Advances in Neuroblastoma Research

Eleventh Conference

June 16-19, 2004

Genova, Italy

An Official Event of "Genoa 2004, European Capital of Culture"

GE NOVA 04

Programme and Abstracts

MAIN TOPICS
Biology
Genetics
Molecular Biology
Translational Research
Clinical Research

WORKSHOPS
Microarray technology
Spinal cord compression
Opsoclonus myoclonus

Associazione Italiana ONLUS
per la Lotta al Neuroblastoma

Welcome to Genova

Blu Jeans
Jesus Passion (XVI Century)
San Lorenzo Cathedral, Genova

It is a pleasure for the local Organizing Committee and for myself to welcome all of you to Genova, especially those who have never been here before. This meeting is located in the Congress Center, once the cotton warehouses, in the heart of our busy harbor.

For us 2004 is both a very important year and a task, because Genova, together with Lille in France, is one of the two European Capitals of Culture.

I wish to thank all of you. I hope that everybody enjoys both the scientific sessions and the social events that have been arranged to provide you with some joyful intervals during these busy days.

I think that most of you do not know the past of our town and Region, so I wish to tell you a few things about the very peculiar history of our Region, a State without land. Since the old ages, Ligurians have been engaged in overseas trade because their narrow strip of land, lying between the sea and the mountains, could not provide them with a living. In the 13th century the Genoese founded the “Banco di San Giorgio” (St. George’s Bank, 1407), which minted the new coins Lira, soldo, denaro: one Lira was equal to 20 soldos and one soldo to 12 denaros. The same system was immediately adopted in England by the British Banks, where the Lira symbol ”£” means pound-sterling, the soldo symbol “s” means shilling and the denaro symbol “d” means penny. Genoese people became very rich but they were jealous of their wealth, though they were generous with their guests. They kept on living in narrow lanes but their houses were luxuriously decorated and furnished, even equipped with warm-air, an old system of central heating. Rubens, who worked in Genova for several years in the so called “gold street” (Via Aurea, now Via Garibaldi), published a book so that the architecture of the town could become known in the northern countries. Many other important painters and artists from other countries worked in Genova in the past, and among them I wish to remind you of Van Dyck. You may visit the exhibit called “Rubens’ age”, witness of our past, in the Ducal Palace, in the heart of our cultural town. But how could these people make so much money? Thanks to their shrewdness and great business and trade ability. For example, in the 15th century the Pope granted them the exclusive privilege of collecting his tithes all over Great Britain because the Genoese bankers renounced to all interest payments. They earned an enormous amount of money from the tax collection since they would buy wholesale goods from England and Holland and sell them retail on the Mediterranean markets. In the 16th and 17th centuries Genoese bankers granted big loans to several countries and even to kings. The King of Spain, who was unable to pay the agreed interest, gave them the privilege of collecting taxes throughout the whole country. Most of the money used by the Kings of Spain to finance Christopher Columbus’s enterprise to discover the new continent of America came from the Genoese banks. The Genoese soul was a merchant’s soul: they even sold to the City of London their own flag (a red cross on a white background) and their Patron, Saint George. This is why they had to add a new patron, St. John the Baptist. Even in these early centuries insuring property, goods, ships was common in Genova in order to avoid any risks. It is interesting to know that the sailors’ trousers were blue jeans, because they discovered that denim lasted longer than the usual material, it was cheap and was produced in small factories in the Ligurian country-side. “Jean” meant “Genoese” in sailors’ language all over the world.

With these few words I wanted to give you an outline of the typical characteristics and skills of Genoese people who differed from the inhabitants of all other Italian regions. While the latter aimed at extending their possessions and building large, luxurious towns, the Genoese only aimed at increasing their financial power and their riches.

Now, I wish to come back to this important international Congress.

Neuroblastoma is the most fascinating and enigmatic of childhood malignancies from both the clinical and biological viewpoints. We clinicians must look at our sick children carefully and systematically, and discuss any peculiar characteristics among our staff members, while bearing ever in mind the statement "observation and experimental research". We must likewise learn how to re-educate ourselves following this renewed marriage of clinical pediatrics and biological research, availing ourselves of and exploiting the rich resources that are available to us, so as to play an important role in building the necessary bridges between biology and clinical care. Nevertheless, our chances of success in this wonderful profession and in this field of biomedicine remain unchanged, and are founded on our conviction in what we are called on to do, on our curiosity, and on our creativeness.

The continuous support of advocacy groups, parents' associations and volunteers is pivotal to our success. Without their selfless assistance many of our challenges would remain unmet. Furthermore, we must not overlook their painstaking efforts to raise the awareness of the general public regarding certain, especially rare, conditions. The outcome of their efforts is usually a flow of funds to offset the health care costs which cannot always be fully covered by public allocations alone. Indeed, these groups also become key players in supplying both state-of-the-art treatment to patients, and research. We truly feel a great sense of indebtedness toward them.

The Italian Neuroblastoma Association, since early 90’s engaged to support research, together with the City of Genova and GE NOVA 04 wishes to thank the “William Guy Forbeck Research Foundation” by offering a symbolic award to the mother of William, an 11 year old boy who died of neuroblastoma.

GE NOVA 04 and myself wish to give another symbolic award to a very special person, Dr. Audrey Elizabeth Evans, a pioneer of Paediatric Oncology, a life completely dedicated to children with cancer, a life for children’s lives.

I would like to express my wish that this meeting shall fulfill other goals as well, primarily that of encouraging future cooperation and an exchange of ideas among the many investigators from the institutions that are represented here today.

Prof Luisa M Massimo
Director Emeritus
Department of Paediatric Haematology and Oncology
Giannina Gaslini Research Children’s Hospital, Genova

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Posters Display

Scientific Programme

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Introductory Remarks *by Audrey Evans*

8:30-10:30 AM **PLENARY SESSION A: Genetics** Hall "Maestrale"

Chairmen: Akira Nakagawara (J) - Manfred Schwab (D)

10:30-11:00 AM Break

11:00 AM-1:00 PM **PLENARY SESSION B: Biology** Hall "Maestrale"

Chairmen: Nai-Kong Cheung (US)- Jerry Melino (I)

1:00-2:00 PM Lunch

2:00-3:30 PM PARALLEL SESSION A: Translational Hall "Scirocco & Libeccio"

Chairmen: Michelle Haber (AUS) – Carol J Thiele (US)

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3:30-4:00 PM Break

4:00-5:00 PM **POSTER VIEWING**
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Chairmen: Frank Berthold (D) – Robert Castleberry (US)

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3:30-4:00 PM Break

4:00-5:00 PM **POSTER VIEWING**
ROOM A Biology - Clinical - Genetics
ROOM B Genetics - Molecular Biology - Translational

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Children´s Hospital, University Clinic Essen, NRW, Germany.

54 **236.1 Microarray expression profiling improves definition of high- and intermediate-risk metastatic neuroblastomas without MYCN amplification**
Shahab Asgharzadeh, Hong Wang, Yujun Yang, Hiro Shimada, Katherine Matthay, Jonathan Buckley, Robert C Seeger
Department of Pediatric Hematology-Oncology, Children's Hospital of Los Angeles, CA, USA.

54 **107.1 MYCN-Status according to FISH: Amplification, Gain, and Non-Amplification**
Ruediger Spitz, Barbara Hero, Matthias Skowron, Karen Ernestus, Frank Berthold
Pediatric Oncology and Hematology, Children\'s Hospital, Cologne, Germany.

54 **070.1 Gene expression profiling suggests a role for haploinsufficiency of 1p35-36 genes in neuroblastoma**
Isabelle Janoueix-Lerosey, Eugene Novikov, Marta Monteiro, Nadège Gruel, Gudrun Schleiermacher, Béatrice Loriod, Catherine Nguyen, Olivier Delattre
INSERM U509, Institut Curie, Paris, France.

55 **049.1 IGFBP-5 in neuroblastoma: Complex regulations and multiple effects.**
Vincenzo Cesi, Barbara Tanno, Roberta Vitali, Fabiola Sesti, Maria Laura Giuffrida, Camillo Mancini, Giuseppe Raschellà
Department of Toxicology and Biomedical Sciences, ENEA Research Center Casaccia, Rome, Italy.

55 **089.1 A novel 1p36.2 located gene, APITD1, with tumor suppressive properties and a putative p53 binding domain, shows low expression in neuroblastoma tumors**
Cecilia Krona[1], Katarina Ejeskär[1+2], Helena Carén[1], Frida Abel[1], Rose-Marie Sjöberg[1], Tommy Martinsson[1]
Department of Clinical Genetics[1], Inst. forHealth of Women and Children, Göteborg University, Sahl, Sweden; Cell and Gene Therapy Group[2], Murdoch Children's Research Institute, Parkville, Australia.

55 **208.1 A novel region for predisposition to neuroblastoma maps to chromosome 12**
Luca Longo[1], Gian Paolo Tonini[1], Simona Coco[1], Marcella Devoto[2], Marco Seri[3], Giovanni Romeo[3], Patrizia Perri[1]
Laboratory of Neuroblastoma[1], National Institute for Cancer Research (IST), Genova; University of Bologna[3]; Bologna, Italy; Nemours Children's Clinic-Wilmington2, DE, USA.

55 **017.1 Multi locus alleotyping defines clinical subtypes of neuroblastoma**
Carmel M McConville[1], Shaheen A Chughtai[1], Tracey Genus[1], Sarah Dyer[2], Judy Powell[3], Pramila Ramani[4]
Paediatrics & Child Health[1] and Department of Epidemiology and Public Health[3], University of Birmingham; Regional Genetics Unit[2], Birmingham Women's Hospital; Department of Pathology[4], Birmingham Children's Hospital, UK.

56 **366.1 Safety and systemic availability of intravenous and oral arsenic trioxide (As2O3) in children with relapsed/refractory neuroblastoma**
Godfrey CF Chan[1], Tommy WH Yuen[1], Giselle TY Cheung[2], WI Wong[3], Diane MW Ng[1], Yuk Lam Kwong[2], Yu Lung Lau[1], Ricky YK Man[3], CR Kumana[2]
Department of Paediatrics & Adolescent Medicine[1], Department of Medicine[2], Department of Pharmacology[3], The University of Hong Kong, China.

56 **383.1 Pitfalls in detection of contaminating neuroblastoma cells by tyrosine hydroxylase RT-PCR due to catecholamine-producing hematopoietic (stem) cells**
Z.Kuçi, G.Seitz, S.Kuçi, M.Schumm, P.Lang, D.Niethammer, G.Bruchelt
University Children's Hospital, D-72076 Tübingen, Germany.

56 **109.1 Tyrosine Hydroxylase expression in blood of patients with neuroblastoma: Analysis by a real time RT-PCR quantitative assay**
Soledad Gallego, Andreu Parareda, Josep Sanchez de Toledo
Department of Pediatric Oncology Unit and Unitat Recerca Biomedica, Hospital Universitari Vall d\'Hebron, Barcelona, Spain.

56 **042.1 Detection of residual neuroblastoma cells using a new four-color flow cytometric assay**
Katrien Swerts, Barbara De Moerloose, Catharina Dhooge, Yves Benoit, Jan Philippé, Geneviève Laureys
Department of Pediatrics, Ghent University Hospital, Belgium.

57 **028.2 Induced doxorubicin resistance in caspase-8/10 silenced neuroblastoma cell lines results in multi-drug resistance that involves early and p53-independent anti-apoptotic mechanisms**
Marjorie Flahaut, Annick Mühlethaler, Jean-Marc Joseph, Katia Balmas Bourloud, Katya Auderset, Nicole Gross
Paediatrics, University Hospital, CHUV, Lausanne, Switzerland.

57 **347.2 IL-12 inhibits AKT activity and induces BID activation in conjunction with complete regression of orthotopic murine neuroblastomas**
Tahira Khan, Julie A Hixon, Jimmy K Stauffer, Erin Lincoln, Timothy C Back, Jon M Wigginton
Pediatric Oncology Branch, NCI-CCR, Frederick, MD, USA.

57 **262.2 Evaluation of efficacy of NK cell therapy in human neuroblastoma-bearing mice**
Roberta Castriconi[1], Michele Cilli[2], Barbara De Giovanni[3], Alessandro Dondero[1], Annalisa Pezzolo[4], Vito Pistoia[4], Claudio Gambini[3], Alessandro Moretta[1], Maria Valeria Corrias[4]
Department of Experimental Medicine[1], University of Genoa, Service of Animal Model[2], IST, Service of Pathology[3] and Laboratory of Oncology[4], Giannina Gaslini Children's Hospital, Genoa, Italy.

57 **330.2 Natural killer (NK) lymphocytes are cytotoxic for multidrug resistant neuroblastoma cells**
Hong-wei Wu, Zamir Warsi, Leonid Metelitsa, Shahab Asgharzadeh, Susan Groshen, Robert Seeger
Division of Hematology/Oncology, Department of Pediatrics, Children's Hospital Los Angeles/USC, CA, USA.

7:00-9:00 PM Opening Ceremony & Welcome Reception

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Chairmen: Jean Bénard (F) - Mirco Ponzoni (I)

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Department of Pediatric Oncology and Hematology, University of Cologne, Germany.

58 **081.1 Selection for p53 mutant cells by cytotoxic agents in neuroblastoma**
Jane Carr, Christine Challen, Julian Board, Katrina M Wood, Andrew DJ Pearson, John Lunec, Deborah A Tweddle
Molecular Biology and Department of Pathology, RVI, Northern Institute for Cancer Research, Newcastle Upon Tyne, UK.

58 **192.1 In vivo resistance to CPT-11 in neuroblastoma: Does pleiotrophin play a role?**
Loreley Calvet, Birgit Geoerger, Alexander Valent, Gilles Vassal
Department of Pharmacology and New Treatments in Cancer, Institute Gustave Roussy, Villejuif, France.

58 **227.1 Interferon-mediated anti-angiogenic therapy for neuroblastoma**
Andrew M Davidoff, Christian J Streck, Catherine YC Ng, Youbin Zhang, Junfang Zhou, Amit C Nathwani
Department of Surgery, St. Jude Children's Research Hospital, Memphis, Tennessee, USA.

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Department of Childhood Cancer Research Unit[1] and Dept. of Oncology & Pathology[3], Karolinska Institute, Stockholm; Department Exp. Pathology. University of Tromso[2], Sweden.

59 **310.1 [131I]meta-iodobenzylguanidine and Topotecan: Experimental combination treatment of tumours expressing the noradrenaline transporter**
Anthony G McCluskey, Emilio Cosimo, Mark N Gaze, Marie Boyd, Robert J Mairs
Radiation Oncology, CRUK Beatson Labs, University of Glasgow, Scotland, UK.

59 **150.1 Anti-tumour effects of anti-G_{D2}-targeted liposomal antisense oligonucleotides result from a CpG-mediated immune stimulatory effect synergising with an anti-c-myb effect**
Chiara Brignole[1], Fabio Pastorino[1], Danilo Marimpietri[1], Gabriella Pagnan[1], Danilea Di Paolo[1], Marta Zancolli1, Theresa M Allen[2],Vito Pistoia[1], Mirco Ponzoni[1]
Laboratory of Oncology[1], G. Gaslini Children's Hospital, Genoa, Italy; Department of Pharmacology[2], University of Alberta, Edmonton, Canada, USA.

10:30-11:00 AM Break

Friday 18, 2004

Oral Presentations

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Chairmen: C Patrick Reynolds (US) - Massimo Romani (I)

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Inge M Ambros, Rita Narath, Peter F Ambros
Tumorcytogenetics, CCRI, St. Anna Kinderspital, Vienna, Austria.

60 **043.1 MDM2 is a direct target of the MYCN oncogene in neuroblastoma**
Andrew Slack, Zaowen Chen, Lisa Hunt, Jason M Shohet
Department of Pediatrics, Baylor College of Medicine, Houston, Texas, USA.

60 **075.1 Deregulated WNT/BCATENIN pathway in neuroblastoma without MYCN amplification**
Vincent Dam[1], Pavel Mazanek[2], Xueyuan Liu[1], John M Maris[1], Kathleen R Cho[3], Michael D Hogarty[1]
Department of Pediatrics[1], The Children's Hospital of Philadelphia, PA; University of Michigan Medical School[3], USA; Children's Hospital Brno[2], Czech Republic.

60 **323.1 The Delta-Notch pathway integrates the wnt pathway, the noradrenalin synthesis route and the neurotrophin pathway in neuroblastoma**
Vera van Limpt[1], Alexander Schramm[2], Alvin Chan1, Angelika Eggert[2], Rogier Versteeg[1]
Department of Human Genetics, Academic Medical Center, University of Amsterdam, The Netherlands; Dept. of Hematology/Oncology, University Children's Hospital of Essen, Germany.

61 **354.1 Differential effects of TrkA or TrkB expression on DNA repair capacity might contribute to the genomic stability of SY5Y neuroblastoma cells**
Johannes H Schulte, Daniela Dobrzinski, Steffi Kuhfittig-Kulle, Elke Feldmann, Alexander Schramm, Harald Stephan, Ludger Klein-Hitpass, Werner Havers, Petra Pfeiffer, Angelika Eggert
Department of Hematology/Oncology, University Children's Hospital of Essen, Germany.

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Kazushige Furuya, Toshinori Ozaki, Takayuki Hanamoto, Akira Nakagawara
Division of Biochemistry, Chiba Cancer Center Research Institute, Japan.

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Karine Million, David Goldschneider, Etienne Blanc, Emilie Horvilleur, Gilda Raguanez, Frédéric Tournier, Jean Benard, Sétha Douc-Rasy
CNRS-UMR 8126, Institut Gustave Roussy, Villejuif, France.

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Division of Biochemistry, Chiba Cancer Center Research Institute, Japan.

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F Bourdeaut[1], D Trochet[2], I Janoueix-Lerosey[1], S. Lyonnet[2], O Delattre[1], J Amiel[2]
Laboratoire de pathologie moléculaire des cancers[1], INSERM U-509, Institut Curie and Unité de recherche sur les Handicaps génétiques de l'Enfant[2], INSERM U-393, Hôpital Necker-Enfants Malades, Paris, France.

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Department of Oncology, Children\'s Hospital of Philadelphia, PA, USA.

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Chiba Cancer Center Research Institute[1] and Nara Institute of Science and Technology[2], Japan; Brain Tumor Research Center[3], Cancer Center, University of California, San Francisco, CA, USA; Hokkaido University[4], School of Medicine; Saitama Cancer Center Research Institute[5]; Hisamitsu Pharmaceutical Co.

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Department of Center for Medical Genetics, Ghent University Hospital, Gent, Belgium

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Jaume Mora[1], Miguel Alaminos[2], Nai-Kong V Cheung[3], Jose Rios[4], William L Gerald[5]
Oncology, Hospital Sant Joan de Deu de Barcelona[1], Centro Nacional de Investigaciones cientificas[2], Madrid, Universitat Autonoma de Barcelona[4], Spain; Memorial Sloan-Kettering Cancer Center[3], New York, USA.

3:30-4:00 PM Break

4:00-5:00 PM **POSTER VIEWING**
ROOM A Biology - Clinical - Genetics
ROOM B Genetics - Molecular Biology - Translational

Friday 18, 2004

Oral Presentations

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Chairmen: Ruth Ladenstein (AT) – Michio Kaneko (J)

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Takeo Tanaka, Tomoko Iehara, Tohru Sugimoto, Minoru Hamasaki, Satoshi Teramukai, Yoshiaki Tsuchida, Michio Kaneko, Tadashi Sawada
Department of Pediatrics and Division of Clinical Research, National Hospital Kure Medical Center, Kure City, Hiroshima, Japan.

64 **123.1 From genotype to phenotype in neuroblastoma: The derivation of clinically different entities**
Frank Berthold[1], Javed Khan[2], Frank Westermann[3], Simon Thorsten[1], Ruediger Spitz[1], Manfred Schwab[3], Matthias Fischer[1], Barbara Hero[1]
Dept. of Pediatric Oncology and Hematology[1], Childrens' Hospital, University of Cologne, Koeln, Germany; NIH2, Bethesda; German Caner Reseach Center[3], Heidelberg.

64 **278.1 Prediction of MYCN amplification in neuroblastoma using serum DNA and real-time quantitative PCR**
Takahiro Gotoh, Hajime Hosoi, Yasumichi Kuwahara, Shinya Osone, Kunihiko Tsuchiya, Tomoko Iehara, Hiroshi Kuroda and Tohru Sugimoto
Department of Pediatrics, Kyoto Prefectural University of Medicine, Kyoto, Japan.

64 **115.1 Association of high-level MRP1 expression with poor clinical outcome in a large prospective study of primary neuroblastoma**
Michelle Haber[1], Janice Smith[1], Sharon Bordow[1], Susan L Cohn[2], Wendy B London[3], Glenn M Marshall[1], Murray D Norris[1]
Department Experimental Therapeutics[1], Children's Cancer Institute Australia for Medical Research, Sydney, NSW, Australia; Northwestern University School of Medicine[2], Chicago, IL; University of Florida and Children's Oncology Group Statistics Department[3], Gainesville, FL, USA.

65 **370.1 131-I-MIBG double infusion with autologous stem cell transplant (ASCT) for neuroblastoma: A new approaches to neuroblastoma therapy (NANT) study**
Katherine K Matthay[1], John Huberty[1], Randall Hawkins[1], Gregory Yanik[2], Martin Charron[3], Susan Groshen[1], Robert C Seeger[1], Suzanne Shusterman[3], John M Maris[3]
Department Pediatrics[1], University of California, San Francisco, CA; University of Michigan[2]; University of Pennsylvania[3], USA

65 **136.1 High risk stage 3 neuroblastoma: Favorable outcome with myeloablative therapy and 13-cis-retinoic acid**
Julie R Park, Judith G Villablanca, Robert C Seeger, Wendy B London, Robert B Gerbing, Patrick C Reynolds, Katherine K Matthay
Department Pediatric Oncology, Children's Hospital and Regional Medical Center/Fred Hutchinson Cancer Research Center, Seattle, Washington, USA and Children's Oncology Group.

3:30-4:00 PM Break

4:00-5:00 PM **POSTER VIEWING**
ROOM A Biology - Clinical - Genetics
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5:00-7:00 PM SELECTED POSTERS — Hall "Scirocco & Libeccio"

Chairmen: Vito Pistoia (I) - Robert C Seeger (US)

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66 **025.1 Id2 is sufficient and necessary for growth and angiogenesis in neuroblastoma**
Anna Lasorella, Gerson Rothschild, Alice Lee, Jessica J Kandel, Darrell J Yamashiro, Antonio Iavarone
Institute for cancer Genetics, Columbia University, NY, USA.

