazine, cytochalasin-D, methyl-beta-cyclodextrin/MβCD, Pitstop2, and EIPA) were then used to assess inhibition of internalization and subsequently sensitization to ADCC in the highest internalizing cell line, LAN1.

Results

Internalization of 14G2a-pHrodo over 24 hours varied significantly across cell lines (AUC range: 50-529) with LAN1 demonstrating the highest internalization AUC (529 +/- 40). Internalization rate was not correlated with GD2 surface expression (r=0.15). However, a moderate inverse correlation was found between antibody internalization and sensitivity to neutrophil-mediated ADCC in the 10 cell lines tested (r=-0.57). Antibody internalization by LAN1 was significantly inhibited by treatment with EIPA, chlorpromazine, and MβCD, all without significant change in surface GD2 expression. Treatment with MβCD (2mM), additionally, was able to sensitize LAN1 to neutrophil-mediated ADCC (52% cytotoxicity) compared to untreated LAN1 (17% cytotoxicity).

Summary/Conclusions

Anti-GD2 antibody internalization varies widely across human neuroblastoma cell lines, is inversely correlated with ADCC sensitivity, and, in a high internalizing cell line, can be effectively modulated by inhibitors of pinocytosis. The finding that membrane cholesterol depletion with MβCD sensitized LAN1 to neutrophil-ADCC suggests that membrane organization of GD2, specifically in relation to cholesterol-rich domains, may play a more significant role in antibody internalization than specific endocytosis processes. Elucidating further the mechanisms underlying differential internalization across cell lines could lead to the development of agents targeting internalization and ultimately augmenting tumor sensitivity to antibody-based immunotherapy.

Type Basic

Presentation Preference Oral presentation

Abstract nr. 487

Activating Transcription Factor 5 (ATF5) Induces Anoikis Resistance by regulation of BCL2-modifying factor (BMF)

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Resistance to anoikis is a pre-requisite step in metastasis and allows tumor cells to survive in systemic circulation and distant organs. We have previously demonstrated that activating transcription factor 5 (ATF5), a member of the ATF/CREB family, is highly expressed in Stage 4 *MYCN*-amplified tumors and that ATF5 acts as a promoter of neuroblastoma metastasis by inducing resistance to anoikis.

Aims

The aim of our current study was to determine the mechanism by which ATF5 promotes anoikis resistance.

Methods/Materials

To determine the molecular mechanism of induction of anoikis, we analyzed the expression of anti-apoptotic BCL-2 family members BCL-2, BCL-XL, MCL-1 and pro-apoptotic BMF, BIM, BAD, BAX, PUMA protein in adherent and suspension BE(2)-C and SK-N-DZ neuroblastoma cell lines, as well as i*n vivo* after intracardiac injection.

Results

Pro-apoptotic BMF expression was upregulated following ATF5 depletion in BE(2)-C and SK-N-DZ under suspension conditions but not in adherent cells.Next, we asked whether ATF5 silencing induces BMF in circulating tumor cell (CTC) *in vivo*. Circulatory BE(2)-C cells with ATF5-depleted were isolated from blood of mice 12 hr after intracardiac injection exhibited an induction of *BMF* mRNA. Ectopic expression of BMF markedly reduced anchorage-independent viabilities of BE(2)-C (65% decrease, $P< 0.001$), and SK-N-DZ (60% decrease, $P<0.01$), as well increasing anoikis in BE(2)-C (100%; $P<0.001$) and SK-N-DZ (80%, $P<0.01$). BMF knockdown by two different siRNAs, on the contrary, increased detached BE(2)-C and SK-N-DZ viabilities by an average of 30% and 25% and reduced anoikis by 30% and 20%. We further addressed if induction of anoikis following