66 **204.1 H-prune, nm23-H1 and nm23-H2: New markers of neuroblastoma progression**
Anna D'Angelo[1], Veruska Aglio[1], Alessandra André[1], Pietro Carotenuto[1], Daniela Spano[2], Lucia Giordani[2], Francesco Lanzi[3], Letterio Runza[4], Gianluigi Arrigoni[4], Gian Paolo Tonini[5], Massimo Zollo[1], Achille Iolascon[2]
T.I.G.E.M.1, FONDAZIONE TELETHON and Università di Foggia e CEINGE[2], Naples; HSR Institute[2] and Children Hospital V. Buzzi[3], Milan; IST[5], Genova, Italy.

66 **309.1 Identification of MYCN transcriptional activity inhibitors yields compounds which preferentially inhibit the growth of neuroblastoma cells**
Xiaohong Lu, Herbie Newell, Andrew DJ Pearson, John Lunec
Northern Institute for Cancer Research, University of Newcastle upon Tyne, UK.

66 **053.1 Methylation patterns in ganglioneuroma and neuroblastoma**
Massimo Romani, Ilaria Gelvi, Ida Casciano, Paola Scaruffi, Angela Di Vinci, Gian Paolo Tonini, Barbara Banelli
Tumor Genetics, Istituto Nazionale per la Ricerca sul Cancro – IST, Genova, Italy.

67 **078.1 KIF1B is a variant-dependent tumor suppressor gene mapped to a 500-kb homozygously deleted region at chromosome 1p36.2 in neuroblastoma**
Arasambattu K Munirajan, Masato Takahasi, Miki Ohira, Toshinori Ozaki, Hajime Kageyama, Kou Miyazaki, Akira Nakagawara
Division of Biochemistry, Chiba Cancer Center Research Institute, Chiba, Japan.

67 **301.1 Response of neuroblastoma to the hypoxic environment**
Celso Castaldo, Fabio Sallustio, Maria Carla Bosco, Maura Puppo, Luigi Varesio
Laboratorio di Biologia Molecolare, Istituto Giannina Gaslini, Genova, Italia.

67 **374.1 Expression of TrkA in SY5Y neuroblastoma cells sensitizes for chemotherapy-induced apoptosis by up regulation of caspase-8**
Hauke Sievert[1], Sonja Kramer[2], Sabine Dreesmann[3], Alexander Schramm[3], Natalya Peretyatko[1], Angelika Eggert[3]
Department of Pediatric Hematology/Oncology[1], University Children's Hospital of Heidelberg; University Children's Hospital of Essen[3], Germany; Chiba Cancer Center Research Institute[2], Japan.

67 **338.1 p53/MEK1/NF-kappaB signaling pathway mediates anticancer drugs induced cell death in N-type neuroblastoma cells**
Xin Bian, Anthony W Opipari, Valerie P Castle
Department of Pediatrics and Department of Obstetrics and Gynecology, University of Michigan Medical School, Ann Arbor, MI, USA.

68 **008.1 Enhancement of targeted radiotherapy in neuroblastoma: A novel gene therapy approach**
Emilio Cosimo, Marie Boyd, Anthony G McCluskey, T Robson, Michael R Zalutsky, Robert J Mairs
Radiation Oncology, University Of Glasgow, UK.

68 **349.1 Genes associated with multi-drug resistance in neuroblastoma cell lines identified by gene expression profiling**
Nino Keshelava, Bo Yang, Xuan Chen, Timothy J Triche, C Patrick Reynolds
Department of Hematology/Oncology, Children's Hospital Los Angeles/USC, CA, USA.

68 **029.2 A clustering of gene hypermethylation distinguishes high and low-risk neuroblastoma patients**
Miguel Alaminos[1], Veronica Davalos[1], Jaume Mora[1], Nai-Kong V Cheung[2], William L Gerald[2], Manel Esteller[1]
Cancer Epigenetics Laboratory[1], Spanish National Cancer Center (CNIO), Madrid, Spain; Memorial Sloan-Kettering Cancer Center[2], New York, USA.

68 **305.1 Antigen specific immunity in neuroblastoma patients: Antibody and T-cell recognition of NY-ESO-1 tumor antigen**
Chiara Castelli[1], Roberto Luksch[2], Elisabeth Stockert[3], Yao-Tseng Chen[3], Paola Collini[2], Tiziana Ranzani[1], Claudia Lombado[1], Piero Dalerba[1], Licia Rivoltini[1], Franca Fossati-Bellani[2], Lloyd Old[3], Giorgio Parmiani[1], Monica Rodolfo[1]
Unit of Immunotherapy of Human Tumors[1] and Department of Pediatric Hematology Oncology[2], Istituto Nazionale Tumori, Milano, Italy; Memorial Sloan Kettering[3], New York, USA.

69 **002.1 Improved outcome in high-risk neuroblastoma after application of intensified multimodal therapy**
Per Kogner, Per Borgström, Bengt Karpe, Göran Lundell, Anna-Lena Hjelm Skog, Jacek Winiarski, Moustapha Hassan
Childhood Cancer Research Unit, Pediatric Surgery, Hematology and General Oncology at Karolinska University Hospital, Karolinska Institutet, Stockholm Sweden.

69 **222.1 Intensive targeted chemoradiotherapy of metastatic neuroblastoma with high-dose iodine-131 labelled meta-iodobenzylguanidine, Topotecan and haemopoietic support**
Mark N Gaze[1], Glenn D Flux[2], Robert J Mairs[3], Frank H Saran[2], Simon T Meller[2]
Meyerstein Institute of Oncology[1], The Middlesex Hospital, Royal Marsden Hospital[2], London; University of Glasgow[3], UK.

69 **072.1 Results of NB 97 SFOP protocol in children > 1 year with a stage 4 neuroblastoma**
Dominique Valteau-Couanet, Jean Michon, Yves Perel, Christophe Bergeron, Hervé Rubie, Carole Coze, Chantal Rodary, Olivier Hartmann
For the SFOP (Société Française de Oncologie Pédiatrique).

69 **303.2 The SIOPEN-R-NET Project: Building a European network for neuroblastoma treatment (HR-NBL-1/ESIOP) and research**
Ruth Ladenstein[1], Ulrike Pötschger[1], Ditha Modritz[1], Günther Schreier[2], Bruno De Bernardi[3]
Department Oncology Department[1], St.Anna Children's Hospital and ARC Seibersdorf[2], Vienna, Austria; Department of Hematology Oncology[3], Giannina Gaslini Children's Hospital, Genova, Italy.

8:30-11:00 PM Gala Dinner

Hall "Maestrale"

8:30-10:30 AM PARALLEL SESSION E: Molecular Biology – Translational

Chairmen: Garrett M Brodeur (US) - Giuseppe Raschellà (I)

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10:30-11:00 AM Break

Saturday 19, 2004

Oral Presentations

8:30–10:30 AM PARALLEL SESSION F: Clinical

Hall "Levante & Ponente"

Chairmen: Adela Cañete (S) - Hervé Rubie (F)

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10:30-11:00 AM Break

11:00-12:30 AM **PLENARY SESSION E: Translational – Clinical** Hall "Maestrale"

Chairmen: Audrey Evans (US) – Andrew DJ Pearson (UK)

12:30 AM Assignment of ANR 2004 Awards
Conclusive Remarks *by Akira Nakagawara*

BIOLOGY

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82 **202.1** **Functional expression and release of ligands for the activating immunoreceptor NKG2D in human neuroblastoma** **#17**
Lizzia Raffaghello[1], Irma Airoldi[1], Ignazia Prigione[1], Marta Camoriano[1], Isabella Levrieri[2], Claudio Gambini[3], Daniela Pende[4], Soldano Ferrone[5], Vito Pistoia[1]
Laboratory of Oncology[1], Laboratory of Analyses[2], Service of Pathology[3], G. Gaslini Children's Hospital, Genoa, Italy; Laboratory of Immunology[4], CBA/IST, Genova, Italy; Department of Immunology[5], Roswell Park Cancer Center, Buffalo, NY, USA.

82 **344.1** **Mismatch repair protein expression in pre and post treatment neuroblastoma** **#18**
Deborah A Tweddle, Lynn Braidwood, Katrina M Wood, Julian R Board, Andrew DJ Pearson, Robert Brown
Department of Molecular Biology and Department of Pathology, Northern Institute for Cancer Research, CR UK Beatson Labs, Newcastle upon Tyne, Tyne and Wear, UK.

82 **245.1** **GD2 loss variants in neuroblastoma** **#19**
Roswitha Schumacher-Kuckelkorn, Sandra Schmidt, Anke Gradehandt, Barbara Hero, Frank Berthold
Department of Pediatric Oncology and Hematology, Childrens'Hospital University of Cologne, Germany.

82 **316.1** **Biological modifiers of RANKL activity prevents osteoclastogenesis induced by neuroblastoma cells** **#20**
Ilaria Amato[1], Luca Battistelli[1], Corinne Calia[1], Sergio Capaccioli[2], Martino Donnini[2], Nicola Baldini[1], Donatella Granchi[1]
Laboratory for Pathophysiology[1], Istituti Ortopedici Rizzoli, Bologna; Department of Experimental Pathology and Oncology[2], Florence, Italy.

83 **080.1** **Hypoxia induced dedifferentiation of neuroblastoma cells: phenotypic persistency after reoxygenation** **#21**
Linda Holmquist, Annika Jögi and Sven Påhlman
Department of Laboratory Medicine, Division of Molecular Medicine, Lund University, University Hospital MAS, Malmö, Sweden.

83 **333.1** **Normalization to averaged expression levels of four control genes results in reliable transcript quantification by real-time RT-PCR in primary neuroblastoma** **#22**
Matthias Skowron
Childrens Hospital, Depatrment of Pediatric Oncology and Hematology, University of Cologne, Germany.

83 **353.1** **NDSP, a novel secreted protein in neuroblastoma** **#23**
Sanjeev A. Vasudevan[1], Susan M. Burlingame[1], Zhiyun J. Liu[1], Parul N. Pate[2], Jianhua Yang[2], Jed G. Nuchtern[1]
M.E. DeBakey Department of Surgery[1] and Department of Pediatrics[2], Baylor College of Medicine, Houston, TX, USA.

CLINICAL

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321.1 **Unequivocal identification of disseminated tumor cells in the bone marrow by immunofluorescence and FISH reveal essential functional and prognostic information** **#24** 84
Peter F Ambros[1], Gabor Mehes[2], Andrea Luegmayr[1], Inge M Ambros[1], Ruth Ladenstein[3], Helmut Gadner[3]
Tumorcytogenetics[1], Department of Pathology[2], Pecs, and Oncology Department[3], CCRI, St. Anna Kinderspital, Vienna, Austria.

241.2 **Immunocytochemical detection of GD2-positive cells in bone marrow on diagnosis of localised neuroblastoma: clinical implications** **#25** 84
Angela Sementa[2], Lawrence Faulkner[3] Daniela Dau[1], Francesca Negri[2] Fabiana Malaguti[2] Cristina Rosanda[2] Riccardo Haupt[1], Claudio Gambini[2] Cinzia Marchi[2] Maria Valeria Corrias[2] Bruno De Bernardi[1], Alberto Garaventa[1]
Department of Hematology-Oncology[1], Labaratory of Oncology and the Service of Pathology Giannina Gaslini Children Research Hospital, Genova[2]; Meyer Children's Hospital Chair of Pediatric University of Florence, Italy[3].

042.3 **Development and standardization of an immunocytochemical assay to detect residual neuroblastoma cells** **#26** 84
Katrien Swerts, Peter F Ambros, Chantal Brouzes, Jose M Fernandez Navarro, Nicole Gross, Dyanne Rampling, Roswitha Schumacher-Kuckelkorn, Angela Sementa, Ruth Ladenstein, Klaus Beiske
For the E-SIOP Neuroblastoma Bone Marrow Studies Sub-Committee.

042.1 **Detection of residual neuroblastoma cells using a new four-color flow cytometric assay** **#27** 84
Katrien Swerts, Barbara De Moerloose, Catharina Dhooge, Yves Benoit, Jan Philippé, Geneviève Laureys
Department of Pediatrics, Ghent University Hospital, Belgium.

125.1 **New advances in neuroblastoma minimal residual research: Five colour flow cytometry** **#28** 85
Cornelia Pieper, Stefanie Zimmermann, Claudia Andreas, Konrad Bochennek, Andrea Brinkmann, Stephanie Grohal, Thomas Klingebiel, Ulrike Koehl
Pediatric Hematology and Oncology, University Hospital of Frankfurt, Germany.

153.1 **Bone marrow infiltration in neuroblastoma: a flow cytometric tri-colour assay** **#29** 85
Fabio Bozzi[1], PaolaCollini[2], Marta Podda[1], Felicita Gambirasio[1], Elena Barzanò[1], Franca Fossati[1], Roberto Luksch[1]
Medical Oncology, Division of Pediatrics[1] and Division of Pathology "B"[2], Istituto Nazionale Tumori, Milan, Italy.

428.1 **Immunocytological evaluation of bone marrow disease in peripheral neuroblastic tumours** **#30** 85
Francesca Negri, Fabiana Malaguti, Francesco Morandi, Roberta Cinti, Claudio Gambini, Cristina Rosanda[1], Angela Rita Sementa
Departments of Anatomic Pathology and Haematology-Oncology[1] , Giannina Gaslini Children's Hospital, Genova, Italy.

320.1 **GD2 synthase mRNA is less specific for detection of neuroblastoma in blood and bone marrow than tyrosine hydroxylase mRNA and dopadecarboxylase mRNA** **#31** 85
Bertil Kågedal[1], Catarina Träger[1], Anita Kullman[1], Per Kogner[2]
Division of Clinical Chemistry[1], Department of Biomedicine and Surgery, Linköping; Childhood Cancer Research Unit1, Stockholm, Sweden.

383.1 **Pitfalls in detection of contaminating neuroblastoma cells by tyrosine hydroxylase RT-PCR due to catecholamine-producing hematopoietic (stem) cells** **#32** 86
Z.Kuçi, G.Seitz, S.Kuçi, M.Schumm, P.Lang, D.Niethammer, G.Bruchelt
University Children's Hospital, D-72076 Tübingen, Germany.

109.1 **Tyrosine Hydroxylase expression in blood of patients with neuroblastoma: Analysis by a real time RT-PCR quantitative assay** **#33** 86
Soledad Gallego, Andreu Parareda, Josep Sanchez de Toledo
Department of Pediatric Oncology Unit and Unitat Recerca Biomedica, Hospital Universitari Vall d\'Hebron, Barcelona, Spain.

106.1 **Quality assurance of reverse transcriptase polymerase chain reaction (RT-PCR) to detect neuroblastoma: ESIOP Neuroblastoma Group** **#34** 86
Sue A Burchill, Maria V Corrias, Bertil Kagedal, Silvestre Oltra, Marit Sletten, Katrien Swerts, Ales Vicha, Annelise Bennaceur, Ruth Ladestein
Cancer Research UK Clinical Centre, St James\'s University Hospital, Leeds, United Kingdom for the E-SIOP Neuroblastoma RT-PCR Group.

042.2 **Detection of residual neuroblastoma cells using quantitative real-time RT-PCR** **#35** 86
Katrien Swerts, Barbara De Moerloose, Catharina Dhooge, Yves Benoit, Jan Philippé, Geneviève Laureys
Department of Pediatrics and Department of Clinical Chemistry, Microbiology and Immunology, Ghent University Hospital, Belgium.

311.1 **Tumour cell clearing in the bone marrow of stage 4 neuroblastoma: Can it predict outcome?** **#36** 87
Ditha Modritz, Ruth Ladenstein, Inge Ambros, Andrea Luegmayr, Helmut Gadner, Peter F Ambros
Children's Cancer Research Institute, St. Anna Kinderspital, Vienna, Austria.

137.1 **Tumor cell detection in apheresis products in pediatric patients with advanced neuroblastoma: Where to go?** **#37** 87
Volker Witt, Ruth Ladenstein, Gerhard Fritsch, Ullrike Pötschger, Helmut Gadner, Peter F Ambros
Department of Pediatric Oncology, St. Anna Kinderspital, Vienna, Austria.

138.2 **Standardised reporting of I-123 mIBG scans for determination of response to induction chemotherapy** **#38** 87
Glenn D Flux, Gary JR Cook, Val J Lewington, Ruth Ladenstein
For the Nuclear Medicine and Physics Sub-Committee of the E-SIOP Neuroblastoma Group.

405.1 **Assessment of chemotherapy response by MIBG scan: a blind quality control** **#39** 87
Maria Rita Castellani[1], Dario Casara[2], Francesco Giammarile[3], Lorenzo Maffioli[4], Vittoria Ruffini[5], Giampiero Villavecchia[6], Francesca Albertini[1], Paola Angelini[7], Roberto Luksch[8], Emilio Bombardieri[1], Bruno De Bernardi[7]
Department of Nuclear Medicine[1] and Department of Pediatric Hematology Oncology[8], Istituto Nazionale Tumori, Milan; Università di Padova[2];Ospedale di Lecco[4]; Policlinico Gemelli[5], Rome; Ospedale Galliera[6], and Giannina Gaslini Children's Hospital[7], Genova, Italy; Centre Leon Berard[3], Lyon, France.

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96 **033.3** **Spinal cord compression (SCC) in neuroblastoma: The Italian experience from 1999-2003**
Paola Angelini, Roberto Luksch, Maria Giuliano, Margherita Nardi, Massimo Abate, Paolo D'Angelo, Angela Tamburini, Katia Tettoni, Monica Cellini, Maurizio Bianchi, Alberto Michelazzi, Bruno De Bernardi, Maria Luisa Garré
for the Italian Cooperative Group on Neuroblastoma(ICGNB).

#75

96 **050.1** **Spinal cord compression in neuroblastoma - experience of Polish Pediatric Solid Tumors Study Group (PPSTSG)**
Walentyna Balwierz[1], Aleksandra Niezgoda[1], Bozena Dembowska-Baginska[2], Danuta Perek[2], Danuta Januszkiewicz-Lewandowska[3], Aleksandra Rybczynska[13], Joanna Klaczynska[4], Jerzy Kowalczyk[4], Elzbieta Drozynska[5], Anna Balcerska[5], Sylwia Koltan[6], Wysocki M.[6], Halina Bubala[7], Danuta Sonta-Jakimczyk[7], Maryna Krawczuk-Rybak[8], Katarzyna Muszynska-Roslan[8], Matysiak M. [9]
Department of Oncology/Hematology, P-A Institute of Pediatrics MC JU in 1Krakow and other centers of PPSTSG: [2]Warsow CZD, [3]Poznan, [4]Lublin, [5]Gdansk, [6]Bydgoszcz, [7]Zabrze, [8]Bialystok, [9]Warsaw, Poland AM.

#76

97 **257.1** **Infantile Dumbbell-type Neuroblastoma**
Tomoko Iehara, Minoru Hamazaki, Takeo Tanaka, Hajime Hosoi, Mitio Kaneko, Tadashi Sawada, Toru Sugimoto
Pediatrics, Kyoto Prefectural University of Medicine, Japan on behalf of the Japanese Infantile Neuroblastoma Cooperative Study Group.

#77

97 **306.2** **Neuroblastoma of paravertebral ganglions penetrating spinal canal - cases report**
Joanna Zawitkowska – Klaczynska, Krzysztof Katski, Joanna Nurzynska-Flak, Jerzy R Kowalczyk
Department Pediatric Hematology and Oncology, Children's Hospital, Lublin, Poland.

#78

69 **303.2** **The SIOPEN-R-NET Project: Building a European network for neuroblastoma treatment (HR-NBL-1/ESIOP) and research**
Ruth Ladenstein[1], Ulrike Pötschger[1], Ditha Modritz[1], Günther Schreier[2], Bruno De Bernardi[3]
Department Oncology Department[1], St.Anna Children's Hospital and ARC Seibersdorf[2], Vienna, Austria; Department of Hematology Oncology[3], Giannina Gaslini Children's Hospital, Genova, Italy.

#79

97 **385.1** **Front-line topotecan and high-dose cyclophosphamide followed by ICE in high-risk neuroblastoma**
Alberto Donfrancesco, Alessandro Jenkner, Aurora Castellano, Ilaria Ilari, Giuseppe M Milano, Carlo Dominici, Luigi De Sio, Paola Fidani
Division of Oncology, Ospedale Pediatrico Bambino Gesù IRCCS, Rome, Italy.

#80

97 **215.1** **Consolidation treatment with chimeric anti-GD2-antibody ch14.18 in children >1 year with metastatic neuroblastoma**
Thorsten Simon[1], Barbara Hero[1], Andreas Faldum[2], Rupert Handgretinger[3], Martin Schrappe[4], Dietrich Niethammer[5], Frank Berthold
Pediatric Oncology and Hematology[1], Children's Hospital, University of Cologne, Koeln; Johannes-Gutenberg-University of Mainz, Department of Pediatric Hematology and Oncology[4], Hannover Medical School, Hannover, Children's University Hospital, Tuebingen[5], Germany; St Jude Children's Research Hospital[3], Department of Hematology-Oncology, Memphis, TN, USA.

#81

98 **143.1** **13 cis- retinoic acid in children with high-risk neuroblastoma: Is more better?**
Shifra Ash[1], Jerry Stein[2], Batia Stark[1], Yacov Goshen[1], Liora Kornreich[3], Zvi Bar-Sever[4], Meora Feinmesser[5], Isaac Yaniv[1]
Pediatric Hematology Oncology[1], Bone Marrow Transplantation Unit[2], Imaging[3], and Nuclear Medicine[4], Schneider Children's Medical
Center of Israel, Petah-Tikva, Israel; Pathology[5], Rabin Medic Center, Beilinson Campus.

#82

98 **228.1** **Treatment results of high-risk neuroblastoma children (1995-2002). A single centre experience**
Josef Malis, Marketa Chanova, Ales Vicha, Eliska Cumlivska, Michal Ryg[l], Hana Krizova, Bela Malinova, Edita Kabickova, Roman Kodet
Department of Pediatric Hematology and Oncology, Charles University, [2]nd Medical Faculty, Prague[5], Czech republic

#83

69 **002.1** **Improved outcome in high-risk neuroblastoma after application of intensified multimodal therapy**
Per Kogner, Per Borgström, Bengt Karpe, Göran Lundell, Anna-Lena Hjelm Skog, Jacek Winiarski, Moustapha Hassan
Childhood Cancer Research Unit, Pediatric Surgery, Hematology and General Oncology at Karolinska University Hospital, Karolinska Institutet, Stockholm Sweden.

#84

98 **303.1** **Risk adapted treatment according to the Austrian Neuroblastoma Trial A-NB94**
Ruth Ladenstein, Ulrike Pötschger, Christian Urban, Franz-Martin Fink, Georg Ebetsberger, Neil Jones, Gabriele Amann, Ernst Horcher, Karin Dieckmann, Helmut Gadner, Peter F Ambros
Oncology Department, St. Anna Children's Hospital, Vienna, Austria.

#85

69 **072.1** **Results of NB 97 SFOP protocol in children > 1 year with a stage 4 neuroblastoma**
Dominique Valteau-Couanet, Jean Michon, Yves Perel, Christophe Bergeron, Hervé Rubie, Carole Coze, Chantal Rodary, Olivier Hartmann
For the SFOP (Société Française de Oncologie Pédiatrique).

#86

98 **237.1** **High-dose chemotherapy consisting of thiotepa and melphalan prior to local surgery for advanced neuroblastoma**
Keiko Kubota, Yoshiko Hashii, Shigenori Kusuki, Sadao Tokimasa, Hiroyuki Fujisaki, Akihiro Yoneda, Takeshi Kusafuka, Masahiro Fukuzawa, Junichi Hara
Developmental Medicine (Pediatrics and Pediatric Surgery), Osaka University Graduate School of Medicine, Japan.

#87

99 **285.1** **Comparison of two induction regimens for high-risk neuroblastoma**
Victoria Castel, Adela Cañete, Purificación García-Miguel, Carmen Melero, Aurora Navajas, Acha T, Samuel Navarro, Rosa Noguera
Spanish Neuroblastoma Group.

#88

99 **381.1** **The use of liposomal busulphan in high-dose therapy of neuroblastoma**
Per Borgström, Jacek Winiarski, Moustapha Hassan, Per Kogner
Pediatric Oncology Research Unit, Karolinska Institute, Stockholm, Sweden.

#89

99 **307.1** **Combinational treatment with chemotherapyt and 131-I-MIBG for advanced neuroblastoma at diagnosis**
Stefano Mastrangelo, Vittoria Rufini, Angela Di Giannatale, Riccardo Riccardi
Division of Pediatric Oncology, Catholic University, Rome, Italy.

#90

69 **222.1** **Intensive targeted chemoradiotherapy of metastatic neuroblastoma with high-dose iodine-131 labelled meta-iodobenzylguanidine, Topotecan and haemopoietic support**
Mark N Gaze[1], Glenn D Flux[2], Robert J Mairs[3], Frank H Saran[2], Simon T Meller[2]
Meyerstein Institute of Oncology[1], The Middlesex Hospital, Royal Marsden Hospital[2], London; University of Glasgow[3], UK.

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#91

99 **308.1** **A single institution study of low dose 1311mIBG therapy for stage 4 neuroblastoma – therapeutic implications**
Sunil J Desai[2], Karina Black[2], Gail Amyotte[1], John W Logus[1], Ciaran Terry[1], Alexander J McEwan[1]
Cross Cancer Institute1 and Stollery Children's Hospital2, Edmonton, Alberta, Canada.

#92

100 **335.1** **Dosimetry in 131I-MIBG mieloablative therapy**
Carlo Chiesa, Francesca Alberini, Maria Rita Castellani, Roberto Luksch, Marta Podda, Bombardieri Emilio
Medicina Nucleare, Istituto Nazionale Tumori, Milano, Italy.

#93

100 **074.1** **Immunotherapy with rHuIL-2 after autologous BMT for neuroblastoma**
Arcangelo Prete, Roberto Rondelli, Franco Locatelli, Sara Pierinelli, Franca Fagioli, Chiara Messina, Alberto Garaventa, Andrea Pession
For the AIEOP BMT Registry.

#94

100 **273.2** **A pilot study of sequential myeloblative autologous stem cell transplantation (MA-AUTOSCT) and immunotherapy with reduced intensity allogenic stem cell transplant (RI-ALLOSCT) for high/risk neuroblastoma**
Darrell J Yamashiro, Mary B Bradley, Lauren A Harrison, Alice Lee Julia, Glade Bender, Olga Bessmertny, James H Garvin, Gustavo Del Toro, Diane George, Mitchell S Cairo
Department of Pediatrics, Columbia University, New York, USA.

#95

100 **361.3** **Intrathecal 131-I-labeled 3F8 antibody in patients (PTS) with GD2-expressing central nervous system (CNS) and leptomeningeal (LM) neoplasms**
Kim Kramer, Nai-Kong V Cheung, John Humm, Ester Dantis, Ira J Dunkel, Suzanne Wolden, Ronald Finn, Brian H Kushner, Yasmin Khakoo, Mark M Souweidane, Pat Zanzonico, Keith Pentlow, Samuel Yeh, Steven M Larson
Department of Pediatrics, Memorial Sloan-Kettering Cancer Center, New York, USA.

#96

101 **034.1** **Laparoscopic adrenal surgery for neuroblastomas in children**
Pascal De Lagausie, Dominique Berrebi, Pascale Philippe-Chomette, Jean-Michel Michon, Catherine Garel, Michel Peuchmaur, Yves Aigrain
Pediatric Surgery, Hopital Robert Debré, Paris, France.

#97

101 **128.1** **Long-term complications from treatment in survivors of high risk neuroblastoma**
Caroline Laverdiere, Nai-kong V Cheung, Brian H Kushner, Kim Kramer, Shakeel Modak, Michael P LaQuaglia, Suzanne Wolden, Kirsten K Ness[2], James G Gurney[2], Charles A Sklar
Department of Pediatrics, Department of Surgery, Department of Radiation Oncology, Memorial Sloan Kettering Cancer Center, New York; Division of Pediatric Epidemiology and Clinical Research2, University of Minnesota, USA.

#98

101 **118.1** **Prospective analysis of complications central venous catheter related in paediatric patients with neuroblastoma. An experience of a single institution**
Cinzia Mazzola[1], Giuseppe Fratino[1], Gian Paolo Cuneo[1], Alberto Michelazzi[1], Angelo C Molinari[2], Elio Castagnola[3], Riccardo Haupt[4], Antonino Rizzo[1]
Department of Paediatric Surgery[1], Department of Paediatric Haematology and Oncology - Thrombosis and Hemostasis Unit[2], Department of Infections
Diseases[3], and Epidemiology and Biostatistics Section[4], Scientific Directorate, Giannina Gaslini Children's Hospital, Genova, Italy.

#99

101 **382.1** **Anemia is a risk factor for cisplatin-induced hearing impairment in children with high-risk neuroblastoma**
Anders E Svedmyr[1], Hans Ehrsson[1], Erik Forestier[1], Per Kogner[2]
Department of Pediatrics[1], Childhood Cancer Research Unit, Umeå; Childhood Cancer Research Unit[2], Karolinska, Hospital, Stockholm, Sweden.

#99 bis

138 **239.3** **Ibuprofen interferes with the capillary gas- chromatography determination of urinary HVA and VMA in neuroblastoma diagnosis**
Levreri I., Caruso U.*, Deiana F., Buoncompagni A.**, De Bernardi B.***, Melioli G., Marchese N.
*Clinical Pathology, * University Dept.of Pediatrics, ** Pediatrics Dept, ***Dept. Of Hematology-Oncology, Giannina Gaslini Children's Institute, Genova, Italy.*

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102 **194.1** **Gene expression profiling of neuroblastoma**
Junko Takita, Masami Ishii, Shuichi Tsutsumi, Yukichi Tanaka, Keisuke Kato, Yasunori Toyoda, Ryoji Hanada, Keiko Yamamoto, Fumio Bessho, Hiroyuki Aburatani, Yasuhide Hayashi
Pediatrics, Graduate School of Medicine University of Tokyo, Japan.

#101

54 **122.1** **Prediction of clinical outcome and biological subclassification of primary neuroblastomas by expression profiling**
Alexander Schramm, Johannes H Schulte, Ludger Klein-Hitpass, Hauke Sieverts, Bernd Berwanger, Holger Christiansen, Thorsten Simon, Frank Berthold, Werner Havers, Patrick Warnat, Benedikt Brors, Jürgen Eils, Roland Eils, Angelika Eggert
Children´s Hospital, University Clinic Essen, NRW, Germany.

#102

102 **371.1** **Gene expression associated with prognosis of neuroblastoma**
Hitoshi Ichikawa[1], Tomohito Yagi[1], Miki Ohira[2], Jun Yasuda[1], Misao Ohki[3], Akira Nakagawara[2]
Cancer Transcriptome Project[1] and Cancer Genomics Project[3] National Cancer Center Research Institute, Tokyo; Division of Biochemistry[2], Chiba Cancer Center Research Institute, Japan.

#103

54 **236.1** **Microarray expression profiling improves definition of high- and intermediate-risk metastatic neuroblastomas without MYCN amplification**
Shahab Asgharzadeh, Hong Wang, Yujun Yang, Hiro Shimada, Katherine Matthay, Jonathan Buckley, Robert C Seeger
Department of Pediatric Hematology-Oncology, Children's Hospital of Los Angeles, CA, USA.

#104

102 **185.1** **Functional genomics in neuroblastoma**
Daniela Spano[1], Veruska Aglio[2], Maria D'Apolito[3], Giuseppe Russo[4], Veronica Servedio[5], Achille Iolascon[1], Massimo Zollo[2]
C.E.I.N.G.E.[1], Università degli Studi di Napoli Federico II, and T.I.G.E.M.[2] Napoli; Servizio di Genetica Medica[3], CSS-IRCCS, San Giovanni Rotondo, Foggia, Italy; Sbarro Institute for Cancer Research and Molecular Medicine[4], Temple University, Philadelphia, PA, USA; Laboratorio di Medicina Molecolare[5], Ospedali Riuniti, Foggia, Italy.

#105

102 **207.1** **Use of CGH array to identify MYCN amplification and chromosome 1P36 deletion in neuroblastoma**
Paola Scaruffi[1], Stefano Parodi[2], Katia Mazzocco[1], Raffaella Defferrari[1], Vincenzo Fontana[2], Stefano Bonassi[2], Gian Paolo Tonini[1]
Laboratory of Neuroblastoma[1], National Institute for Cancer Research (IST) and Environmental Epidemiology and Biostatistics[2], National Institute for Cancer Research (IST), Genoa, Italy.

#106

54 **070.1** **Gene expression profiling suggests a role for haploinsufficiency of 1p35-36 genes in neuroblastoma**
Isabelle Janoueix-Lerosey, Eugene Novikov, Marta Monteiro, Nadège Gruel, Gudrun Schleiermacher, Béatrice Loriod, Catherine Nguyen, Olivier Delattre
INSERM U509, Institut Curie, Paris, France.

#107

103 **367.1** **Gene expression profiling of neuroblastoma cell lines treated identifies genes upregulated by retinoic acid that are overexpressed in favorable neuroblastoma primary tumors**
Eiso Hiyama[1,3], Keiko Hiyama[2], Takeshi Kobayashi[3], Hiroaki Yamaoka[1,3], Masahiko Nishiyama[2], Taijiro Sueda[3], C. Patrick Reynolds[4]
Department of Pediatric Surgery[1], Hiroshima Univ. Hosp, Department Translational Cancer Research[2], RIRBM, Hiroshima Univ., Hiroshima; Department of Surgery[3], Graduate School of Biomedical Science, Hiroshima Univ., Hiroshima, Japan; Childrens Hospital Los Angeles[4], USA.

#108

103 **329.1** **Identification of a gene signature discriminating stage 4S and stage 4 neuroblastoma using serial analysis of gene expression**
Matthias Fischer, André Oberthuer, Yvonne Kahlert, Benedikt Brors, Frank Berthold
Department of Pediatric Oncology and Hematology, University Children's Hospital, Cologne, Germany.

#109

103 **395.1** **Gene expression differences among stages in stroma-poor neuroblastoma**
Orit E Oppenheimer[1], Adam B Olshen[1], Alaminos Miguel[1], Mora Jaume[2], Nai-Kong V Cheung[1], Gerald L William[1]
Department of Pediatric Hematology Oncology[1], Memorial Sloan Kettering Cancer Center, New York, USA; Hospital Sant Joan de Deu de Barcelona[2], Spain.

#110

55 **208.1** **A novel region for predisposition to neuroblastoma maps to chromosome 12**
Luca Longo[1], Gian Paolo Tonini[1], Simona Coco[1], Marcella Devoto[2], Marco Seri[3], Giovanni Romeo[3], Patrizia Perri[1]
Laboratory of Neuroblastoma[1], National Institute for Cancer Research (IST), Genova; University of Bologna[3]; Bologna, Italy; Nemours Children's Clinic-Wilmington[2], DE, USA.

#111

103 **223.2** **Genome-wide transcriptome analysis for neuroblastoma gene discovery and refinement of clinical risk prediction**
Qun Wang, Sharon Diskin, Suzanne Shusterman, Eric Rappaport, Yael P Mosse, Deepa Khazi, Cynthia Winter, George Hii, Edward F Attiyeh, Rebecca King, Nicholas Rhodin, Gregory Grant, Katherine K Matthay, Wendy B London, John M Maris
Department of Oncology, Children's Hospital of Philadelphia, PA, USA.

#112

104 **392.1** **Methylation of multiple CpG islands in neuroblastomas with poor prognosis**
Toshikazu Ushijima, Masanobu Abe, Miki Ohira, Atsushi Kaneda, Yukiko Yagi, Yoshihiro Kitano, Takashi Sugimura, Akira Nakagawara
Carcinogenesis Division, National Cancer Center Research Institute, Tokyo, Japan.

#113

104 **321.2** **Expression profiling of aggressive and benign neuroblastic tumors: is there a common pattern?**
Peter F Ambros[1], Cornelia Stock[1], Eva Bozsakyova[1], Inge M Ambros[1], Andrea Luegmayr[1], Ruth Ladenstein[2], Helmut Gadner[2]
Tumorcytogenetics[1] and Oncology Department[2], CCRI, St. Anna Kinderspital, Vienna, Austria.

#114

104 **287.1** **A ran-binding protein, RanBPM, stabilizes p73 and enhances its pro-apoptotic activity: Its possible role in spontaneous regression of neuroblastoma**
Sonja Kramer, Toshinori Ozaki, Kou Miyazaki, Akira Nakagawara
Division of Biochemistry, Chiba Cancer Center Research Institute, Japan.

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#115

104 **207.2** **Identification of genes differently expressed in favorable and unfavorable histology neuroblastoma tumors**
Paola Scaruffi[1], Stefano Moretti[2], Stefano Bonassi[2], Gian Paolo Tonini[1]
Laboratory of Neuroblastoma1, National Institute for Cancer Research (IST); Department of Mathematics, University of Genoa and Environmental Epidemiology and Biostatistics[2], Italy.

#116

105 **154.1** **Characterization of amplicons in neuroblastoma cell lines and tumours by microarray-based comparative genomic hybridization**
Anne Fix, Martine Peter, Gaelle Pierron, Alain Aurias, Olivier Delattre, Isabelle Janoueix-Lerosey
INSERM U509, Institut Curie, Paris, France.

#117

105 **251.3** **Isolation of normal neuroblastoma precursor cells: tools for comparative gene expression profiling**
Katleen De Preter[1], Jo Vandesompele[1], Pierre Heimann[2], Nadine Van Roy[1], Frank Speleman[1]
Center for Medical Genetics[1], University Hospital Ghent; Department of Medical Genetics[2], University Hospital Erasme, Brussels, Belgium.

#118

105 **046.3** **Genome-wide analysis of gene expression in neuroblastomas detected by mass screening**
Alexander Krause[1], Valérie Combaret[2], Isabelle Iacono[2], Bruno Lacroix[1], Christelle Compagnon[1], Christophe Bergeron[2], Philippe Leissner[1], Bruno Mougin[1], Alain Puisiex[2]
Human Genetics Department[1], BioMérieux SA, Marcy l'Etoile, and Unité d'Oncologie Moléculaire[2], Centre Léon Bérard, Lyon, France.

#119

105 **346.1** **Prediction of clinical outcome using gene expression profiling and artificial neural networks for patients with neuroblastoma**
Jun S Wei[1], Braden T Greer[1], Frank Westermann[2], Seth M Steinberg[1], Chang-Gue Son[1], Qing-Rong Chen[1], Craig C Whiteford[1], Bilke Sven[1], Krasnoselsky L Alexei[1], Cenacchi Nicola[1], Catchpoole Daniel[3], Berthold Frank[4], Schwab Manfred[2], Khan Javed[1]
Department of Pediatric Oncology Branch [1], National Cancer Institute, National Institutes of Health Gaithersburg, MD and The Children's Hospital at Westmead3, USA; German Cancer Research Center[2] and Klinik für Kinderheilkunde der Universitätzu Köln[4], Germany.

#120

106 **090.1** **CGH in chemoresistant neuroblastoma cell lines**
Ales Vicha[1], Jiri Bednicek[1], Tomas Eckschlager[1], Cinatl Jaroslav[1], Cinatl Jindrich[2]
Pediatric Hematology and Oncology[1] 2nd, Medical Faculty and Faculty Hospital Motol, Prague, Czech Republic; Institute virology JV Goethe medical centre, Frankfurt, Germany.

#121

106 **348.1** **Phylogeny of neuroblastoma tumor progression from comparative genomic hybridization data**
Sven Bilke, Quing-Rong Chen, Jun S Wei, Craig C Whiteford, Nicola Cenacchi, Alexei L Krasnoselsky, Braden Greer, Chang-Gue Son, Frank Westerman, Frank Berthold, Manfred Schwab, Daniel Catchpoole, Javed Khan
Department of Pediatric Oncology Branch, National Institutes of Health, Gaithersburg, Maryland, USA

#122

106 **360.1** **Genome-wide micro-array analysis of neuroblastoma links genomic alterations to expression profiles**
Alexei L Krasnoselsky, Sven Bilke, Craig C Whiteford, Qing-Rong Chen, Jun S Wei, Frank Westermann, Frank Berthold, Manfred Schwab, Javed Khan
Department of Pediatric Oncology Branch, National Cancer Institute, Gaithersburg, Maryland, USA.

#123

106 **179.1** **Expression of the PPM1D gene is associated with poor prognosis in Neuroblastoma**
Yuyan Chen[1], Junko Takita[1], Ryoji Hanada[2], Keiko Yamamoto[2], Yukichi Tanaka[3], Yasunori Toyoda[4], Yasuhide Hayashi[5]
Department of Pediatrics[1], University Tokyo,; Division of Hematology/Oncology[2], Saitama Children's Medical Ctr., Saitama; Division of Pathology[3] and Division of Hematology[4], Kanagawa Children's MedicalCtr., Kanagawa; Gunma Children's Medical Ctr.[5], Gunma, Japan.

#124

107 **056.1** **Alternative pathways of MYCN gene copy number increase in primary neuroblastoma tumors**
Alexander Valent[1], François Lozach, Marine Guillaud-Bataille, Barbara Spengler[2], Marie-José Terrier-Lacombe, Dominique Valteau-Couanet, Olivier Brison, Jean Bénard, Alain Bernheim
Génomique Cellulaire des Cancers[1], Institut Gustave Roussy, Villejuif, France; Laboratory of Neurobiology[2], Department of Biological Sciences, Fordham University, Bronx, NY, USA.

#125

54 **107.1** **MYCN-status according to FISH: Amplification, gain, and non-amplification**
Ruediger Spitz, Barbara Hero, Matthias Skowron, Karen Ernestus, Frank Berthold
Pediatric Oncology and Hematology, Children\'s Hospital, Cologne, Germany.

#126

107 **363.1** **RNA Interference to elucidate the mechanism of cell cycle regulation by N-myc in neuroblastoma cells**
Chan-Wook Woo, Hope Cassano, Carol J Thiele[1]
Cell & Molecular Biology Section, Pediatric Oncology Branch, National Cancer Institutes, National Institutes of Health, Bethesda, MD, USA.

#127

55 **049.1** **IGFBP-5 in neuroblastoma: complex regulations and multiple effects.**
Vincenzo Cesi, Barbara Tanno, Roberta Vitali, Fabiola Sesti, Maria Laura Giuffrida, Camillo Mancini, Giuseppe Raschellà
Department of Toxicology and Biomedical Sciences, ENEA Research Center Casaccia, Rome, Italy.

#128

107 **251.1** **Combined subtractive cDNA cloning and array CGH: An efficient approach for identification of overexpressed genes in DNA amplicons**
Katleen De Preter, Filip Pattyn, Geert Berx, Kristine Strumane, Björn Menten, Frans Van Roy, Anne De Paepe, Frank Speleman, Jo Vandesompele
Center for Medical Genetics, University Hospital Ghent, Belgium.

#129

107 **328.1** **Gene expression patterns in neuroblastoma cells mediated by TrkA and TrkB expression and activation**
Jane E Minturn, Ruth Ho, Eric Rappaport, Audrey E Evans, Garrett M Brodeur
Division of Oncology, Children's Hospital of Philadelphia, PA, USA

#130

108 **373.1** **Application of proteomics to identify novel targets of Trk-signaling in neuroblastoma cells**
Angelika Eggert[1], Ognjan Apostolov[1], Barbara Sitek[2], Kathy Pfeiffer[2], Kai Stühler[2], Helmut E Meyer[2], Werner Havers[1], Alexander Schramm[1]
Department Hematology/Oncology[1], University Children\'s Hospital of Essen; Medical Proteome Center Bochum[2], Germany.

#131

108 **264.1** **Genes regulating drug sensitivity in human neurobalstoma**
Vitaliya Sagulenko, Frank Westermann, Manfred Schwab
Tumor Genetics, German Cancer Research Center, Heidelberg, Germany.

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108 **273.1** **ID2 promotes angiogenesis in neuroblastoma**
Alice Lee, Anna La sorella, Gerson Rothschild, Antonio Iavarone, Jessica J Kandel, Darrrell J Yamashiro
Pediatrics, Columbia University, New York, USA.

#133

66 **025.1** **Id2 is sufficient and necessary for growth and angiogenesis in neuroblastoma**
Anna Lasorella, Gerson Rothschild, Alice Lee, Jessica J Kandel, Darrell J Yamashiro, Antonio Iavarone
Institute for cancer Genetics, Columbia University, NY, USA.

#134

108 **288.1** **Down regulation of gene-expression by N-myc in Neuroblastoma**
Arjen Koppen, Rachida Ait-Aissa, Antoni PA Hendrickx, Rogier Versteeg, Linda J Valentijn
Human Genetics, Academic Medical Center, Amsterdam, The Netherlands.

#135

109 **162.1** **MYCN signalling pathways in vivo and in vitro**
Frank Westermann, Benedikt Brors, Jun Wie, Rainer Koenig, Ruprecht Wiedemeyer, Kai Henrich, Matthias Fischer, Andre Oberthuer, Frank Berthold, Javed Khan, Manfred Schwab
Department. Tumour Genetics (B030), German Cancer Research Centre, Heidelberg, Germany.