ATF5 depletion was mediated by endogenous BMF function. We treated detached BE(2)-C and SK-N-DZ cells, expressing shATF5-1 or shATF5-2, with siBMF-1 or siBMF-2 and siGFP (control). *BMF* knockdown, resulted in the significant inhibition of anoikis induction in ATF5-silenced cells. We next investigated how ATF5 suppresses BMF in suspension settings. BMF has been shown to be a target of transcription factor FOXO3. ATF5 knockdown increased FOXO3 levels in BE(2)-C and SK-N-DZ suspension cells. Additionally, FOXO3 silencing, by two different siRNAs, abated the induction of BMF following ATF5 depletion and reduced ATF5 silencing-induced anoikis. Next, we targeted ATF5 with a dominant negative peptide inhibitor CP-d/n-ATF5 which is a novel cell penetrating (CP) peptidomimetic drug consisting of a cell penetrating domain fused with a dominant negative ATF5 (CP-d/n-ATF5) construct. CP-d/n-ATF5 reduced anchorage-independent viabilities and increased anoikis of BE(2)-C and SK-N-DZ. CP-d/n-ATF5 treatment induced BMF only in suspension culture. Lastly, we examined the effect of CP-d/n-ATF5 *in vivo*. BE(2)-C or SK-N-DZ cells were intracardically injected into nude mice and treated with CP-d/n-ATF5 or vehicle for 12hr. CP-d/n-ATF5 treatment reduced number of BE(2)-C ($P<0.001$) and SK-N-DZ ($P<0.01$) CTCs and increase anoikis in BE(2)C and 2-fold SK-N-DZ CTCs.

Summary/Conclusions

Our studies demonstrate that ATF5 induces anoikis resistance of tumor cells *in vitro*and of CTC *in vivo*, increasing their survival thereby facilitating metastasis. Mechanistically, ATF5 induces anoikis resistance by suppressing BMF. Therapeutically targeting ATF5, using a dominant negative peptide inhibitor, CP-d/n-ATF5, increases anoikis sensitivity by increasing BMF. CP-d/n-ATF5 has the potential to be an anti-metastatic therapy and thus improve outcomes for metastatic neuroblastoma.

Type Basic

Presentation Preference Rapid Fire Poster Presentation

Abstract nr. 488

Evaluation of ATR inhibitors in preclinical models of high-risk neuroblastoma

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Topic Progress in treatment and challenges for preclinical studies

Background/Introduction

ATRX gene mutations are found in older high-risk neuroblastoma (NB) patients and correlate with poor outcome. ATRX is a chromatin remodeling protein important for deposition of histone variant H3.3 at telomeres and G-quadruplex structures in the DNA that can impede DNA replication. Adult cancer cell lines with ATRX mutations have been shown to be sensitive to ATR inhibitors (ATR_is), but little has been explored in the pediatric setting. ATR is essential for maintenance of telomeres and other functions including repairing damaged DNA. As MYCN amplified NB cells incur replicative stress through DNA damage related to elevated levels of reactive oxygen species, targeting ATR in that setting may be beneficial as well.

Aims

To evaluate the efficacy of ATR inhibitors (VX-970 and AZD6738) as single agents and in combination with liposomal irinotecan (nal-IRI) in preclinical models of high risk neuroblastoma.

Methods/Materials

Orthotopic xenografts (O-PDXs) were created by injecting luciferase labeled NB cells into the para-adrenal space of nude mice. Pharmacokinetic studies on NB tumor-bearing mice were performed for all drugs to determine matched human AUC-guided dosing. A total of 68 O-PDXs derived from 2 high-risk NB patients, one with MYCN amplification (SJNBL046_X) and one with ATRX mutation (SJNBL047443_X), were randomized and enrolled into 6 treatment groups: control, nal-IRI, VX-970, AZD6738, VX-970 + nal-IRI, AZD6738 + nal-IRI. The combinations with nal-IRI were chosen for comparison as other studies have shown ATR_is sensitize cancer cells to topoisomerase I inhibitors. Four courses of blinded placebo-controlled therapy were planned on a clinically relevant schedule. Ultrasounds were done to measure tumor volume with each course, and bioluminescence was measured weekly to evaluate tumor response. Mice were classified as progressive disease if tumor approached 20% body weight at any point in the study.

Results

Single agent use of VX-970 or AZD6738 did not show a significant survival advantage in either O-PDX tested compared to control mice that did not receive chemo. Mice receiving ATR_is combined with nal-IRI survived longer than mice receiving ATR_is alone in both O-PDXs; however there was not a significant difference overall compared to mice receiving single agent nal-IRI. The average survival days for all groups are shown in the table below.

Summary/Conclusions

Comprehensive preclinical testing using multiple O-PDX models of NB that represent the clinical and molecular spectrum of high-risk disease is feasible and warranted to explore potential novel agents. While neither ATR_i tested showed significant survival advantage in the O-PDXs tested, this study serves as a benchmark for future combination testing. Other preclinical trials using alternative agents to target these pathways such as talazoparib (PARP inhibitor), AZD1390 (ATM inhibitor), CX-5461 (DNA G-quadraplex stabilizer) in combination with ATR_is and other standard NB agents (cyclophosphamide and topotecan) are currently underway and data will be available for presentation at the annual meeting.