#136

109 **355.1** **Loss of hCas function, a novel neuroblastoma differentiation related gene, via LOH at chromosome 1p36.22 and functional silencing by N-myc**
Xuezhong Yang, Linda Stapleton, Valery Bliskovsky, Gautam Rajpal, Kathleen Cullion, Beverly Mock, Thomas Ried, Carol J Thiele
Pediatric Oncology Branch, National Cancer Institute, Bethesda, MD, USA.

#137

109 **399.1** **A constitutional translocation t(1;17)(p36.2;q11.2) in a neuroblastoma patient disrupts NBG1, a putative tumor suppressor gene**
Frans Van Roy[1], Karl Vandepoele[1], Nadine Van Roy[2], Katrien Staes[1], Geneviève Laureys[3], Geert Berx[1], Frank Speleman[2]
Department for Molecular Biomedical Research[1], Department of Medical Genetics[2], Department of Pediatric Oncology[3], VIB - Ghent University, Belgium.

#138

67 **078.1** **KIF1B is a variant-dependent tumor suppressor gene mapped to a 500-kb homozygously deleted region at chromosome 1p36.2 in neuroblastoma**
Arasambattu K Munirajan, Masato Takahasi, Miki Ohira, Toshinori Ozaki, Hajime Kageyama, Kou Miyazaki, Akira Nakagawara
Division of Biochemistry, Chiba Cancer Center Research Institute, Chiba, Japan.

#139

55 **089.1** **A novel 1p36.2 located gene, APITD1, with tumor suppressive properties and a putative p53 binding domain, shows low expression in neuroblastoma tumors**
Cecilia Krona[1], Katarina Ejeskär[1+2], Helena Carén[1], Frida Abel[1], Rose-Marie Sjöberg[1], Tommy Martinsson[1]
Department of Clinical Genetics[1], Inst. forHealth of Women and Children, Göteborg University, Sahl, Sweden; Cell and Gene Therapy Group[2], Murdoch Children's Research Institute, Parkville, Australia.

#140

109 **340.1** **Detailed characterization of a 1p36.3 region consistently deleted in neuroblastoma**
Peter S White, Patricia M Thompson, Takahiro Gotoh, Jun Igarashi, Erin R Okawa, Cindy Winter, John M Maris, Garrett M Brodeur
Department of Pediatrics, University of Pennsylvania, Philadelphia, PA, USA.

#141

110 **337.1** **Functional and expression analysis of CHD5, a candidate neuroblastoma suppressor on 1p36**
Jun Igarashi, Peter S White, Erin R Okawa, Qin Wang, John M Maris, Garrett M Brodeur
Division of Oncology, The Children's Hospital of Philadelphia, Pennsylvania (PA), USA.

#142

110 **282.1** **HsCAMTA1 and FLJ10737 within a commonly deleted region at 1p36 in neuroblastoma**
Kai-Oliver Henrich[1], Frank MW Westermann[1], Axel Benner[2], Frank Berthold[3], Manfred Schwab[1]
Deutsches Krebsforschungszentrum, Division of Tumour Genetics[1], Heidelberg; Central Unit Biostatistics[2], Deutsches Krebsforschungszentrum; Univ.-Kinderklinik Köln, Abteilung für Pädiatrische Onkologie[3], Germany.

#143

110 **240.1** **Methylation studies of genes located in the chromosome region 1p36.2 in neuroblastoma tumors**
Helena Carén[1], Susanne Fransson[1], Rose-Marie Sjöberg[1], Katarina Ejeskär[1], Luke Hesson[2], Farida Latif[2], Tommy Martinsson[1]
Department of Clinical Genetics[1] and Section of Medical and Molecular Genetics[2], Department of Paediatrics and Child Health, Göteborg University, Sahlgrenska Univ Hospital, Sweden.

#144

110 **162.2** **Combination of loss of heterozygosity and ploidy analysis clearly distinguishes between regions of allelic imbalance and allelic loss at 1p36 in neuroblastoma**
Frank Westermann, Young-Gyu Park, Ruediger Spitz, Kai Henrich, Volker Ehemann, Karen Ernestus, Henriette Kurth, Frank Berthold, Manfred Schwab
Department of Tumour Genetics (B030), German Cancer Research Centre, Heidelberg, Germany.

#145

111 **213.1** **LOH analysis on microdissected neuroblastic cells reveals undetected interstitial deletions at chromosome 1p36 in neuroblastoma**
Simona Coco[1], Luca Longo[2], Patrizia Perri[1], Carla Marino[3], Claudio Gambini[3], Katia Mazzocco[1], Raffaella Defferrari [1] , Gian Paolo Tonini [2]
Laboratory of Neuroblastoma[1], Italian Neuroblastoma Foundation; Laboratory of Neuroblastoma[2], National Institute for Cancer Research (IST); Service of Pathology[3], Gaslini Children's Hospital, Genoa, Italy.

#146

111 **075.3** **Analysis of MCL1 as a candidate oncogene within the 1Q region of gain in high-risk neuroblastomas**
Xueyuan Liu[1], Kelly C Goldsmith[1], Vincent Dam[1], Brian T Morgan[1], Pavel Mazanek[2], Yael P Mosse[1], Christopher S Hackett[3], Murray D Norris[4], Michelle Haber[4], William A Weiss[3], Michael D Hogarty[1]
Department of Pediatrics[1], The Children's Hospital of Philadelphia, PA; University of California at San Francisco[3], CA, USA; Children's Hospital Brno[2], Czech Republic; Children's Cancer Institute Australia for Medical Research[4]

#147

111 **071.2** **cDNA macroarrays analyses of p73-target genes reveal that Wnt8A is up-regulated by DeltaN-p73 in SH-SY5Y cells**
David Goldschneider, Etienne Blanc, Karine Million, Hedi Haddada, Gilda Raguenez, Jean Benard, Sétha Douc-Rasy
CNRS-UMR 8126, Institut Gustave Roussy, Villejuif, France.

#148

111 **224.2** **p53 and JNK Apoptotic Pathways in Neuroblastoma**
Pamela P McKenzie, Linda C Harris
Department of Molecular Pharmacology, St. Jude Children\'s Research Hospital, Memphis, TN, USA

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#149

112 **087.3** **High expression of novel HECT-type E3 ubiquitin ligases, NEDL1 and NEDL2, which target Dishevelled-1 and p73, respectively, is associated with favorable neuroblastomas with spontaneous regression**
Kou Miyazaki[1], Toshinori Ozaki[1], Junko Hirato[2], Akira Nakagawara[1]
Division of Biochemistry[1], Chiba Cancer Center Research Institute; Department of Pathology[2], Gunma University School of Medicine Japan.

#150

112 **238.1** **Shc family expression in neuroblastoma**
Elena Terui, Tadashi Matsunaga, Hideo Yoshida, Katsunori Kouchi, Hiroaki Kuroda, Tomolo Hishiki, Takeshi Saitoh, Shin-ichi Yamada, Hiroshi Shirasawa, Naomi Ohnuma
Department of Pediatric Surgery and Department of Microbiology, Chiba University, Japan.

#151

112 **260.1** **Hunting for a 3p tumor suppressor gene in neuroblastoma: a combined genomic and functional approach**
Jasmien Hoebeeck[1], Jo Vandesompele[1], Bram De Wilde[1], Evi Michels[1], Nadine Van Roy[1], Valérie Combaret[2], Nurten Yigit[1], Els De Smet[1], Claire Hoyoux[3], Geneviève Laureys[1], Anne De Paepe[1], Frank Speleman[1]
Center for Medical Genetics[1], Ghent University Hospital and Centre Hospitalier Régional de la Citadelle[3], Liege, Belgium; Centre Léon Bérard[2], Lyon, France.

#152

112 **315.1** **MSX1: A target from the MEIS1 oncogene located on the 4p16 tumour suppressor region**
Ingrid M Revet[1], Gerda Jorritsma[1], Patrizia Perri[2], Rogier Versteeg[1], Dirk Geerts[1]
Human Genetics[1], Academic Medical Centre, Amsterdam, The Netherlands; Laboratory of Neuroblastom[2] – Italian Neuroblastoma Foundation, Genoa, Italy

#153

113 **276.1** **Somatic genetic events occurring in infant with neuroblastoma during the first year of life**
Gian Paolo Tonini[1], Katia Mazzocco[2], Raffaella Defferrari[2], Simona Coco[2], Luca Longo[1], Patrizia Perri[2], Paola Scaruffi[1], Francesca Negri[3], Claudio Gambini[3], Bruno De Bernardi[4], Andrea Di Cataldo[5]
[1]Laboratory of Neuroblastoma, National Institute for Cancer Research (IST), [2] Laboratory of Neuroblastoma, Italian Neuroblastoma Foundation, [3]Department of Pathology and [4]Hematology/Oncology, Gaslini Children's Hospital, Genoa, [5]Department of Pediatric Hematology/Oncology, University of Catania, Italy.

#154

113 **223.1** **Additional evidence for linkage of hereditary neuroblastoma to 16p13 and discovery of cooperating mutations**
Yael P Mosse, Marci B Laudenslager, Deepa Khazi, George Hii, Huaqing Zhao, Eric Rappaport, Patricia Dahia, Garrett M Brodeur, Barbara L Weber, John M Maris
Department OF Oncology, Children's Hospital of Philadelphia, PA, USA.

#155

113 **169.1** **High-resolution Array-based Genomic Profiling of the Critical Region of Chromosome 17 Gain**
Jo Vandesompele, Björn Menten, Nadine Van Roy, Evi Michels, Els De Smet, Stefan Vermeulen, Anne, De Paepe, Geneviève Laureys, Frank Speleman
Center for Medical Genetics, Ghent University Hospital, Belgium.

#156

113 **105.1** **Regions syntenic to human 17q are gained in mouse and rat neuroblastoma cell lines**
Maria Lastowska[1], Yeun-Jun Chung[2], Ngan C Cheng[3], Michelle Haber[3], Murray D Norris[3], Ursula R Kees[4], Andrew DJ Pearson[1], Michael S Jackson[1]
The University of Newcastle upon Tyne[1] and The Wellcome Trust Sanger Institute[2], Hinxton, Cambridge, UK; Children's Cancer Institute Australia[3], Sydney, NSW 203[1], and The University of Western Australia[4], Perth, Australia.

#157

114 **281.1** **Interphase FISH reveals intratumoral heterogeneity of chromosome 17(q) status in neuroblastoma: implications for risk assessment**
Nadine Van Roy[1], Jo Vandesompele[1], Karen Depourcq[1], Els De Smet[1], Geneviève Laureys[2], Ivo De Wever[3], Anne De Paepe[1], Frank Speleman[1]
Department Medical Genetics[1] and Pediatric Oncology[2], University Hospital, Gent; Oncological surgery section[3], kuLeuven, Leuven, Belgium.

#158

114 **044.1** **Molecular cloning of unbalanced chromosome 17 breakpoints in neuroblastoma cell lines reveals distinct underlying mechanisms**
Gudrun Schleiermacher[1], Franck Bourdeaut[1], Isabelle Janoueix-Lerosey[1], Valérie Combaret[2], Alain Aurias[1], Olivier Delattre[1]
INSERM U509[1], Institut Curie, 26 rue d\'Ulm, Paris; and Laboratoire de Biologie Cellulaire[2], Centre Léon Bérard, Lyon, France.

#159

114 **092.1** **Quantitative analysis and detailed mapping of 17q gains in neuroblastomas using array CGH**
Marine Guillaud-Bataille[1], Alexander Valent[1], Christine Perot[1], Pascal Soularue[2], Amandine Pitaval[2], Hugues Roest Crollius[3], Hugues Ripoche[4], Jean Bénard[5], Vladimir Lazar[6], Marie-José Terrier-Lacombe[7], Gilbert Lenoir[6], Xavier Gidrol[2], Philippe Dessen[4], Alain Bernheim[1], Gisèle Danglot[1]
Laboratoire de génomique cellulaire des cancers CNRS UMR 81251, Bioinformatique4, Service de Génétique5, Génomique fonctionnelle6, Anatomopathologie7, Institut Gustave Roussy (+9), Villejuif; Génomique fonctionnelle2, CEA, Centre national de séquençage3, Evry, France.

#160

114 **029.1** **EMP3, a putative tumor suppressor gene mapping at 19q13.3, is epigenetically silenced In neuroblastoma by promoter hypermethylation and may have prognostic value**
Miguel Alaminos[1], Veronica Davalos[1], Nai-Kong V Cheung[2], William L Gerald[2], Manel Esteller[1]
Cancer Epigenetics Laboratory[1], Spanish National Cancer Center (CNIO), Madrid, Spain; Memorial Sloan-Kettering Centre[2], New York, USA.

#161

115 **243.1** **LIM-only protein, LMO3, is up-regulated and acts as an oncogene in aggressive neuroblastomas by physically interacting with a neuronal bHLH transcription factor, HEN2**
Mineyoshi Aoyama, Toshinori Ozaki, Hiroyuki Inuzuka, Daihachiro Tomotsune, Junko Hirato, Yoshiaki Okamoto, Hisashi Tokita, Akira Nakagawara
Division of Biochemistry, Division of Animal Science, Chiba Cancer Center Research Institute and Department of Pathology, Gunma University Japan.

#162

115 **302.1** **Imbalance of transcripts encoding mitochondrial pro- and anti-apoptotic members in unfavorable neuroblastoma**
Frida Abel[1], Rose-Marie Sjoberg[1], Staffan Nilsson[2], Per Kogner[3], Tommy Martinsson[1]
Department of Clinical Genetics[1], Gothenburg University, Sahlgrenska University Hospital East, Institution of Mathematical Statistics[2], Chalmers University of Technology, Gothenburg; Childhood Cancer Research Unit[3], Karolinska Institute, Karolinska Hospital, Stockholm, Sweden.

#163

115 **312.1** **Tumor suppressor genes methylation in neuroblastoma: RASSF1 gene is almost always methyled in primary tumors**
Mariana B Michalowski[1], Florence De Frapoint[1], Sylvie Michelland[1], Valerie Combaret[2], Marie-Christine Favrot[1], Dominique Plantaz[1]
UF Cancerologie Biologique et Biotherapie[1], Centre Hopitalier Universitaire de Grenoble, Grenoble cedex 9 and Centre Leon Berard[2], Lyon, France.

#164

115 **251.2** **A putative haplo-insufficient contribution of SDHD to high stage neuroblastoma tumorigenesis**
Katleen De Preter, Jo Vandesompele, Jasmien Hoebeeck, Caroline Vandenbroecke, Joel Smet, Annick Nuyts, Geneviève Laureys, Valerie Combaret, Nadine Van Roy, Rudy Van Coster, Marleen Praet, Anne De Paepe, Frank Speleman
Center for Medical Genetics, University Hospital Ghent, Belgium.

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116 **356.1** **Akt pathway mediates BDNF/TrkB resistance to chemotherapy in neuroblastoma cells** **#165**
Zhijie Li, Jerry Jaboin, Carol J Thiele
Pediatric Oncology Branch, National Cancer Institute, Bethesda, Maryland, USA.

116 **339.2** **Tight regulation of retroviral gene expression by combination of advanced tetracycline-transactivator and repressor proteins** **#166**
Petra Obexer, Michael J Ausserlechner, Bernhard Meister, Reinhard Kofler
Department of Pediatric Oncology, Tyrolean Cancer Research Institute, Innsbruck, Austria.

116 **230.1** **NTRK1 gene polymorphisms in 30 primary neuroblastoma tumours** **#167**
Lipska BS[1,3], Drozynska E[2], Kardas I[1], Limon J[1]
Department of Biology and Genetics[1], Institute of Pediatrics[2], Postgraduate School of Molecular Medicine[3], Medical University of Gdansk, Poland.

116 **097.1** **Notch-1 signaling in neuroblastoma cells** **#168**
Marie Stockhausen, Jonas Sjölund, Christina Manetopoulos, Håkan Axelson
Department of Molecular Medicine, Laboratory Medicine, U-MAS, Malmö, and Unit for Molecular Medicine, U-MAS, Lund, Sweden.

117 **099.1** **Comparative analysis of transcription factor function in pre B and neuroblastoma cells** **#169**
Christina Manetopoulos, Anna Lagergren, Håkan Axelson, Mikael Sigvardsson
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Paediatrics[1], CHUV University Hospital, Lausanne, Switzerland; Departement de Pediatrie[2], UPRES EA3535, Institut Gustave Roussy, Villejuif, France.

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Department of Pediatrics and The Robert H. Lurie Comprehensive Cancer Center, Northwestern University, Feinberg School of Medicine, Chicago, IL, USA.

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Department of Surgery, Gunma Children\'s Medical Center, Gunma, Japan.

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Department of Pediatric Oncology and Hematology, and Molecular Tumor Biology and TumorImmunology, University of Cologne, Germany.

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Department of Pediatrics, Kyoto Prefectural University of Medicine, Graduate School of Medical Science, Kyoto, Japan.

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Department of Pediatric Hematology and Oncology[1], Tohoku University School of Medicine, Sendai, Miyagi, Japan; Cell & Molecular Biology Section/Pediatric Oncology Branch[2], National Cancer Institute, Bethesda, USA.

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Karolinska Institutet, Childhood Cancer Research Unit, Woman and Child Health, Stockholm, Sweden.

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Department of Pediatrics[1], University of Bologna, S. Orsola Hospital, Bologna; Department of Organic and Industrial Chemistry[2], University of Parma, Italy.

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Division of Paediatric Oncology[1] and Division of Haematology[3] Catholic; Department of Oncology [2] - R&D Sigma Tau, Pomezia, Rome; Mario Negri[4], Milan, Italy.

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Roberta Castriconi1,3, Alessandra Dondero1, Barbara Carnemolla2, Raffaella Augugliaro2, Claudia Cantoni1, 3, Francesca Negri3, Maria Valeria Corrias3, Lorenzo Moretta1, 3, Alessandro Moretta1, Cristina Bottino3
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Roberta Castriconi[1], Michele Cilli[2], Barbara De Giovanni[3], Alessandro Dondero[1], Annalisa Pezzolo[4], Vito Pistoia[4], Claudio Gambini[3], Alessandro Moretta[1], Maria Valeria Corrias[4]
Department of Experimental Medicine[1], University of Genoa, Service of Animal Model [2], IST, Service of Pathology[3] and Laboratory of Oncology[4], Giannina Gaslini Children's Hospital, Genoa, Italy.

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Hong-wei Wu, Zamir Warsi, Leonid Metelitsa, Shahab Asgharzadeh, Susan Groshen, Robert Seeger
Division of Hematology/Oncology, Department of Pediatrics, Children's Hospital Los Angeles/USC, CA, USA.

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133 **058.1** **Preclinical evaluation of autologous dendritic cells transfected with tumor mRNA or loaded with apoptotic cells for immunotherapy of high risk neuroblastoma**
Silvija Jankovic[1], Rolf D Pettersen[1], Stein Sæbøe Larssen[2], Finn Wesenberg[1], Mette RK Olafsen[1], Gustav Gaudernack[2]
Department of Pediatric Research1, National Hospital of Norway, Oslo, Norway; Section for Immunotherapy[2], The Norwegian Radium Hospital, Oslo, Norway.

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Stefan Fest[1], Nicole Huebener[1], Yan Zeng[1], Anne Strandsby[1], Silke Weixler[1], Gerhard Gaedicke[1], Angelica Riemer[2], Elisabeth Förster-Waldl[2], Erika Jensen-Jarolim[2], Holger N Lode[1]
General Pediatrics, Experimental Oncology, Charite, Universitätsmedizin Berlin, Campus Virchow, Germany; Universtity Vienna, Austria.

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134 **134.1** **Production of dimeric small immunoproteins specific for neuroblastoma-associated antigen GD2**
Marco Bestagno[1], Marzia Occhino[1], Lizza Raffaghello[2], Oscar R Burrone[1], Vito Pistoia[2], Maria Valeria Corrias[2]
Immunology Department[1], ICGEB, Trieste; Laboratory of Oncology[2], Giannina Gaslini Children's Hospital, Genova, Italy.

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134 **221.1** **Isolation and characterisation of peptide mimotopes of the neuroblastoma-associated carbohydrate antigen GD2 ganglioside**
Irena Horwacik[1], Dominik Czaplicki[1], Katarzyna Talarek[1], Aleksandra Kowalczyk[1+2], Hanna Rokita[1], Danuta Kozbor[2]
Faculty of Biotechnology[1], The Jagiellonian University, Krakow, Poland.; Department of Immunology[2], Roswell Park Cancer Institute, Buffalo, NY, USA.

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68 **305.1** **Antigen specific immunity in neuroblastoma patients: Antibody and T-cell recognition of NY-ESO-1 tumor antigen**
Chiara Castelli1, Roberto Luksch2, Elisabeth Stockert3, Yao-Tseng Chen3, Paola Collini2, Tiziana Ranzani1, Claudia Lombado1, Piero Dalerba1, Licia Rivoltini1, Franca Fossati-Bellani2, Lioyd Old3, Giorgio Parmiani1, Monica Rodolfo1
Unit of Immunotherapy of Human Tumors1 and Department of Pediatric Hematology Oncology2, Istituto Nazionale Tumori, Milano, Italy; Memorial Sloan Kettering3, New York, USA.

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134 **261.1** **Combined targeted GM/CSF and IL/2 therapy switches innate to adaptive anti-neuroblastoma immunity**
Holger N Lode, Nicole Huebener, Yan Zeng, Stefan Fest, Torsten Dreier, Silke Weixler, Stephen D Gillies, Ralph A Reisfled, Gerhard Gaedicke
Pediatrics, Experimental Oncology, Charité, CVK, Berlin, Germany.

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135 **345.1** **IL-27 mediates complete regression of primary and/or metastatic murine neuroblastoma tumors: role for CD8+ T cells**
Rosalba Salcedo[1], Cynthia Hahn[1], Jimmy Stauffer[1], Erin Lincoln[2], Julie Hixon[1], Robert Kastelein[3], Jon M. Wigginton[1]
Pediatric Oncology Branch[1], NCI-CCR and IRSP[2], SAIC-Frederick, Frederick, MD and Department of Immunology[3], DNAX Research Institute, Palo Alto, CA, USA.

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57 **347.2** **IL-12 inhibits AKT activity and induces BID activation in conjunction with complete regression of orthotopic murine neuroblastomas**
Tahira Khan, Julie A Hixon, Jimmy K Stauffer, Erin Lincoln, Timothy C Back, Jon M Wigginton
Pediatric Oncology Branch, NCI-CCR, Frederick, MD, USA.

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135 **347.3** **IL-18 Induces complete regression of orthotopic primary and metastatic murine neuroblastoma tumors and potentiates antitumor immune reactivity in combination with IL-2**
Julie A Hixon[1], Jimmy K Stauffer[1], Erin Lincoln[1], Timothy C Back[1], Zdenka Jonak[2], Jon M Wigginton[1]
Department Pediatric Oncology Branch, NCI-CCR and IRSP[1], SAIC-Frederick, MD; Glaxo-Smithkline Pharmaceuticals[2], King of Prussia, PA, USA.

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135 **186.1** **Neuroblastoma immunotherapy with IL-12 and IL-15-gene modified NB cells**
Michela Croce, Anna Maria Orengo, Raffaella Meazza[2], Claudio Gambini[2], Barbara De Giovanni, Vito Pistoia, Maria Valeria Corrias, Silvano Ferrini
Laboratory of Immunopharmacology, Istituto Nazionale per la Ricerca contro il Cancro; Laboratori of Oncology and Service of Pathology, Giannina Gaslini Children's Hospital Genova.

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Laboratory of Oncology[1] and Laboratory of Clinical and Experimental Immunology[2], Giannina Gaslini Children's Hospital and Laboratory of Immunopharmacology[3], Istituto Nazionale per la Ricerca sul Cancro, Genova; Department of Oncology and Neurosciences[4], G. D'Annunzio University, Chieti; Italy.

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Laboratory of Oncology[1], G. Gaslini Children's Hospital, Genoa; Department of Pharmaceutical Sciences[2], University of Bologna, Italy.

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Olaf Witt[1], Johannes Schulte[2], Lothar Schweigerer[1], Jörg Hoheisel[3]
Department of Pediatrics I1, Children\'s Hospital, University of Goettingen[2], Pediatric Oncology, University of Essen, and DKFZ[3], Functional Genome Analysis, Heidelberg, Germany.

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Cancer Epigenetics Laboratory[1], Spanish National Cancer Center (CNIO), Madrid, Spain; Memorial Sloan-Kettering Cancer Center[2], New York, USA.

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La Sapienza University[1], Bambino Gesù Children's Hospital[2], Rome; RLC-NHS-Trust Alder Hey[3], Liverpool; University of Kiel[4], Kiel; Regina Elena Cancer Institute[5]; ENEA Research Center Casaccia[6], Rome; UK, Germany & Italy.

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Laboratory of Oncology[1], Service of Pathology, G. Gaslini Children's Hospital, Genoa, Italy; Department of Experimental and Diagnostic Medicine, Section of Pathology, University of Ferrara, Italy.

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Department of Biochemistry and Pediatrics, University of Tromsø, Norway.

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Laboratory of Oncology[1] and Service of Pathology[2] G. Gaslini Children's Hospital, Genoa, Italy.

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Martina Korfei, Klaus T Preissner, Manfred Kaps, Franz Blaes
Department of Biochemistry, Justus-Liebig-University, Giessen, Germany

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Cancer Research Center of Hawaii[1], University of Hawaii at Manoa, Honolulu, Hawaii, USA; Department of Biomedical Sciences[2], University of California, Riverside, USA.

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School of Biological Sciences, University of Liverpool, UK.

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Marina Lanciotti, Paola Di Michele, Simona Coco, Riccardo Haupt, Luca Boni, Carlo Dufour, Alberto Garaventa, Gian Paolo Tonini
Department Pediatric Hemato-Oncology, Giannina Gaslini Children's Hospital and Laboratory of Neuroblastoma, National Institute Cancer Research (IST), Genova, Italy.

Published-only Abstracts

Application of Microarray-Based Technology to Neuroblastoma

Wednesday June 16, 2004

Workshop

3:00 – 7:00 PM **Hall "Scirocco & Libeccio"**

The completion of the human genome sequence has provided a framework for the molecular analysis of cancer. Microarray technology allows for simultaneous analysis of thousands of genomic and/or transcriptomic alterations within tumor cells. In the most recent Advances in Neuroblastoma Research meeting, many groups presented papers utilizing these evolving tools. The aim of the Workshop is intended to focus on the great potential, but also the possible pitfalls of this promising technology, on how it can be applied to the molecular analysis of neuroblastoma, and on its integration with other technologies, with the aim to develop a better understanding of the alterations of this paediatric tumour, and to identify novel diagnostic markers and therapeutic targets.

10' **Introduction to microarray technology: Basic principle and pre-clinical approach**
Gian Paolo Tonini, *National Institute for Cancer Research, Genoa, Italy*

Gene expression - Session chaired by Rogier Versteeg (NL)

30' **Global gene expression analyses in neuroblastoma: What do we learn from different techniques?**
Frank Westermann, *German Cancer Research Centre, Heidelberg, Germany*

15' **Quantitative profiling.**
Rogier Versteeg, *University of Amsterdam, Amsterdam, Netherlands*

15' **Use of oligonucleotide microarrays versus SAGE versus proteomics in neuroblastoma cell culture models: Can we compare the data and what do we learn from them?**
Angelika Eggert, *University Children's Hospital of Essen, Essen, Germany*

15' **Designing a specific Neuroblastoma Oligonucleotide Microarray**
Matthias Fischer, *University Children's Hospital, Cologne, Germany.*

15' **Identifying subsets of metastatic neuroblastomas and cell lines with oligonucleotide microarray expression profiling.**
Robert C Seeger, *Childrens Hospital Los Angeles Research Institute, Los Angeles, USA*

15' **Response of neuroblastoma to hypoxia: An approach with microarray technology**
Luigi Varesio and Gian Paolo Tonini, *Giannina Gaslini Children's Hospital and National Institute for Cancer Research, Genova, Italy*

15' **Coffee Break**

DNA gain and loss - Session chaired by John M Maris (USA)

30' **The impact of integrated analysis of genetic and genomic alterations using microarrays in neuroblastoma**
Akira Nakagawara, *Chiba Cancer Center Research Institute, Chiba, Japan.*

30' **Comparison of arrayCGH versus oligonucleotide microarrays (Affymetrix GeneChip® Mapping 10K Array and Assay Set) for high-resolution analysis of DNA copy number in neuroblastoma**
Frank Speleman, *Gent University Hospital, Gent, Belgium*

15' **Dissecting the genome of human neuroblastomas with array CGH**
Yael Mosse, Philadelphia Childrens Hospital, Philadelphia, USA

15' **Use of CGHarray to identify DNA gain and loss in neuroblastoma.**
Paola Scaruffi and Stefano Moretti, *National Institute for Cancer Research, Italian Neuroblastoma Foundation, Genova, Italy*

15' **Study of genetic rearrangements in neuroblastoma tumors using commercial pangenomic CGH arrays and dedicated laboratory-made BACs arrays.**
Alexander Valent, *Institut Gustave Roussy, Paris, France*

15' **Conclusion**
John M. Maris, *The Children's Hospital of Philadelphia, Philadelphia, USA*

Workshop

Spinal Cord Compression

Saturday June 19, 2004

2:00 - 5:00 PM **Hall "Levante"**

A minority of children with neuroblastoma (mostly localised) present with symptomatic spinal cord compression. Although this complication does not influence the eventual outcome, it may heavily compromise the quality of life, if not timely recognised and promptly treated. However, there is little agreement on the optimal way to treat this condition. Neurosurgeons, oncologists and sometimes radiotherapists often compete on who should act first. There is also little information on the long-term side results of these treatments.
This Workshop intends to focus on the unclarified issues of this complication and lay the foundations of a more exhaustive meeting that might take place in one year.

Chairpersons **Bruno De Bernardi, Dominique Plantaz**

2:00 PM ***Introduction***: Audrey Evans *(Philadelphia, USA)*

2:10 ***Literature Review***: Dominique Plantaz *(Grenoble, France)*

2:25 ***Pathophysiology***: Jean-Guy Passagia *(Grenoble, France)*

2:40 ***Neuroradiology***: Paolo Tortori Donati *(Genova, Italy)*

2:50 ***Case series with comments by neurosurgeons and radiotherapists***

Walentyna Balwierz *(Warsaw, Poland)*
Joanna Begent *(London, UK)*
Frank Berthold *(Koeln, Germany)*
Sue L Cohn *(Chicago, USA)*
Kim Kramer *(New York, USA)*
Dominique Plantaz *(Grenoble, France)*
Maria Luisa Garré *(Genova, Italy)*

Neurosurgeons
Armando Cama *(Genova, Italy)*
Jean-Guy Passagia *(Grenoble, France)*

Radiotherapist
Guido Sotti *(Padova, Italy)*

4:50 ***Late Effects***: Paola Angelini *(Genova, Italy)*

5:00 **Conclusion**

Opsoclonus Myoclonus

Saturday June 19, 2004

Workshop

2:00 - 5:00 PM **Hall "Ponente"**

This Workshop intends to focus on the pathogenesis, clinical features and therapeutic approach of opsoclonus-myoclonus syndrome (OMS), with emphasis on the cases occurring in association with neuroblastoma. Diagnosis and follow-up of neurological manifestations will be covered in detail in view of the novel therapeutic protocol recently proposed by the COG and based upon the use of the immunosuppressive and cytotoxic agent cyclophosphamide. This topic will be the matter of a specific presentation pointing to the feasibility of cyclophosphamide treatment in neuroblastoma-associated OMS. Hopefully, the Workshop will allow to reach a consensus on different aspects of this severe and disabling syndrome, for which no efficacious treatment has been identified so far. International collaboration on such a rare disorder is warranted to radically change the prognosis of OMS.

2:00 ***K.K. Matthay***: Overview of NB-associated OMS: diagnosis, prognosis, immunological aspects and long term outcome

2:20 ***M. Pike***: Diagnostic criteria and neurological evaluation of OMS patients

2:40 ***W. Mitchell***: Follow up, late complications and treatment of OMS patients

3:00 ***V. Pistoia***: Lymphoid infiltration in OMS-associated neuroblastoma

3:15 ***F. Blaes***: Immunopathogenesis of OMS

3:30 ***P. De Alarcon***: US COG-protocol for NB-associated OMS

3:45 ***B. Hero***: Use of cyclophosphamide in the treatment of OMS

4:00 ***M. Pike and W. Mitchell***: The neurologists' opinion on COG protocol

4:20 **General discussion**

5:00 **Conclusion**

Abstracts

Oral Presentations

Oral Presentations

Plenary Session A
Genetics

Ref ID: 068.1

Molecular signature to predict the prognosis of neuroblastoma and its application to a diagnostic microarray of clinical use

Miki Ohira[1], Shigeyuki Oba[2], Yohko Nakamura[1], Eriko Isogai[1], Setsuko Kaneko[3], Takahiro Hirata[4], Hiroyuki Kubo[4], Takeshi Goto[4], Saichi Yamada[5], Yasuko Yoshida[5], Shin Ishii[2], Akira Nakagawara[1]

Division of Biochemistry[1], Chiba Cancer Center Research Institute; Graduate School of Information Science[2], Nara Institute of Science and Technology, Ikoma; Department of Pediatric Surgery[3], University of Tsukuba School of Medicine, Tsukuba; Hisamitsu Pharmaceutical Co. Inc.[4], Tokyo; Micro Ceramics Laboratory, R & D Center, NGK Insulators, LTD, Nagoya, Japan

Neuroblastoma is an enigmatic tumor with heterogeneous clinical behaviors including maturation, regression, and aggressive growth. Although recent progress in therapeutic strategies against advanced neuroblastoma has improved patient survival, long-term outcomes still remain very poor. The prediction of cancer prognosis is one of the most urgent demands to initiate the suitable treatment of neuroblastoma. To this end, we constructed an in-house, microceramic pump-based ink-jet-printed cDNA microarray carrying 5,340 genes obtained from primary neuroblastoma cDNA libraries and applied it for the analysis of 136 tumors. A probabilistic output computational analysis using 87 learning samples has selected 70 genes which constructed a classifier for patient outcome, and provided a correct prognosis in 27 of 29 test samples (93%). Fifty-four out of 70 top-ranked genes were also independently significant predictors of patient's outcome, and they included TUBA1, ENO1, EEF1G, and DDX1. Kaplan-Meier analysis using the classifier indicated 5-year survivals for 23% and 90% of patients with unfavorably and favorably predicted neuroblastomas, respectively ($p<10-5$). Of clinical interest, the intermediate risk type of neuroblastoma, whose cumulative survival rate was 79%, was divided into two subgroups with better (86%) and worse (40%) prognoses by the posterior value of 0.5 ($p<10-5$). Furthermore, our microarray prediction exhibited the best balance between sensitivity (96%) and specificity (90%) among prognostic factors including MYCN amplification and TrkA expression in the receiver operating characteristic (ROC) curves. Based on these results, we made a practical DNA chip carrying top-ranked 200 genes for clinical use, which gave a highly comparable result to that obtained from the 5,340 cDNA microarray. Thus, our microarray system made its practical use feasible at the bedside to predict the prognosis of the patient with neuroblastoma.

Ref ID: 330.3

Oligonucleotide Microarray Expression Profiles Identify Subsets of Patients with Ultra High- and High-Risk Metastatic Neuroblastoma

Robert C Seeger, Hong Wang, Yujun Yang, Katherine K Matthay, Jonathan Buckley, Shahab Asgharzadeh

Division of Hematology/Oncology, Department of Pediatrics, Children's Hospital Los Angeles/USC, CA, USA.

Overall survival (OS) of patients with high-risk metastatic neuroblastoma (stage 4, diagnosed after 18 mos) has improved so that subgroups are apparent within this category. Accurate definition of subgroups at the time of diagnosis would have a major impact upon developing potentially more effective therapy. We hypothesized that gene expression profiles would identify patients with different outcomes. Primary tumors from 82 patients with stage 4 disease diagnosed after 18 mos of age with OS of 32% at 5 yrs were studied. Expression profiles were obtained with Affymetrix U133A+B arrays. Data were pre-processed using dChip and analysed with Genetrix software. MYCN-amplified (MYCN-A) and non-amplified (MYCN-NA) tumors were used to test the ability of analytic methods to distinguish biologically different tumors. Unsupervised principal component analysis identified clusters of MYCN-A and MYCN-NA tumors, and supervised nearest shrunken centroids (NSC) with leave-n-out analysis distinguished MCYN-A and MYC-NA groups. Genes highly expressed by MYCN-A tumors included MYCN, NCYM, HEI10, and TWIST whereas those highly expressed by MYCN-NA tumors included brain specific protein, SDC3, CYB561, NTRK1, and MYC. Next, NSC analysis was used to identify genes that distinguished tumors causing disease progression and death in the first year from those associated with survival 5-10 yrs. When genes so identified were used for Cox regression (adjusted for MYCN status), highly expressed genes associated with rapid PD and death included PCOLCE, SRP19, FUBP1, HDGF, TCF4, TOPBP1, and BCL11B. Conversely, highly expressed genes associated with long-term survival included LGI1, ADAMTS8, S100B, PPFIA2, and SST. This study identifies genes whose expression may predict outcome for patients with metastatic disease that is indistinguishable at diagnosis by clinical criteria. This should improve risk assessment and lead to more specific and effective therapy for these children.

Ref ID: 286.1

N-myc, Cdc42 and nm23 genes function in a differentiation pathway blocked by copy number defects in neuroblastoma

Linda J Valentijn, Heather A Root, Rogier Versteeg

Human Genetics, Academic Medical Center, Amsterdam, The Netherlands.

Chromosomal aberrations found in neuroblastoma tumors include amplification of N-myc on chromosome 2p24, deletion of one allele of chromosome 1p35-36 and gain of chromosome 17q. These three aberrations are correlated. Almost all neuroblastomas with N-myc amplification have a deletion of 1p36 and extra copies of chromosome 17q. We have used serial analysis of gene expression (SAGE) to identify all genes regulated by N-myc in neuroblastoma. Here we show that Cdc42, which is located on chromosome 1p36, is down-regulated by N-myc in human neuroblastoma cell lines and tumors. We introduced Cdc42 into neuroblastoma cell line SHEP-21N, which expresses N-myc under a tetracycline-dependent promoter. Cdc42 is a G-protein that is only active in the GTP-bound form. Constitutively active Cdc42 strongly induced differentiation. However, the wild type Cdc42 only induced differentiation when N-myc was switched off. Therefore, N-myc not only down-regulates the Cdc42 mRNA expression, but also regulates the activity of the Cdc42 protein. Previously, we described that N-myc up-regulates the expression of the nm23-H1 and -H2 genes, which map to chromosome 17q. Here we show that the inhibitory effect of N-myc on Cdc42-induced differentiation is mediated by the nm23 genes. They can fully reconstitute the effect of N-myc and probably function, directly or indirectly, as GTPase-activating proteins (GAPs) for Cdc42. The mechanism of inactivation of Cdc42 in neuroblastoma might represent a novel paradigm for tumor suppressor gene inactivation. The three chromosomal defects, N-myc amplification, LOH of 1p36 and gain of 17q, converge to block the Cdc42-mediated differentiation pathway. Cdc42 is not fully destroyed like classical tumor suppressor genes, but can be reactivated.

Ref ID: 155.1

MEIS1 functions as a neuroblastoma oncogene

Dirk Geerts, Nathalie Schilderink, Gerda Jorritsma, Ingrid M Revet, Joris Smaling, Rogier Versteeg

Department of Human Genetics, Academic Medical Centre, University of Amsterdam, The Netherlands.

BACKGROUND AND AIMS: Homeobox genes encode transcription factors that control embryonic development by transcriptional regulation of large sets of target genes. Aberrant expression of homeobox genes is involved in genetic diseases and in cancer. We discovered genomic amplification of the MEIS1 homeobox gene in IMR32, and subsequently demonstrated high level expression of MEIS1 and MEIS2 in most neuroblastoma cell lines investigated, as well as in many tumours. We decided to study the role of the MEIS genes in neuroblastoma pathogenesis.
METHODOLOGY: MEIS1 expression in neuroblastoma cell lines was manipulated by transfection with the MEIS1E dominant-negative splice variant. High MEIS1E expression caused impaired proliferation, and increased contact inhibition and cell death, indicating the importance of MEIS1 expression for neuroblastoma cell growth. To identify the MEIS1 downstream target genes, the gene expression profiles of several transfectants was determined using SAGE (serial analysis of gene expression) and DNA micro-array technology.
RESULTS AND CONCLUSIONS: Differential expression as a result of MEIS1E expression was found for genes involved in chromatin binding, mRNA processing, cell cycle control, and neuronal development. We now focus on two important categories of MEIS1 downstream genes using siRNA-mediated knockdown and inducible expression of selected targets: 1) Target genes from genomic locations known to be aberrant in neuroblastoma, and 2) Target genes encoding developmentally important transcription factors (e.g. other homeobox genes and chromatin binding proteins). For both categories, we confirmed interesting MEIS1 downstream target genes. Surprisingly, all 4 clusters of the HOX homeobox genes, that encode MEIS cofactor proteins, showed highly aberrant expression in neuroblastoma compared to normal adrenal gland. Additionally, several of these HOX genes were shown to be regulated by N-Myc. Elucidation of the MEIS1 regulatory hierarchy in this way will lead to a comprehensive understanding the role of the MEIS1 gene in neuroblastoma tumorigenesis.

Ref ID: 046.2

Discrimination between good and bad prognosis neuroblastoma by serum protein expression profiles analysis

Valérie Combaret, Christophe Bergeron, Stéphanie Brejon, Isabelle Iacono, Myriam Cubizolles, Sylvie Negrier, Alain Puisieux

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Neuroblastoma (NB) is the most common pediatric extracranial solid tumour. A variety of serum markers have been used to assist in the diagnosis of NB, and some of them as ferritin and LDH may be useful to predict outcome. We used new proteomic tools to identify other serum markers with prognostic impact. Using surface-enhanced laser desorption/ionisation (SELDI) time-of-flight mass spectrometry (Ciphergen Biosystems), we screened for differentially expressed proteins in the serum of 13 patients with good prognosis NB and 18 patients with bad prognosis NB. Serum samples were profiled on various retentate chromatography arrays (anion exchange, cation exchange, metal affinity and reverse phase), using different binding conditions for each array type. The ProteinChip® Reader (model PBS IIc) with Ciphergen ProteinChip® Software 3.1 was used to acquire the data. Protein peak clustering and classification analysis were performed using the Biomarker Wizard tool and Biomarker Patterns Software 4.0, for univariate and multivariate analyses respectively. The best results were obtained on the H50 ProteinChip® surface (reverse phase retentate chromatography) where an average of 47 protein peaks, with masses ranging from 3,146 to 28,734 were resolved. Twenty of these were found to be differentially expressed in good and bad prognosis NB. The serum protein expression profiles were used to train and develop decision-tree classification algorithms, the best classification tree being generated by using only two peptides peaks (11,467 Da and 17,272 Da). This classification model achieved 100% sensitivity and 84.6 % specificity in discriminating good from bad prognosis NB. Purification, followed by trypsin digestion and peptide mapping, permitted to identify the protein with a Mr of 11,467 as the serum amyloid A (SAA). Using ELISA, we are currently testing the SAA concentration in serum of neuroblastoma patients to evaluate its prognostic value.

Ref ID: 364.1

Discovery of antiangiogenic targets for high-risk neuroblastoma using a high-density oligonucleotide-based approach

Suzanne Shusterman, Rebecca L King, Eric Rappaport, Qun Wang, Sharon Diskin, Michael Morowitz, Rosalind Barr, Nicholas Rhodin, John M Maris

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There is a strong correlation between tumor vascularity and aggressive disease phenotype in neuroblastoma, but the most relevant antiangiogenic targets are not known. We have therefore quantified mRNA expression of 101 highly annotated primary neuroblastoma samples using the Affymetrix U95Av2 platform to discover the angiogenesis-related genes differentially expressed in high-risk patients. In addition, to determine which angiogenesis-related genes show increased expression during the phase of tumor recovery following chemotherapy, mice with established human neuroblastoma-derived xenografts were treated with cyclophosphamide (450 mg/kg) or placebo. Starting at treatment initiation, tumors were harvested at 4 day intervals and mRNA was quantitated with the Affymetrix platform. For both data sets, inter-chip expression data was modeled using Probe Profiler and strict QC metrics were confirmed. Differential expression of angiogenesis-related genes was then determined using a variety of software tools (SAM, PaGE) and filtered using an angiogenesis-specific gene list. In the primary tumor series, several proangiogenic (including METAP2, VEGFR2, and PDGFRA) and antiangiogenic (BAIAP2 and BAIAP3) gene transcripts were found to distinguish metastatic primary tumors from localized tumors with extremely high confidence (false discover rate <1%). In the murine model, at the time of maximal tumor regression following cyclophosphamide, there was decreased expression of several proangiogenic gene transcripts including HIF1µ and METAP2. Coincident with tumor regrowth following chemotherapy, there was increased expression of proangiogenic factors including VEGF and METAP2. Targeting METAP2 enzymatic activity with a novel reversible inhibitor resulted in dramatic diminution of tumor growth rate in xenograft models ($P < 0.001$) and cured MYCN transgenic mice with established tumors. These data show that angiogenic factors are differentially expressed in relation to clinical phenotype and in response to chemotherapy. It also shows the power of transcriptome analysis for guiding the rational selection of antiangiogenic targets for clinical development.