	SJNBL046_X (MYCN amplified)	**SJNBL047443_X (ATRX mutation)**
Treatment Group	**Avg. days on study**	**Avg. days on study**
Control	25.3	34.3
nal-IRI	51.2	39.4
VX-970	30.7	32.1
AZD6738	30.8	27.8
VX-970 + nal-IRI	48	41.8
AZD6738 + nal-IRI	64	44.4

Type Translational

Presentation Preference Rapid Fire Poster Presentation

Advances in Neuroblastoma Research (ANR) Meeting
ANR 2020
Amsterdam
11-14
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Abstract nr. 489

Utilizing metabolomic profiling to uncover therapeutic vulnerabilities in neuroblastoma

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Biological heterogeneity of neuroblastoma poses strong therapeutic challenges, with overall survival rates for high-risk patients close to 50% despite multimodal therapy. Molecular risk factors include amplification of the transcription factor *MYCN*, the neuronal form of *MYC*, which is a known master regulator of cellular metabolism.

Aims

Investigate the role of *MYCN* in reprogramming neuroblastoma metabolism.

Methods/Materials

Fresh-frozen human primary neuroblastoma samples (*MYCN* amplified / non-amplified) were provided by the COG biobank. Water soluble metabolites were extracted and analyzed using a high resolution quadrupole-orbitrap mass spectrometer operating in untargeted negative and positive ion mode. Liquid chromatography separation was achieved via hydrophilic interaction chromatography and data was investigated for metabolite pool size differences using the MAVEN and the R software packages. Expression of metabolic enzyme pathways associated with significantly changed metabolites in *MYCN* amplified tumors was performed using R2 on the dataset published by Kocak et al. 2013. The 129X1/SvJ TH-MYCN murine transgenic neuroblastoma model was utilized for functional studies. Mice were catheterized in the jugular vein and ^{13}C metabolite tracers were infused to reach steady state. During experiments mice were freely moving and tissues and serum was analyzed following the above mentioned method. Tumor and inter organ cooperativity in proline biosynthesis as well as energy production was analyzed on whole body level.

Results

Significantly increased pool size was detected for several purine and pyrimidine nucleotide species and the amino acid proline. Gene expression analysis confirmed *MYCN* correlated upregulation of nucleotide biosynthesis, folate 1C-pathway and proline metabolism in primary neuroblastoma tumors. *In vivo* follow up studies utilizing the TH-*MYCN* neuroblastoma model showed a shortage of one-carbon unit building blocks in late stage tumors signified by accumulation of intermediate products of purine biosynthesis. Proline levels in mouse tumors were more than three-fold increased compared to any other mouse organ. *In vivo* stable isotope tracing revealed proline uptake from bloodstream as a primary source of tumor proline and transporters were identified as potential inhibitor targets. The major contribution to intratumoral *de novo* biosynthesis was from circulating glutamine. This amino acid was also a dominant source of ATP production as determined by fractional contribution of citric acid cycle carbons.

Summary/Conclusions

In summary our work provides the first systems level characterization of neuroblastoma metabolism to identify *MYCN* amplification correlated metabolite pool size changes. Our data supports recent preclinical findings on increased antifolate sensitivity of *MYCN* amplified neuroblastoma. Moreover we provide evidence for upregulated proline metabolism as one of the new potential therapeutic targets currently under investigation

Type Basic

Presentation Preference Oral presentation

Abstract nr. 490

Flow Cytometry for Neuroblastoma

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Topic Other

Background/Introduction

Flow cytometry is a technique that can be used to detect neuroblastoma cells. There is a need to be able to monitor minmal residual disease in neuroblastoma and this can be used as adjunct to other tests

Aims

We review our experience with the use of flow cytometry in diagnosis and residual disease monitoring. We looked at the relationship with the use of histomorphology .

Methods/Materials

Specimens collected from patients with high risk neuroblastoma from bone marrow, skull lesion and lymph node. Bone marrow samples were collected in sodium heparin tubes. Other lesions were placed in normal saline following biopsy and sent to the flow cytometry laboratory within an hour. Flow cytometry was performed upon harvesting in most samples. Some were banked and subsequently thawed and checked for viability. Markers used to detect neuroblastoma were GD2, CD56 and CD81. CD45, CD33 were also included to distinguish neuroblastoma cells from those of hematopoietic cells. Bone marrow aspirates and bone marrow biopsies were obtained at the same time. For bone marrow biopsies, neuroblastoma cells were identified based on morphology and immunohistochemical stains for NSE, chromogranin and synaptophysin.