Ref ID: 342.1

Probabilistic Analysis of cDNA Array-CGH Profiles Identifies Genomic Alterations Specific to Stage and MYCN-Amplification in Neuroblastoma

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Characterization of genomic alterations is critical to our understanding of tumorigenesis and cancer progression. Here we performed Array-comparative genomic hybridization (A-CGH) on cDNA microarrays containing 42,000 elements in neuroblastomas (NBs) from stage 1, stage 4 without MYCN amplification (stage 4-) and stage 4 with MYCN amplification (stage 4+). We identified 6 independent amplicons on 2p and precisely defined boundaries for all amplicons. We confirmed the previously reported amplified genes on 2p and identified novel amplification of several other genes (NCOA1, ADCY3, PPP1CB, ALK, CGI-127, LBH, CAPN13, GalNac-T10, EHD3, XDH, SRD5A2, CGI-27, AMP18, TEM8) and ESTs. In addition we identified another amplicon on 12q14-q15, bounded by PRO2268 and RAB3IP containing one previously reported amplified gene (MDM2) as well as several novel amplifications (CPM, CPSF6, LYZ, GAS41, SNT-1, CCT2, VMD2L3, and RAB3IP). We next applied a probabilistic approach to detect single copy alterations and identified genomic alterations specific to stage and MYCN-amplification. Only a few of imbalances were found in all three subgroups. Remarkably stage 4+ disease appeared to have relatively few genomic alterations compared with Stage 1 and 4-, implying that MYCN amplification is sufficient to drive these tumors to an aggressive phenotype.

Oral Presentations

Plenary Session B
Biology

Ref ID: 020.1

Gangliosides link fenretinide-induced ceramide to 12-lipoxygenase-dependent apoptosis of neuroblastoma

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Ceramide has been implicated as a common intermediate of many apoptotic pathways. Metabolism of ceramide results in the formation of gangliosides which have been implicated in ROS generation and apoptosis. Fenretinide [N-(4-hydroxyphenyl)retinamide] has been reported to induce apoptosis of neuroblastoma cells via increased levels of intracellular ceramide but the link between ceramide induction and subsequent apoptosis remains unclear. We have shown that fenretinide increases the activity of acidic sphingomyelinase in SH SY 5Y neuroblastoma cells resulting in the hydrolysis of sphingomyelin and ceramide accumulation. Fenretinide increased glucosylceramide synthase activity in these cells and a glucosylceramide synthase inhibitor blocked fenretinide-induced apoptosis. This suggests that ceramide induced in response to fenretinide is subsequently metabolized into glycosylsphingolipids and gangliosides. Fenretinide increased levels of the ganglioside GD3 and knockdown of GD3 synthase by RNA interference blocked the fenretinide-dependent increase in GD3, ROS and apoptosis. The addition of exogenous GD3 to SH-SY5Y neuroblastoma cells induced ROS and apoptosis, which was abrogated by a specific 12-LOX inhibitor. GD2 levels also increased in response to fenretinide, but exogenous GD2 did not induce apoptosis. These results suggest that GD3 is a key signaling intermediate inducing apoptosis in response to fenretinide via the activation of 12-Lipoxygenase. This apoptotic pathway mediated by gangliosides may represent a new target for future drug development. Furthermore, the induction of GD2 suggests that fenretinide might also enhance the response of neuroblastoma to anti-GD2 therapy.

Ref ID: 075.2

BH3-domain peptidomimetics activate apoptosis and elucidate death pathways in neuroblastoma

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Apoptosis evasion contributes to chemotherapy resistance in neuroblastoma (NB). Bcl2 family members are critical mediators of apoptosis and interact through Bcl2 homology (BH) domains. Prodeath multidomain members (Bax, Bak) are engaged by activated BH3-only proteins such as Bid and Bad to induce death. Antideath members (Bcl2, BclX, Mcl1) compete for BH3 proteins to prevent apoptosis. BH3 mimetic peptides (BH3mps) are 25-35mer peptides of prodeath BH3-domains and are sufficient for genetically tractable death induction [Letai, Cancer Cell 2002]. We assessed BCL2 family expression using the Affymetrix U95Av2 genechip in 100 primary NBs (N=20 with MYCN amplification). Primary NBs with MYCN-amplification expressed less BAD, BNIP3 and BIK compared with non-amplified NBs (all $p<0.01$) supporting a reduced BH3-domain burden. We then investigated BH3mps (r8BidBH3, r8BadBH3; modified by arginine 8mers to transduce cell membranes) for their ability to engage apoptosis in NB cell lines in vitro. An alternate r8BidBH3 (r8BidAltBH3) has two substituted highly conserved residues to control for nonspecific membrane disruption. Cell lines were treated with 10-50 microM of BH3mps alone and in combinations to assess synergy. r8BidBH3 or r8BadBH3 alone induced apoptosis while r8BidAltBH3 had markedly diminished efficacy. CASP8 negative and MYCN-amplified cell lines were more sensitive to r8BH3mps. Cell lines with absent CASP8 expression may be more sensitive as r8BH3mps engage apoptosis downstream of their apoptotic defect. Concomitant exposure to r8BadBH3 and r8BidBH3 demonstrated synergy with apoptosis at ineffective monotherapy concentrations. Preliminary studies of r8BadBH3 given with cytotoxics shows similar effects. r8BadBH3 may displace endogenous BH3 proteins and/or exogenous r8BidBH3 to engage apoptosis. Assessment of r8BH3mp conformation (alpha-helicity) and affinity for multidomain Bcl2 proteins is ongoing, as are investigations with BH3mps and isolated NB mitochondria to genetically query the status of these pathways and define candidate therapeutic targets.

Ref ID: 032.2

Bone Marrow Mesenchymal Stem Cells are Essential for Osteoclast Activation in Bone Invasion by Human Neuroblastoma

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Bone metastases is observed in 75% of patients with advanced neuroblastoma but its mechanism has been so far poorly understood. We have recently shown that neuroblastoma cells recruit osteoclasts to invade the bone and create osteolytic lesions in vivo (Cancer Research 63:3026-3031, 2003). In osteolytic metastases seen in breast cancer and multiple myeloma, osteoclast precursor cells (OPC) are stimulated by factors like parathyroid hormone related peptide (PTHrP), the ligand for the receptor activator of NFkB (RANKL), IL-1, IL-6, macrophage colony stimulating factor (M-CSF) or monocyte inhibitory protein-1a (MIP-1a) produced by tumor cells. Surprisingly, we found no evidence for the expression of these factors in neuroblastoma cells that form osteolytic lesions in vivo. Consistently when cultured in the presence of OPC, neuroblastoma cells had little effect on their maturation and activation. However, when human bone marrow mesenchymal stem cells (BM-MSC) were added to the culture, a 3.5 fold increase in osteoclast activation was observed. We then detected a 45.1 fold increase in IL-6 expression by BM-MSC when cultured in the presence of neuroblastoma cells, and discovered that IL-6 was responsible for osteoclast activation since its effect was suppressed in the presence of an anti-human IL-6 blocking antibody. Stimulation of IL-6 expression in BM-MSC involved EGFR mediated signaling. Thus BM-MSC provide an alternate pathway for osteoclast activation in bone metastasis in neuroblastoma by being the major source of the osteoclast activating protein IL-6.

Ref ID: 111.1

"Cross-talk" between Schwann cells and neuroblasts influences tumor differentiation and angiogenesis

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BACKGROUND: Neuroblastoma (NB) tumors consist of two main cell populations: neuroblastic/ganglionic cells and Schwann cells. Previous in vitro studies have shown that Schwann cells produce factors that are capable of supporting the survival and differentiation of NB cell lines and inhibiting angiogenesis. We have recently developed an in vivo model to study the "cross-talk" between these cell types and show that Schwann cells influence neuroblast differentiation and tumor angiogenesis.
METHODS: Following exposure of the left sciatic nerve of athymic nude mice (n=19), SMS-KCNR NB cells (5x105/5 µl) were injected intrafascicularly. Control animals (n=12) were inoculated outside the sciatic nerve with the same number of SMS-KCNR cells. Animals were sacrificed 6-12 weeks following tumor inoculation, and the tumors were embedded in paraffin and sectioned for histologic and immunohistochemical staining. S-100 and GAP-43 antibodies were used for detecting Schwann cells and NB cell differentiation. Anti-mouse CD31 antibody was used to highlight endothelial cells. The mean vascular density (MVD) was quantified by counting ten consecutive high power fields and expressed as MVD/mm2.
RESULTS: Schwann cell infiltration was detected by S-100 staining in all 19 NB xenografts that developed within the sciatic nerve, whereas only 3 of 12 control xenografts contained small number of Schwann cells ($p<0.001$). Histologic evidence of neuroblast differentiation was seen in the xenografts engrafted in the nerve, with cells displaying more abundant cytoplasm and expressing increased levels of S-100 and GAP-43 compared to neuroblasts in control tumors. In addition, the MVD was significantly lower in the xenografts that originated in the sciatic nerves compared to controls (50±13.07/mm2 vs. 113±32.85/mm2, respectively; $p<0.0001$).
CONCLUSION: Interactions between Schwann cells and NB cells in vivo results in neuroblast differentiation and inhibited angiogenesis. "Cross-talk" between Schwann cells and neuroblasts may influence the more benign clinical behavior that is characteristic of Schwannian stroma-rich/stroma-dominant NB tumors.

Ref ID: 189.1

Protein kinase C isoforms and glutathione levels: a molecular switch between proliferation and apoptosis in human neuroblastoma cells

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BACKGROUND AND AIMS: Intracellular glutathione (GSH) is critical in balancing the effects of alkylating agents and reactive oxygen species (ROS); GSH levels are crucial for neuroblastoma survival and antioxidant thiol depletion is demonstrated to be of therapeutic value in the clinical treatment of this cancer. Recently, we provided evidence that changes in GSH/GSSG ratio and ROS production in GSH-depleted SK-N-BE(2)-C cells induce early apoptotic features modulated by the enzymatic activity of Protein Kinase C delta, a key intermediate in the signal cascade leading to cell growth arrest and programmed cell death. Our actual aim is to investigate the influence of GSH levels on the enzymatic activity and expression of PKC isoenzymes involved in cell differentiation and growth of different human neuroblastoma lines.
METHODOLOGY AND RESULTS: Cell content of GSH was measured by h.p.l.c. analysis; the pattern and the activation state of PKC isoforms were evaluated by Western Blot and immunoprecipitation respectively. The comparative study showed that LAN 5 and SHSY5Y cell lines have the lowest amount of GSH, GIMEN and SK-N-BE-2-C intermediate levels and ACN are more enriched of the antioxidant thiol. PKC-alpha/PKC-delta ratio was equal in SHSY5Y, SK-N-BE-2-C and GIMEN and was modified in other neuroblastoma lines; in particular, the proliferative isoform, PKC-alpha, was highly expressed in LAN 5, while marked levels of the apoptotic kinase, PKC-delta, were found in ACN cells.
CONCLUSIONS: Both changes in cell GSH content and intracellular redox state might influence the activity and levels of specific PKC isoforms playing a critical role in differentiation, proliferation and apoptosis. Therefore, oxidative modulation of these molecular targets might represent a new approach in neuroblastoma clinical treatment (Grants by COFIN 2002 and FIRB 2001).

Ref ID: 069.1

Wnt-5a gene expression in human metastatic malignant neuroblasts

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Disseminated forms of neuroblastoma (NB), an embryonal malignancy, pose a major challenge for pediatric oncology. To identify NB metastatic-related genes, cDNA array analysis was performed in the human IGR-N-91 NB metastatic xenograft model (Blanc et al., AJP, 2003), which consisted of malignant neuroblasts derived from a primary tumor xenograft (PTX) and from metastatic sites in the mouse, i.e., bone marrow (BM) and myocardium (Myoc). Of the down-regulated development-related genes in metastatic BM and Myoc neuroblasts, we focused our interest on Wnt-5a, since we found a significant decrease in Wnt-5a mRNA in unfavorable versus favorable categories in 40 NB primary tumors ($P < 0.007$). Wnt-5a, a member of the Wnt signaling pathway, mainly associated with patterning decisions in the embryonic nervous system - has been reported to be involved in metastatic melanoma progression (Weeraratna et al., Cancer Cell, 2002) and invasive ductal breast cancer (Jönsson et al., Cancer Res., 2002) via adhesion and migration alterations. As retinoic acid (RA) is involved in the development of neural crest derived tissues upon differentiation, we wondered if RA might reverse the aberrant negative regulation of Wnt-5a in metastatic neuroblasts. IGR-N-91 sublines underwent neuronal differentiation (neuritic extension, reduced MYCN gene expression) following treatment with 10 µM RA treatment for 6 days. In these conditions, Wnt-5a expression increased significantly (real-time RT-PCR, western blot and immunocytochemistry). Moreover, while b-catenin expression remained unchanged, PKC-q a protein kinase C isoform involved in actin cytoskeleton reorganization, was evidenced and paralleled Wnt-5a levels. Wnt-5a transient overexpression did not induce neurite outgrowth or reduce MycN levels. Ongoing research is attempting to determine whether Wnt-5a gene acts as a morphogen or as a tumor suppressor gene during neuroblastoma tumorigenesis.

Ref ID: 177.1

Nucleoside diphosphate kinase A/NM23-H1 is a metastasis promoter and not a suppressor in human neuroblastoma

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Human nucleoside diphosphate kinase A (NDPK-A), encoded by the nm23-H1 gene, acts as a metastasis suppressor in certain tumors. However, this is conflicting with a positive correlation between the metastatic potential and NDPK-A level in other human tumors, such as neuroblastoma. In fact, amplification and overexpression of nm23-H1 have been reported in patients with advanced stages of neuroblastoma. Moreover, we previously discovered a unique mutation resulting in the S120G substitution of NDPK-A (NDPK-A(S120G)) in advanced neuroblastomas. This mutation alters the stability and protein-protein interactions of NDPK-A. To test whether NDPK-A causally promotes neuroblastoma metastasis, we established stable transfectants that constitutively overexpress NDPK-A and NDPK-A(S120G) as well as a florescent orthotopic xenograft animal model of human neuroblastoma. Ectopic NDPK-A was stably expressed and maintained during tumorigenesis and metastasis, as observed in re-established cell lines from the primary and secondary tumor foci of the animals. Without affecting cell proliferation and tumorigenesis, overexpressed NDPK-A or NDPK-A(S120G) significantly increased the incidence and colonization of neuroblastoma metastasis in animal lungs, which was accompanied by abrogation of neuronal differentiation induced by retinoic acid, and by enhancement in cloning efficiency, cell survival, and colony formation. Unlike the wild type, NDPK-A(S120G) was able to reduce cell adhesion while increasing cell migration, rendering it more effective in metastasis promotion. We demonstrate for the first time that NDPK-A is a metastasis promoter, at least in human neuroblastoma derived from the NB69 cell line containing no N-myc amplification. These findings not only suggest a prognostic value of NDPK-A in neuroblastoma patients, but also caution NDPK-A-targeted treatments for patients with different tumor types.

Oral Presentations

Parallel Session A
Translational

Ref ID: 062.1

Mechanisms of embryonal tumour initiation

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The mechanisms causing persistence of embryonal cells, which later give rise to tumours is unknown. One factor which contributes to the genesis of the embryonal childhood tumor, neuroblastoma, is the MYCN proto-oncogene. Here we show that normal mice developed neuroblast hyperplasia in paravertebral ganglia at birth, which completely regressed by 2 weeks of age. In contrast, ganglia from MYCN transgenic (TH-MYCN) mice demonstrated a marked increase in neuroblast hyperplasia and MycN expression during week 1. Regression of neuroblast hyperplasia was then delayed and incomplete prior to neuroblastoma tumour formation at 6 and 13 weeks in homo- and hemizygote mice, respectively. Paravertebral ganglia cells cultured from perinatal TH-MYCN mice, exhibited 3-10 fold resistance to nerve growth factor (NGF) withdrawal, compared to normal mice. Both low and high affinity NGF receptors were expressed in perinatal neuroblast hyperplasia, but not in neuroblastoma tumour tissue. MYCN transgene amplification was present at low levels in perinatal neuroblast hyperplasia from both homo- and hemizygote TH-MYCN mice. However, only in hemizygous mice did tumor formation correlate with a stepwise increase in the frequency of MYCN amplification. These data suggest that inappropriate perinatal MycN expression in paravertebral ganglia cells, initiated tumorigenesis by altering the physiologic process of neural crest cell deletion. Persisting embyronal neural crest cells underwent further changes, such as MYCN amplification and repression of NGF receptor expression, during tumor progression. Our studies provide a unique model for studying perinatal factors influencing embryonal tumour initiation.

Ref ID: 156.1

Expression of the therapy relevant makers EGF receptor, PDGF receptor and c-Kit in neuroblastoma determined with a multitissue array

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AIMS: Alternative therapy is needed for advanced neuroblastoma patients with relapse or progress after chemotherapy. Recently, tyrosine kinase inhibitors have been shown to be potent alternatives for treatment. We investigated neuroblastomas for expression of the tyrosine kinases epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR) and c-Kit.
METHODOLOGY: 160 patients treated in the German neuroblastoma trials NB90/95 (n=72) and NB 97 (n=88) were investigated. From the paraffin embeded neuroblastomas a multitissue array was designed and immunohistochemitry was performed for EGFR, PDGFR and c-Kit. The slides were evaluated semiquantitatively.
RESULTS: All but one case were investigated prior to treatment. 154 neuroblastomas were evaluable for EGFR expression. However, none expressed EGFR. PDGFR was positive in 116 from 148 cases (78%) demonstrating a homogenous staining in most tumor cells. In 47 from 148 cases (32%) c-Kit was expressed in the tumor cells. From the 47 c-Kit positive cases 45 neuroblastomas also expressed PDGFR, while 2 cases were negative for PDGFR. There was no statistically significant correlation between PDGFR or c-Kit expression and event free survival or overall survival or stage. In addition, no correlation existed between the Shimada classification (favourable versus unfavourable) and expression of PDGFR or c-Kit.
CONCLUSIONS: EGFR seems to be not expressed in neuroblastic tumors and therefore no usefull therapeutic target. C-Kit and PDGFR are expressed in neuroblastomas and exspecially PDGFR was expressed in a high percentage of neuroblastomas suggesting the potential usefulness of tyrosine kinase inhibitors in the treatment of advanced neuroblastoma.

Ref ID: 032.3

Combination therapy of zoledronate and low-dose cyclophosphamide imporves survival in a xenograft model of bone invasion in neuroblastoma

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Bone metastasis is a significant cause of mortality and morbidity in children with neuroblastoma. Using a novel bone invasion model in which immunodeficient mice were injected with human neuroblastoma cells into the femur, we have documented that 100% of the animals developed with a median time of 5 weeks severe osteolytic lesions caused by osteoclast activation (Cancer Research 63:3026-3031, 2003). Here we have tested the effect of ibandronate and zoledronate, two bisphosphonate compounds, potent inhibitors of osteoclasts for their therapeutic efficacy in this model. Whereas treatment with ibandronate delayed the formation of osteolytic lesions by 2 weeks, treatment with zoledronate prevented the formation of osteolytic lesions in 81% of the treated mice. However zoledronate did not increase overall survival due to local tumor invasion outside the bone and distant metastasis. We then tested whether the addition of low dose cyclophosphamide (25 mg/kg/day, added to drinking water) could improve survival by preventing local invasion outside the bone. We observed that the addition of cyclophosphamide significantly improved survival and delayed local invasion ($p<0.003$). The data support a clinical trial testing a combination of zoledronate and low dose cyclophosphamide in neuroblastoma patients with bone metastases.

Ref ID: 113.1

Measuring circulating neuroblastoma cells by quantitive RT-PCR: correlation with paired bone marrow and standard disease markers

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BACKGROUND AND AIMS: Histologic examination of bone marrow (BM) is an accepted clinical standard to detect metastatic neuroblastoma (NB). Circulating tumor cells in peripheral blood (PB) derive from depots other than BM, and its measurement may provide added information in cancer management.
METHODOLOGY: 120 patients with stage 4 NB were evaluated for NB cells in PB by quantitative RT-PCR (qRT-PCR) of GD2 synthase mRNA with a sensitivity of one NB cell in one million normal cells. The findings were compared with qRT-PCR of their simultaneously sampled marrow aspirates and 5 standard modalities of disease detection according to INSS criteria: histology, CT/MRI, bone scan, MIBG scan, and urinary HVA/VMA.
RESULTS: Detection of GD2 synthase transcript was found in 62 patients: 11 in both BM and PB, whereas the remaining 51 patients were BM+ only (n=38) or PB+ only (n=13) by qRT-PCR. Paired samples which were BM+PB+ had the highest transcript levels. When extent-of-disease was scored by the number of positive evaluation modalities ranging from 0 to 5, 91% of BM+PB+ patients had evidence of disease in 3 or more modalities. All BM-PB+ patients had less evidence of disease, with a positive disease score <=2.
Patients with positive marker in BM, regardless of the PB RT-PCR status, correlated with more evidence of disease by disease score. Kaplan-Meier analysis indicated a trend of poorer survival for patients who were BM+PB+ than those who were BM-PB-.
CONCLUSIONS: PB monitoring for NB may complement but not replace BM studies. Since blood sampling is less invasive than BM sampling, its clinical utility for routine surveillance deserves further investigation.

Ref ID: 103.2

Therapeutic activity of STI571 in neuroblastoma xenografts

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STI571 (imatinib mesylate, Gleevec) is a selective inhibitor of several structurally related receptor tyrosine kinases including c-Kit and PDGF-R_/_. We investigated in vitro and in vivo STI571 antitumor activity in neuroblastoma. Four human neuroblastoma cell lines (SK-N-BE2c, SK-N-DZ, HTLA230, RNGA) were studied for candidate targets by RT-PCR: PDGR_ and PDGR_ mRNAs were expressed in all cell lines, whilst c-kit__ mRNA was expressed in all but one (RNGA). In clonogenic assays, STI571 induced a dose-dependent inhibition of colony formation in all cell lines although with different sensitivities, IC50 ranging from 7.1 to 21.7 mM. The antitumor activity of STI571 was also studied in xenografts in nude mice from the 4 cell lines. Treatment was started either before tumors were palpable (prevention design) or after tumors were measurable (therapeutic design). STI571 was administered orally (100, 200 and 400 mg/kg/day) twice a day for 5 consecutive days for two consecutive weeks. The carrier vehicle was used as a control. Significant tumor growth inhibition was demonstrated in the xenografts obtained from all the cell lines except RNGA, using both the experimental designs. Tumor growth inhibition was independent from STI571 dosage: Log10 Cell Kill (LCK) and Tumor Weight Inhibition (TWI) values were respectively >9.9 and >1 in all mice. No obvious toxicity was observed in any of the treated mice. Pharmacokinetic and pharmacodynamic evaluation of the three dosages was also performed. These findings indicate that STI571 has a significant antitumor activity in neuroblastoma preclinical models and suggest that c-Kit may be the critical molecular determinant of this activity. Clinical evaluation is now appropriate.

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Ref ID: 102.1

Arsenic trioxide-induced death of neuroblastoma cells involves activation of bax and does not require p53

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BACKGROUND AND AIMS: Based on clinical studies showing that arsenic trioxide (As2O3), via an apoptotic mechanism, and with minimal toxicity, induces complete remission in patients with refractory acute promyelocytic leukemia, and that multidrug resistant and p53-mutated neuroblastoma cells are sensitive to As2O3 both in vitro and in vivo, we searched for molecular mechanisms involved in the As2O3-induced neuroblastoma cell death.
METHODOLOGY: We have studied the effect of As2O3 on the expression and cellular localization of proteins involved in drug-induced death in two neuroblastoma cell lines with intact p53, and two with mutated p53, the latter two displaying multidrug-resistance.
RESULTS: As2O3 provoked Bax expression in all tested neuroblastoma cell lines, including SK-N-BE(2) cells with mutated p53 and LA-N-1 cells, which have a deleted p53. In all cell lines exposed to As2O3, p21 Bax was proteolytically cleaved in a calpain dependent way into the more pro-apoptotic p18 Bax, which was detected exclusively in a mitochondria-enriched subcellular fraction. As2O3 also caused an increase of cytoplasmic cytochrome c, translocation of AIF to the nuclei, and a slight activation of caspase 3. However, inhibition of caspase 3 did not prevent cell death, while inhibition of Bax cleavage was associated with a decreased As2O3-induced cell death.
CONCLUSIONS: We show that multidrug-resistant neuroblastoma cells die following exposure to As2O3, independent of functional p53, suggesting activation of a cytotoxic pathway different from that induced by conventional chemotherapeutic agents. We further propose that proteolytic activation of Bax is an important event in As2O3-induced cell death.

Oral Presentations

Parallel Session B
Clinical

Ref ID: 336.1

Evidence for an age cut-off higher than 365 days for neuroblastoma risk group stratification in the Children's Oncology Group (COG)

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In COG, risk group and therapy for neuroblastoma patients is currently stratified based on the factors stage, MYCN, ploidy, Shimada histology and age at diagnosis with a cutoff of 365 days. Patients <365d,
depending on risk factors, tend to be assigned to lower risk and receive less therapy than patients >365d. The excellent long-term outcome of a subset of patients 1-2yrs of age suggests increasing the age cutoff, but how far? A more accurate determination of a data-driven prognostic and biologically rational age cutoff is ongoing. To date, retrospective analysis of POG biology study 9047 was performed in 1660 patients (1990-1999) with sufficient data. The median age was 483d, with 11% of patients >5 years. 42% of patients were <365d, 58% were >365d of age, with 4year EFS rates + SE of 85%+2% (n=703) and 51%+2% (n=957), respectively (p<0.0001). Multivariate analysis revealed an age cutoff of 365d (p<0.0001) to be independent prognostic factor after adjustment for MYCN (p<0.0001) and stage (p<0.0001). Adjusting for MYCN and stage, plots of relative risk versus age cutoff were used to visualize the continuous nature of the prognostic contribution of age. From a data-driven standpoint, age cutoff of 510d was chosen to maximize relative risk between the two age groups, and 149 patients (4year EFS: 80%+4%) were switched to lower risk group. 51% of patients were <510d, 49% were >510d of age, with 4year EFS rates + SE of 84%+2% (n=852) and 46%+2% (n=808), respectively (p<0.0001). In selecting a definitive new age cutoff, comparison will be made to analogous analyses of more homogenously treated patients from the Children's Cancer Group, and consideration given to the risk/benefit of future treatment scenarios.

Ref ID: 015.2

Influence of age on stage 4 neuroblastoma

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for the Italian Cooperative Group on Neuroblastoma (ICGNB).

BACKGROUND: Recent reports indicate that the traditional time cut-off of stage 4 neuroblastoma (less and more than one year at diagnosis) could be re-designed.
MATERIAL AND METHODS: The AA have reviewed the cases of stage 4 neuroblastoma diagnosed from January 1979 till December 2001 in the Italian centres participating in the ICGNB. The following four age groups were considered: 0-11mos, 12-17mos, 18-23mos, and >24mos. Two periods were identified: 1979-1984 (periodA), and 1985-2001 (periodB) based on the activation in 1985 of high-dose chemotherapy regimens associated with haematopoietic stem cell rescue.
RESULTS: A total of 720 cases were eligible, 167 and 553 of period A and B, respectively. There were 68 children aged 0-11mos (10 in periodA, 58 in periodB), 69 aged 12-17mos (11 vs 58), 77 aged 18-23mos (17 vs 60), and 499 aged >24mos (129 vs 370). MYCN oncogene was tested in 303 cases (all related to periodB)and found amplified in 74 (24%) (10, 17, 11, and 36 cases in the 4 age groups, respectively).

Survival	Periods	Age Groups				
		All	0-11mo	12-17	18-23	24-172
5-yEFS	Both	21.8	71.2	19.4	17.8	15.4
	A	8.5	50.0	30.0	12.5	3.1
	B	25.8	75.0	17.8	18.8	19.8
5-ySUR	Both	28.4	72.7	23.4	23.6	22.9
	A	10.5	50.0	40.0	12.5	5.0
	B	33.8	76.7	21.1	26.4	29.4

Survival in relation to age and MYCN status

		5-ySUR				
MYCN	Cases	All	0-11mos	12-17	18-23	24-172
Normal	229	41.3	92.6	51.9	28.1	32.7
Amplified	74	20.1	30.0	5.0	36.3	20.8

CONCLUSION. Our data indicate that outcome of children aged 12-17mos does not differ from those aged 18-23 and >24mos. However, children aged 12-17mos tended to fare better if MYCN gene copy number was normal, and significantly worse if MYCN was amplified.

Ref ID: 258.1

Localised and unresectable neuroblastoma in infants : Excellent outcome with low-dose primary chemotherapy.

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on the behalf of the Infants Neuroblastoma European Study (INES).

PURPOSE: To evaluate the efficacy of moderate-dose chemotherapy in infants with localised and unresectable neuroblastoma (NB) and confirm the good results of the French pilot study (Ref Rubie et al Br J Cancer 2003).
PATIENTS AND METHODS: All consecutive infants with localise unresectable NB and no MYCN amplification were eligible for the INES-Trial 99.1. Primary tumour was deemed as unresectable if the o imaging data suggested any risk with immediate resection. Diagnostic procedures and staging were done according to INSS recommendations. For children who had no threatening symptom (e.g. vital risk or dumbbell NB with neurologic deficit), chemotherapy consisted of low-dose cyclophosphamide (5 mg/kg/d x 5 days) and vincristine (0.05 mg/kg at day 1) – CV and repeated 1 to 3 times every 2 weeks until surgical excision could be safely performed. No post operative treatment was given. Those infants who had threatening symptoms or an inadequate response to CV received Carboplatin and etoposide (CBP-VP) and possibly Vincristine-Cyclophosphamide-Doxorubicin (CadO).
RESULTS: Between November 1999 and October 2003, in excess of 600 infants under 12 months of age with NB were registered with the study, of whom 112 had a localised and unresectable NB without MYCN amplification. At the time of the current analysis there were 77 evaluable patients of whom 24 had a threatening symptom and received 2 courses of CBP-VP16 alone (n=11). A further 13 also received 2 courses of CAdO according to the protocol. CV alone (2-4 courses) was administered to 18, followed by (CBP-VP) (n=22) or CAdO (n=13) according to the protocol. Treatment was well tolerated, with little significant toxicity. Surgery was attempted in the majority of patients following chemotherapy. All children are doing well but longer and fuller follow up is required.
CONCLUSION: In infants presenting with localised and unresectable NB without MYCN amplification or any threatening symptoms, anthracycline-containing regimens could be avoided in 75% of patients without jeopardising their long-term outcome.

Ref ID: 131.1

Observation of infants with stage 2 or stage 3 single copy MYCN neuroblastoma without chemotherapy

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Infants with localized neuroblastoma without MYCN amplification are known to have a very good prognosis, but chemotherapeutic treatment may be harmful to them. NB97 study design: Standard risk chemotherapy was only scheduled for infants with critical symptoms. Infants with completely resected primary (surgery group) or with incompletely resected or biopsied tumors (observation group) did not receive chemotherapy and were postoperatively only observed. RESULTS: So far, 132 infants were registered (chemotherapy: 55, surgery: 22, observation: 55). Progression or relapse was seen in 32 patients (chemotherapy: 8/55, surgery: 5/22, observation: 19/55). 8/32 patients showed metastases at the time of progression. Regression without cytotoxic treatment was seen in 32/55 patients, with complete regression so far in 17 patients. In 2 patients, substantial residual primary was observed more than 3 years. In both patients, the tumor lost mIBG uptake, in one the residual was resected and showed marked differentation. Aberrations of 11q were found in 3/5 investigated patients with metastatic and in 1/14 patients with local progression, but not in 12 patients with regression. Di-/tetrasomy was seen in 4/5 investigated patients with metastatic relapse (local relapse: 5/13; regression: 2/12). Aberrations in 1p, TrkA and CD44 expression were not helpful to discriminate patients with events. Compared to the previous NB90 trial (standard risk chemotherapy for all infants), EFS was inferior due to the cases with progression (3-year-EFS: NB97:0.74±0.04, NB90:0.88±0.03, p=0.02). In contrast, overall survival was not impaired (3-year-OS: NB97:98±1, NB90:0.91±0.04, p=0.08).
CONCLUSION: As regression is commonly seen in infants with localized neuroblastoma, chemotherapy may be reserved for patients with threatening symptoms or unfavorable biologic markers.

Ref ID: 334.1

Age contines to be a powerful predictor of EFS and OS in Neuroblastoma

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HYPOTHESIS: Patients with NB who are older at diagnosis have a higher risk of relapse and death than younger patients.
METHODS: 1251 patients enrolled on Children\'s Cancer Group protocols 3881 (low/intermediate risk) or 3891 (high risk) between 1989-96. Comparison of EFS and OS rates using a logrank test was performed for age groups [<18mo(n=665), 18mo-5yrs(n=476), 5-12yrs(n=99) and >12yrs(n=11)] overall and within the subsets of stage, MYCN, and histology. The 18mo cutoff was chosen based on evidence that toddlers with stage 4 NB have a more favorable outcome than pts >18mo; otherwise, age cutoffs were arbitrarily identified to permit visualization of potential outcome differences with increasing age.
RESULTS: Patients <18mo have statistically significantly (p<0.0001) higher EFS (77%+2%) and OS (86%+2%) than: patients 18mo-<5 yrs (EFS 40%+2%, OS 51+2%); patients 5-<12yrs (EFS 37%+5%,OS 45%+5%); and patients >12yrs (EFS 27%+23%,OS 34%+20%). The same was true for EFS and OS within the subset of stage 4 patients only, and OS for patients with stages 1-3. Although there is a trend for EFS and OS rates to decrease with increasing age, no other statistically significant differences were found between the other age groups. Statistically significant EFS and OS differences were found within the following subsets: MYCN single copy-all age groups differed except 18mo-<5yrs versus 5-<12yrs; MYCN amplified-only OS for <18mo versus 18mo-<5yrs; Stage 4-<18mo differed from all other ages; Stages 1-3-for OS, <18mo differed from all other ages.
CONCLUSION: Age continues to be a powerful prognostic predictor for infants and children with NB. Further statistical analysis, using age as a continuous variable, and multivariate
analyses are ongoing.

Ref ID: 215.2

New Definition of Low Risk Neuroblastoma Using Stage, Age, and 1p and MYCN Status

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BACKGROUND: Regression is well known in neuroblastoma but risk patients among localized and stage 4S neuroblastoma are poorly defined yet. Data from localized and stage 4S neuroblastoma patients without MYCN-amplification were analyzed to define a new, extended low risk group which does not require postoperative chemotherapy.
METHODS AND PATIENTS: 908 stage 1-3/4S patients without MYCN amplification were included. The prognostic impact of age, stage, serum LDH activity, and alterations of chromosomes 1p, 11q, and 3p were analyzed retrospectively by univariate and multivariate analysis.
RESULTS: By univariate analysis, alterations of chromosomes 1p and 11q were correlated with poor event free survival (EFS) and overall survival (OS). Chromosome 3p alterations were prognostic only for EFS. Age and stage were found prognostic for EFS and OS. Stage 3 patients >2 years showed the worst outcome and were excluded from multivariate analysis. By multivariate analysis, status of 1p (p=0.009, hazard ratio [hr] 4.0) and 11q (p=0.028, hr 3.1) proved prognostic for EFS, but only 1p status (p=0.022, hr 3.6) for OS. The new low risk group was defined as: no MYCN-amplification, either stage 1, stage 2 without 1p alterations, stage 3 £2 years without 1p alteration, or stage 4S. These patients had a better EFS (3-y-EFS 88.0±1.3% vs. 51.7±6.5%, logrank p<0.001) and OS (3-y-OS 97.4±0.6% vs. 86.4±4.5%, logrank p<0.001) than stage 2 & 3 with 1p alterations or stage 3 >2 years.
CONCLUSION: Among non-stage-4 neuroblastoma patients, an enlarged low risk group for wait and see strategy may be defined be stage, age, 1p and MYCN status.

Ref ID: 239.1

Malignant neuroblastic tumors (NTs) in the adolescent (10-18 y). The Italian experience with 51 cases

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For the Italian Cooperative Group Neuroblastoma.

BACKGROUND: NTs prevail in the first years of life with peak incidence around 2. NTs in adolescence are rare, and the information concerning their clinical and biological characteristics and outcome is limited. Patients. Of 2006 cases of NTs enrolled in the Italian NB Registry between 1979–2002, 61 (3%) were adolescents of whom 51 had NB/GNB and 10 GN.
RESULTS: Of 51 adolescents with NB/GNB, 27 were female, 24 male. 48 were younger and 13 older than 14 years, median age 136 m. Primary tumor was in abdomen (37 cases), thorax (9), pelvis (2), thoraco-abdominal (2). One had no primary. 22 (43%) had localized (5, stage 1; 4, stage 2; 13, stage 3) and 29 (57%) metastatic disease. Histology was centrally reviewed in 30/46; 20 tumors were classified stroma-poor (16 of the undifferentiated and 4 of the differentiated subtype) and 10 stroma-rich (6 intermixed, 4 nodular). 4/34 pts had MYCN amplification, 11/24 had diploid/tetraploid DNA, 9/22 had 1p deletion/imbalance. In stage 4 disease metastatic sites included lung (6), liver (3), brain (2). Adolescents were treated the same protocols than younger children.
For all pts 5-y OS and EFS was 41% and 19%. In localized disease (stage 1-3) OS and EFS were 53% and 46%; in stage 4 were 32% and 0%. Among prognostic factors only LDH level above1000 UI/L was significantly associated with worse outcome (OS 52% vs 0%; p=0.0005). Others factors could not be properly studied for low numerosity. Of note that 3/4 pts with MYCN+ died of disease.
CONCLUSIONS: Adolescents with malignant NT tend to have a poor outcome also in localized disease. The reasons of a so bad outcome remain unknown and could be explained only with cooperative multinational studies.

Ref ID: 122.1

Prediction of Clinical Outcome and Biological Subclassification of Primary Neuroblastomas by Expression Profiling

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The development of risk-adapted and more effective therapy strategies in neuroblastoma requires further improvements in accurate risk assessment. Although numerous prognostic factors have been identified, precise risk evaluation in individual neuroblastoma patients remains difficult. To define a reliable predictor for event-free survival after first-line therapy and to identify gene signatures characteristic for clinical and biological subgroups, we performed expression profiling of 68 primary neuroblastomas of all stages by Affymetrix HG-U95Av2 arrays. Expression data from subgroups were analyzed using support vector machines with a radial basis function (SVM-rbf), k-nearest neighbors (KNN) algorithms or multiple decision trees. Significance analyses of microarrays (SAM) was applied to search for genes and gene patterns differentially expressed between subgroups. Surprisingly, expression profiles of stage 4 and stage 4s neuroblastomas could not clearly be distinguished by any of the mathematical methods applied. In contrast, MYCN-amplification as well as high expression of the neurotrophin receptor TrkA demonstrated a strong association with specific gene expression patterns. Recurrence of neuroblastoma within two years of diagnosis was correctly predicted in 81% of cases using SVM-rbf or KNN. Sensitivity and specificity of the predictor were 72% and 89%, respectively. Gene patterns associated with event-free survival were found to be rather homogeneous, while patterns for recurrent disease appeared to be diverse. We conclude, that SVM-rbf and KNN are suitable tools for prediction of clinical outcome and biological subclassification in neuroblastoma. Predictive gene signatures have to be evaluated prospectively in larger study cohorts.

Ref ID: 236.1

Microarray expression profiling improves definition of high- and intermediate-risk metastatic neuroblastomas without MYCN amplification

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Age at diagnosis is a key variable in risk-based stratification of metastatic neuroblastomas without MYCN amplification (MYCN-NA). We investigated whether expression profiling with oligonucleotide microarrays (Affymetrix U133A+B) can improve prediction of outcome independent of age. Unsupervised principal component analysis of data from 83 primary stage 4 MYCN-NA neuroblastomas obtained at diagnosis revealed distinct clusters from <12 mos and >24 mos patients at the extremes and >12-24 mos in intermediate and overlapping areas. Supervised nearest shrunken centroids analysis with leave-n-out cross validation identified 490 genes from 39,000 probe sets that defined new high- and intermediate-risk neuroblastomas based on event-free survival (EFS) and not age at diagnosis. The high-risk group (<12 mos n=2; >12-24 mos n=6 and; >24 mos n=31) had EFS of 15%, while the intermediate-risk group (<12 mos n=26; >12-24 mos n=14 and; >24 mos n=4) had EFS of 91%. This analysis identified several genes associated with poor EFS (e.g., HSP90, Pro1073, TrkB, BDNF, MAGE-A4, MAGE-A9, MAGE-A10, and MET) and others with good EFS (e.g., TrkA, HRK, and EPN2). Highlighting chromosomal positions of genes based on Cox regression outcome analysis demonstrated “hot spots” on 11q12, 17q21 correlating with poor EFS and 1p-36.3, 17p11 and 19p correlating with good EFS. In summary, this study identifies gene expression patterns that can improve definition of high- and intermediate-risk MYCN-NA metastatic neuroblastomas and suggests potential therapeutic targets.

Ref ID: 107.1

MYCN-Status according to FISH: Amplification, Gain, and Non-Amplification

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BACKGROUND: While the role of MYCN amplification (MNA) for predicting outcome is undisputed since years, the phenomenon of gene copy excess below the amplification threshold (<5-fold, =MYCN gain) is hardly described and its relevance unclear.
METHODS: To discuss biologic characteristics and clinical impact of MYCN gain versus amplified or non-amplified neuroblastoma we investigated the MYCN status of 659 tumours uniformly analysed with fluorescence in situ hybridisation.
RESULTS: In our cohort the portion of amplified tumours was 18% (116/659). Additional 38 tumours (6%) displayed a MYCN gain. Both amplified and gain tumours were associated with advanced stage disease, increased patients’ age and further chromosomal alterations. Whereas 85% of amplified neuroblastoma displayed aberrations in 1p, MYCN gain tumours correlated with 11q alterations (67%). MYCN expression level was not different between non-amplified tumours and those with gain, but significantly increased in amplified samples (2-113-fold). MNA versus non-amplification discriminated between good and poor outcome independent of stage, age and the degree of amplification. However, within the group of amplified tumours patients with localized disease and age <1 year showed a significantly better outcome than children with stage 4 disease and >1year. Though MYCN gain was associated with poor event-free-survival in localized stages (P=0.005) this appeared to be related to associated genetic aberrations and not by the MYCN gain itself. A survival difference of neuroblastomas with gain and single copy MYCN could not be delineated.
CONCLUSION: MNA predicts poor outcome for neuroblastomas of all stages and age. MYCN gain is also a characteristic feature of advanced stage tumours and higher age of patients, but was not associated with increased MYCN expression and appeared not to be discriminative in predicting outcome.

Ref ID: 070.1

Gene expression profiling suggests a role for haploinsufficiency of 1p35-36 genes in neuroblastoma

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Deletion of the chromosome 1p36 region is a frequent abnormality in neuroblastoma. To gain further insights into the role of this alteration in oncogenesis, we have constructed a specific cDNA microarray representing most known genes and ESTs from the 1p35-36 region and analyzed the expression profiles of 15 neuroblastoma cell lines and 28 neuroblastoma tumours. Hierarchical clustering using expression levels of 320 cDNAs from 1p35-36 separated localized or 4S cases without 1p deletion from advanced stages and cell lines. Supervised learning classification enabled to reliably predict the status of chromosome 1p according to its expression profile. Interestingly, the number of genes or ESTs which presented a significantly decreased expression in samples with 1p deletion was much higher than that exhibiting the opposite profile suggesting that 1p deletion results in a gene dosage effect on a subset of genes critical for the development of 1p-deleted neuroblastoma. Several genes presumed to have functions in neural differentiation (CDC42, VAMP3, CLSTN1), signal transduction in neural cells (GNB1) and cell cycle regulation (STMN1, RPA2, RBAF600, FBXO6, MAD2L2) exhibited a decreased expression in samples presenting 1p deletion. The identification of such genes provides baseline information for further studies to elucidate how these genes could individually or collectively play a critical role in neuroblastoma tumourigenesis.

Ref ID: 049.1

IGFBP-5 in neuroblastoma: complex regulations and multiple effects.