Results

Twenty bone marrow specimens were tested from eight patients. Two patients had diagnostic bone marrow tests due to initial diagnosis of leukemia. The rest of the patients has received treatment previously, two with both chemotherapy and Dinutuximab beta. Five bone marrow specimens were read as negative on flow cytometry but were positive on histology. However the number of cells noted were low levels at two percent or less (rare cells). Three were positive with flow cytometry at llow levels (0.03%, 0.19%, 0.12%). One bone marrow specimen from a patient whose disease progressed after Dinutixumab beta showed tumor cells with partial dim expression of GD2. However we did not have the diagnostic sample for this patient.
Two samples were obtained form patients who had relapsed disease post chemotherapy, with one

having had five cycles of Dinutuximab beta.

Summary/Conclusions

Flow cytometry adds value to measuring disease leve in the bone marrow. This can be used as an adjunct with histology. However, the sensitivity of the test depends on the number of viable cells obtained. Neuroblastoma is known to cause pathcy involvement of the bone marrow which could explain some of the inconsistencies betweent the tests.

Detection of expression of the GD2 antigen by tumor cells may guide in the decision to treat with anti GD2 antibody.

Type Basic

Presentation Preference Traditional poster

Abstract nr. 491

The role of the neurotrophin receptors, TrkA/NTRK1 and TrkB/NTRK2, in modulation of radiation responses in neuroblastoma.

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Topic Neuroblastoma tumour biology and omics: from bench to the clinic

Background/Introduction

Radiotherapy is an integral part of treatment in many cancer types including neuroblastoma (NB). High expression of TrkA/NTRK1 has been found to correlate with excellent prognosis of NB, while high TrkB/NTRK2 expression is associated with unfavorable outcome. Both, TrkA/NTRK1 and TrkB/NTRK2 are members of the neurotrophin receptor family regulating cell fate decisions to favour survival, differentiation or cell death, but their role in modulating radiation resistance is not well characterized.

Aims

The aim of the project how TrkA/NTRK1 and TrkB/NTRK2 and their downstream targets are involved in radioresistance of(NB).

Methods/Materials

We previously established inducible TrkA/NTRK1 or TrkB/NTRK2 overexpression in the human neuroblastoma cell lines, SH-SY5Y and SK-N-AS. To investigate the effects of TrkA/NTRK1 and TrkB/NTRK2 expression and activation in response to irradiation (IR), we monitored changes in cell-cycle distribution by PI-staining and H3pS10-assay. The effects of TrkA/NTRK1 and TrkB/NTRK2 overexpression and activation in combination with different irradiation intensities were analyzed by MTT and colony formation assay.

Results

Induction and activation of both NTRKs, TrkA/NTRK1 and TrkB/NTRK2 was unaffected by

irradiation. Phenotypic and molecular characterization of the radiation response indicated a defective G2/M checkpoint in TrkA/NTRK1 -overexpressing cells, indicated by a higher mitotic index upon ionizing radiation (IR). This effect could be rescued by addition of the NTRK-inhibitor, Loxo-101, suggesting a direct impact of TrkA/NTRK1 on the response to IR. Likewise, ATM- or ATR-inhibition results in an increased mitotic index, which suggests a defect in the damage-sensing pathway. By contrast, TrkB/NTRK2 expression enhanced short-term and long-term survival upon IR. The responsible pathways were examined by inhibitor treatments and preliminary findings suggest involvement of the PI3K-pathway.

Summary/Conclusions

Our experimental data indicate that both, TrkA/NTRK1 and TrkB/NTRK2, are modulators of the radiation response. While increased TrkA/NTRK1 activity in SH-SY5Y cells led to DNA damage independent cell-cycle progression, increased TrkB/NTRK2 signaling was characterized by an increased short-term and also long-term survival upon irradiation. Both findings are in line with clinical data, which correlated high TrkA/NTRK1 expression with a favorable and high TrkB/NTRK2 expression with an unfavorable prognosis. However, further investigation of the molecular downstream mechanisms of these receptors is important to understand underlying principles and to identify potential therapeutic targets.

Type Basic

Presentation Preference Traditional poster