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The relevance of IGF axis in neuroblastoma has been clearly established. IGF Binding Proteins (IGFBPs) are a family of six proteins that regulate the tethering of IGFs to their receptor. We have previously demonstrated the link between IGFBP-5 and Myb in neuroblastoma and now we are investigating other modes of IGFBP-5 regulation and the effects of increase or depletion of IGFBP-5 protein. We characterized different cis-acting elements, located within 300 bp from the transcription start, that regulate IGFBP-5 promoter. We demonstrated that several transcription factors (SMAD, Sp, C/EBP, Myb and CBP/P300), mediate activation or repression triggered by proliferation and differentiation stimuli. In addition, IGF2 increased IGFBP-5 transcription by activating PI3K/Akt pathway and retinoic acid was able to phosphorylate BMP-activated SMADs, increasing their binding to the promoter. Protein binding at the 5' UTR of IGFBP-5 mRNA suggested also post-transcriptional regulation of this gene. Furthermore, we gathered evidences that intracellular IGFBP-5 protein is degraded by the 26S proteasome. We carried out experiments in which the endogenous levels of IGFBP-5 in neuroblastoma cells were decreased by stable transfection of expression vectors (pMIREV) driven by U6 promoter that produce microRNA targeted to IGFBP-5. We detected several effects of IGFBP-5 reduction in IGFBP-5-interfered cells: proliferation appeared markedly decreased (assessed by growth curves and viability assays); anchorage-independent growth was diminished in soft-agar colony assays; apoptosis was sharply increased (Hoechst staining, PI staining, Caspase 3/7 activation assay); retinoic acid-mediated neuronal differentiation was impaired as assessed by morphology and immuno-detection of neural-differentiation markers. Altogether these data strengthen the importance of IGFBP-5 in neuroblastoma, suggesting new strategies for future clinical applications.

Ref ID: 089.1

A novel 1p36.2 located gene, APITD1, with tumor suppressive properties and a putative p53 binding domain, shows low expression in neuroblastoma tumors

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Neuroblastomas generally lack TP53 mutations and no other tumor suppressor gene consistently inactivated has yet been identified in this childhood cancer. Characterization of a new gene, denoted APITD1, in the neuroblastoma tumor suppressor candidate region in chromosome 1p36.22 reveals that APITD1 contains a predicted TFIID-31 domain, representing the TATA box binding protein associated factor, TAFII31, which is required for p53 mediated transcription activation. The genomic organization of APITD1 was determined, and two different transcripts were shown to be ubiquitously expressed, one with an elevated expression in fetal tissues. Primary neuroblastoma tumors of different stages showed either weak or no APITD1 expression. DNA sequencing of the coding regions and the promoter region in 44 neuroblastoma tumors did not reveal any mutations indicating that the coding sequence of APITD1 is well conserved. APITD1 was functionally tested by adding APITD1 mRNA to neuroblastoma cells (SK-N-AS and SK-N-BE), which reduced the cell growth by 90 % as compared to control cells, suggesting APITD1 to have a role in a cell death pathway. We suggest that low expression of this gene, in defective cells, impair the ability for apoptosis through the p53-pathway. Based on its cytogenetic location, 1p36.2, and its biological features in primary neuroblastoma tumors, APITD1 could therefore be considered as a candidate tumor suppressor gene. Further functional studies of the APITD1 RNA and protein, and possible interaction with other genes, are ongoing.

Ref ID: 208.1

A novel region for predisposition to neuroblastoma maps to chromosome 12

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Neuroblastoma (NB) usually occurs as sporadic tumor although 1-2% of cases have a familial recurrence. Linkage studies carried out by us and other groups excluded linkage of NB to some genes implicated in other neurocristopathies and the 1p36 region, frequently deleted in NB. Although evidence of linkage was reported at 16p in North American families, our analysis excluded linkage to this interval in European families, suggesting genetic heterogeneity of NB. A new familial NB case has recently increased the informativeness of an Italian family that presently includes five affected children in the last generation. To identify new regions for NB predisposing genes we performed a genome-wide screening of this family using 382 polymorphic markers that define a 10 cM resolution genetic map. Haplotype reconstruction indicated two chromosomal regions that co-segregate in all five patients: one is located on chromosome 2p (D2S162-D2S2259) and another at 12pter-12q13.13 (D12S352-D12S368). Multi-point linkage analysis excluded the majority of analyzed intervals including the 4p16, previously identified as candidate region by linkage analysis when this family consisted of four patients. Our analysis provided a not conclusive lod-score of 1.65 at D2S305 and yielded a lod-score of 2.97 at D12S1617. These findings indicate a novel region for predisposition to NB located on chromosome 12. To narrow down the interval of interest we are currently genotyping this and a newly recruited family using additional markers. Furthermore, loss of heterozygosity analysis will be undertaken on sporadic NB cases. Database search will highlight genes that may be candidate by location and function.

Ref ID: 017.1

Multi-locus Alleotyping Defines Clinical Sub-types of Neuroblastoma

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Progress in the prognostic assessment of neuroblastoma and in the development of new therapeutic strategies is dependent on a clear understanding of tumour genetics. A wide variety of different genetic abnormalities has been described in neuroblastoma e.g. MYCN amplification, 1p deletion, 11q deletion and 17q gain, and when investigated individually, each has been correlated to some degree with adverse outcome. Nevertheless only MYCN amplification has proven to be useful as a routine clinical marker. Tumour phenotype is the result of multiple rather than single genetic changes however. Consequently in an investigation of tumour loss of heterozygosity (LOH) we have constructed composite alleotypes incorporating genotypes from 96 loci on 5 chromosomal arms (1p, 3p, 4p, 11q, 14q) and have used this in a cluster analysis, together with data on MYCN status, to investigate in more detail the relationship between tumour genotype and clinical phenotype. Our results clearly differentiate between MYCN amplified tumours which also show frequent loss of distal chromosome 1p, and a further group of tumours in which a stepwise accumulation of genetic alterations is associated with progression from low to high stage disease. The initiating genetic event in this group is loss of chromosome 11q, whilst subsequent loss of chromosomes 3p and/or 1p (targeting more distal genes than in MYCN amplified tumours) contributes to an invasive and metastatic phenotype. This stepwise sequence of events is consistent with the later age at diagnosis, and generally less rapid disease course in this tumour group compared with MYCN amplified tumours. Our results also provide the possibility to differentiate between a proportion of localized neuroblastomas which have the potential to progress (as determined by the occurrence of 11q loss) and others which display the more limited growth potential associated with this usually benign tumour.

Oral Presentations

Selected Posters 1

Ref ID: 366.1

Safety and Systemic Availability of Intravenous and Oral Arsenic Trioxide (As2O3) in Children with Relapsed / Refractory Neuroblastoma

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Objective: We conducted a phase I study to evaluate the safety and bioavailability of intravenous (IV) and per oral (PO) As2O3 in children with neuroblastoma.
Method: Eleven children with neuroblastoma were recruited (mean age 2.5 yrs [range 2.1 to 5 yrs, M:F=5:6]). All were previously treated with modified N6/N7 chemotherapy regimen, 3F8 immunotherapy, local irradiation plus auto-PBSCT (n=6), topotecan (n=2) & others (n=1). A maximum of 5 courses would be given and each consisted of 14 days of daily As2O3 alternated with 14 days of rest. In course one, 0.15mg/Kg As2O3 was given as 1 hour IV infusion and then shifted to PO route on Day 2, the rest of the course was by IV route. Blood samples for pharmacokinetic study were taken 0,1,2,4,6 & 8 hours on Days 1 & 2 and 0 hour on Day 3. Blood plasma and cellular fractions were freshly separated and arsenic concentrations were determined in duplicate by inductively coupled plasma-mass spectrometry.
Results: A total of 33 courses of As2O3 were given (3 received 5 courses). The estimated 3 yrs progression free survival was 18% by Kaplan-Meier estimation (2/11 survived at 3yrs, median survival 11 months). In 5/11 tested, plasma and cell samples showed comparable area under the curve attributable to IV or PO dosing. However, the profile of concentrations (peak 400-600 nM) suggested that higher dosage is needed to achieve adequate anti-tumor effect based on in-vitro data for neuroblastoma (2000 nM). No significant side effects were found.
Conclusions: Children could achieve similar plasma and cellular concentration of As2O3 by either IV or PO route. Using the current dosage, the peak and trough concentration of As2O3 were lower than anticipated concentrations required in-vitro.

Ref ID: 383.1

Pitfalls in detection of contaminating neuroblastoma cells by tyrosine hydroxylase RT-PCR due to catecholamine-producing hematopoietic (stem) cells

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RT-PCR analysis of compounds of the catecholamine metabolism (especially tyrosine hydroxylase, TH) is considered to be suitable for detection of contaminating neuroblastoma cells in hematopoietic stem cell preparations. Because of the heterogeneity of neuroblastoma cells, we used for this analysis not only primers for TH, but also for DOPA-decarboxylase, dopamine-ß-hydroxylase, and the noradrenaline transporter. Additionally, primers for tyrosinase were included because in some neuroblastoma cells DOPA production is catalysed preferentially by this enzyme instead of TH. Using this panel of primers, a moderate sensitive detection of the heterogeneous neuroblastoma cells is possible with single RT-PCR, that enables a clear discrimination from hematopoietic (stem) cells[1:1000]. In order to detect a smaller number of contaminating neuroblastoma cells we used nested RT-PCR. Under our experimental conditions, all the respective "neuroblastoma" markers were also positive in mononuclear blood cells, in apheresis preparations (G-CSF mobilized peripheral blood cells) and in highly purified CD34+ and CD133+ stem cells. Our results are generally in line with reports demonstrating both production and uptake of catecholamines by hematopoietic cells [e.g. Marino et al, Exp. Hematology 27 (1999)]. This raises the question whether the RT-PCR analysis of catecholamine metabolism is principally suitable and selective enough to detect a contamination of hematopoietic stem cell preparations with a small number of neuroblastoma cells. Interestingly, by using the TH primers for nested PCR described by Lode [Eur.J.Cancer, 33 (1997)] we found in 15 out of 19 investigated samples one TH signal that appears to be an alternative splice variant in hematopoietic cells but not in neuroblastoma cells. This enables a better discrimination between both cell types.

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Ref ID: 028.2

Induced doxorubicin resistance in caspase-8/10 silenced neuroblastoma cell lines results in multi-drug resistance that involves early and p53-independent anti-apoptotic mechanisms

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Neuroblastoma (NB) is a childhood neoplasm which heterogeneous behaviour can be explained by differential regulation of apoptosis. We previously proposed that caspase 8 silencing in N-type NB cells was in part responsible for death receptor but not chemotherapeutics-mediated resistance. To identify the mechanisms underlying drug resistance, we created doxorubicin (Dox) resistant variant NB cells (DoxR). Caspase-8 silenced, MYCN amplified cell lines, or a single MYCN copy cell line were made resistant to high doses of Dox. The resulting DoxR variants achieved variable levels of resistance, the N-type IGR-N91 cells being the most resistant and the S-type SH-EP the least. DoxR variants were cross resistant to etoposide cisplatin and taxol but remained sensitive to staurosporin, a broad PK inhibitor. The DoxR cells expressed similar levels of caspases-3, 7, 8, 9, Apaf-1, Fadd, Rip, survivin, Bcl2, Bcl-xL, Bax, Smac/Diablo as parental cells, but were unable to activate or cleave these molecules in response to Dox. Likewise, Dox-mediated mitochondrial membrane potential collapse was only observed in the parental cells. Over expression of MDR gene-encoded PgP-1 transporter protein was detected in the DoxR cells. Although verapamil, a MDR1 inhibitor, partially restored sensitivity to Dox, PgP1-mediated drug efflux cannot account for 100% resistance, as DoxR cells were cross-resistant to cisplatin, which is not a a substrate of PgP1. P53 and target genes MDM2 and p21 were rapidly induced by Dox in parental cells, but remained stable or undetectable in the DoxR cells. Pifithrin, a p53 inhibitor, had no effect on DoxR cells in response to Dox. In conclusion, the acquired high and stable drug resistance appears to be mediated by MDR and by p53-independent and early apoptosis-related events that need to be identified.

Ref ID: 347.2

IL-12 Inhibits AKT Activity And Induces BID Activation In Conjunction With Complete Regression Of Orthotopic Murine Neuroblastomas

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Several studies have now described specific molecular defects and/or functional blockade of receptor-mediated apoptosis pathways in human neuroblastoma cells, including loss of caspase-8, FAS, or TRAIL-receptor expression and/or overexpression of prosurvival factors. The present studies demonstrate that systemic administration of IL-12 mediates complete regression of advanced orthotopic intradrenal TBJ neuroblastoma tumors in conjunction with increased local T cell infiltration, inhibition of tumor neovascularization, and ultrastructural changes consistent with tumor and vascular endothelial cell apoptosis. These changes are accompanied by marked increases in circulating IFN-gamma and sFAS-L protein, and enhanced expression of the genes encoding caspase-8, TRAIL, FAS/FASL and TNFR-p55 within the local tumor microenvironment. Although IL-12 treatment induces both endothelial and neuroblastoma cell apoptosis in vivo, and endothelial cells (EOMA) are highly sensitive to FAS/FAS-L or IFN-gamma +/- TNF-alpha induced apoptosis in vitro, TBJ tumor cells appear to be intrinsically-resistant to receptor-mediated apoptosis. Treatment with cycloheximide sensitizes TBJ to undergo receptor-mediated apoptosis in vitro, suggesting the presence of short-lived inhibitors of apoptosis in these cells. We have now demonstrated that TBJ cells constitutively overexpress serine-phosphorylated activated AKT compared to normal murine adrenal gland and that inhibitors of the PI3K/AKT pathway can sensitize otherwise-resistant TBJ cells to undergo receptor-mediated apoptosis in vitro. In vivo, administration of IL-12 markedly inhibits serine-phosphorylation of AKT within TBJ tumors and induces the cleavage and mitochondrial translocation of pro-apoptotic BID. These studies demonstrate potent antitumor activity by IL-12 against murine neuroblastoma tumors, and provide the first evidence that IL-12 may down-regulate a potentially critical survival pathway and mechanism of tumor self-defense in vivo.

Ref ID: 109.1

Tyrosine Hydroxylase expression in blood of patients with Neuroblastoma: Analysis by a real time RT-PCR quantitative assay

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Molecular detection of minimal residual disease by a sensitive methodology could contribute to a better treatment in children with neuroblastoma. To detect circulating neuroblastoma cells we developed a quantitative assay for the analysis of Tyrosine Hydroxylase (TH).
METHODS: We analyzed 70 samples of peripheral blood (PB) and 13 leucapheresis products (PBSC) from 25 patients with neuroblastoma in advanced stages (8 stage 3, and 17 stage 4). TH mRNA was analyzed by a RT-PCR assay using TaqMan technology. For each test sample the amount of TH, and its endogenous reference gene 18S, were determined. Normalized TH value was obtained by dividing TH/18S. Twenty samples of PB from donors were used for normalizing TH, and values <37,05 were considered negative.
RESULTS: With a median follow-up of 47,3 months(range 15-96 months), 9 patients (36%) relapsed. TH expression was detected in all but one patient in PB at diagnosis. During treatment 6 patients cleared tumor cells, while at the end of treatment or during follow-up 18 patients were positive for circulating tumor cells. Nine of these patients relapsed while none of the negative patients did. Actuarial 5-year event-free survival was 100% for TH-negative patients and 40% for TH-positive (p<0.01). Patients with TH-positive PBSC had also a worse prognosis than patients negative for TH. Actuarial 5-year event-free survival was 60% for PBSC negative patients and 32% for positive patients (p=0.05).
CONCLUSIONS: TH-positive patients after treatment seem to have a worse prognosis compared with patients with undetectable TH. Further investigation into the detection of circulating tumor cells during follow up of patients with neuroblastoma is warranted.

Ref ID: 042.1

Detection of residual neuroblastoma cells using a new four-color flow cytometric assay

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BACKGROUND: Detection of bone marrow (BM) involvement is critical for accurate staging and risk assessment in neuroblastoma. Since the therapeutic consequences of the BM findings may be far-reaching, the need for reliable and sensitive detection methods becomes evident. Therefore, two four-color flow cytometric (FC) assays using different combinations of CD9, CD81, CD56, CD45 and anti-GD2 were developed.
METHODS: Thirty-eight BM samples, 17 biopsies and 5 peripheral blood stem cell (PBSC) preparations from 28 neuroblastoma patients were analyzed and the number of CD9+/CD81+/CD45-/CD56+ or anti-GD2+/CD81+/CD45-/CD56+ cells was determined. The results were compared with those of an anti-GD2 immunocytochemical assay.
RESULTS: All tumor samples were CD9+/CD81+/CD45-/CD56+. All except one showed GD2 expression. The results between the FC and the immunocytochemical assay were concordant in 34 out of 43 BM samples and PBSC preparations. Twenty-seven BM samples and 3 PBSC preparation were double negative and four BM samples were double positive. A strong correlation between both assays was found (p=0.006). Seven BM and 2 PBSC samples scored positive for the immunocytochemical assay but were negative for the FC tests. These samples were taken during therapy and after immunocytochemical analysis only a few anti-GD2 positive cells considered as neuroblastoma cells were found.
CONCLUSIONS: FC can be used to detect neuroblastoma cells in a simple, quick and cost-effective way. The sensitivity of the FC assays was lower than that of the immunocytochemical test but it is still possible to screen for residual cells in a reliable way. Further investigations are necessary to determine the clinically relevant detection limit.

Ref ID: 262.2

Evaluation of efficacy of NK cell therapy in human neuroblastoma-bearing mice

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Several lines of evidence suggest that NK cell therapy may represent a successful approach in stage 4 NB patients. However, no adequate murine preclinical models are available to test homing, safety and efficacy of IL2-activated NK cell infusion. Different concentrations of 4 human NB cell lines, with phenotypes closely similar to those of freshly isolated metastatic NB cells, were injected in NOD/scid mice, which completely lack endogenous NK cells. Presence of macro and micro metastasis was monitored in all terminated animals. Following 1x106 cells i.v. injection of HTLA cells, massive bone marrow infiltration, accompanied by renal micrometastasis, was produced in all injected animals, leading them to death. The other cell lines showed variable tumorigenicity and patterns of metastatization. Homing of human IL2-activated NK cells was evaluated by infusing CFSE-labelled NK cells. Blood and organs from mice terminated at different times were then analysed by cytofluorimetry or fluorescence microscopy. NK cells were mainly detected in blood, spleen and bone marrow, but also in lung, liver and kidney. Injection of up to 4x106 NK cells/mouse showed no early or late toxicity. Efficacy of IL2-activated NK cells in reducing NB growth in NB-bearing mice was initially evaluated as single dose infusion of 4x106 NK cells per mouse 24 hr after 1x106 NB cell injection. Survival was significantly prolonged, different NK cell doses and different schedules of administration are currently under evaluation.

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Ref ID: 330.2

Natural Killer (NK) Lymphocytes Are Cytotoxic for Multidrug Resistant Neuroblastoma Cells

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Recurrent tumors, which often are multi-drug resistant, are a significant problem for children with metastatic neuroblastoma. To evaluate the potential of NK cell immunotherapy, we studied NK cytotoxicity against drug-resistant neuroblastoma cell lines, mechanisms of cytotoxicity, and in vivo anti-tumor activity. Purified NK cells (>98%) from normal adults were cultured with IL-2 or IL-2 + IL-15 and then incubated with calcein-AM labeled neuroblastoma cells for 6 hr after which residual calcein fluorescence was quantified. Twelve cell lines were highly sensitive, 4 were intermediate, and 5 were relatively insensitive. Principal component analysis of microarray expression data for 18 of these lines demonstrated 3 clusters correlating with cytotoxicity. Sensitivity was independent of drug resistance, MYCN amplification/expression, and MHC class I expression. By contrast, neuroblastoma expression of ligands for NKG2D (a NK cell activating receptor) MICA, MICB, ULBP-1, -2, and –3 (Taqman PCR) correlated with cytotoxicity (P=0.018). Flow cytometry confirmed that 4 cell lines expressed 2-3 of these ligands and that 8 others expressed ULBP-3. Monoclonal antibody M580 against NKG2D blocked cytotoxicity against LA-N-1 neuroblastoma cells in a dose-dependent manner. Microarray data suggested that CD58 (co-stimulatory ligand) has a role in cytotoxicity, and antibodies against CD2 (CD58 receptor) and CD58 partially blocked killing. Human neuroblastoma tumors formed by NK sensitive CHLA-255 cells in NOD/SCID mice were completely eliminated by intravenous infusion of IL-2 + IL-15 activated NK cells. We conclude that drug resistance does not affect NK activity against neuroblastoma cells and that cytotoxicity against some is NKG2D dependent. These data suggest that strategies to activate NK cells and target them to neuroblastoma cells may contribute to therapy of this disease.

Oral Presentations

Plenary Session C
Translational

Ref ID: 129.1

The tumor-associated antigen PRAME is universally expressed in high stage neuroblastoma and associated with poor outcome

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PURPOSE: The tumor-associated antigen PRAME (Preferentially Expressed Antigen in Melanom) is frequently expressed in a variety of cancers.However, no information is available about its expression in neuroblastoma. We therefore evaluated and quantified PRAME expression in neuroblastoma tumours and assessed its impact on patients' outcome.
METHODOLOGY: Analysis of PRAME expression in a total of 101 patients with neuroblastoma was assessed by both RT-PCR screening, Northern Blotting and Quantitative Real-Time RT-PCR (QPCR). Subsequently, association with tumor stage, patients' age at diagnosis and MYCN amplification was determined. Moreover, impact of PRAME expression on both Event-Free Survival (EFS) and Overall Survival (OS) was evaluated.
RESULTS: RT-PCR screening detected PRAME expression in 93% of primary neuroblastoma and 100% of patients with advanced disease (stage 3 and 4). Both Northern Blotting and QPCR analysis revealed extraordinarily high oscillation of PRAME expression levels and showed highly significant association of higher levels of PRAME expression with both advanced tumor stages ($p<0.01$) and patients' age at diagnosis ($p<0.01$). Finally, grouping of patients according to their QPCR values revealed significant impact on patients' outcome as 3-year EFS was 0.92 (±0.07) for patients with QPCR values <100, 0.71 (±0.08) for patients with values from 100-20.000 and 0.45 (±0.10) for patients with values >20.000 ($p=0.03$). 3-year overall survival for these groups was 1.00, 0.86 (±0.06) and 0.77 (±0.08), respectively ($p=0.10$).
CONCLUSIONS: PRAME expression in neuroblastoma is extraordinary common and was universally seen in patients with advanced stage disease in our study. Furthermore, significant impact of PRAME expression on patients' outcome was shown. Thus, PRAME may present a particularly attractive target for immunotherapeutic strategies in neuroblastoma.

Ref ID: 081.1

Selection for p53 mutant cells by cytotoxic agents in neuroblastoma

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BACKGROUND: Most high-risk neuroblastomas (NB), stage 4 >1 yr, or MYCN amplified initially respond to cytotoxic therapy, but the majority relapse with chemoresistant disease. p53 mutations are infrequent in NB at diagnosis (2%), however a higher incidence has been reported in relapsed tumours and cell lines and are associated with chemoresistance. To explore this further DNA was analysed from 14 paired pre-treatment and relapse and in addition 6 paired pre and post-treatment tumour samples for the presence of p53 mutations using both direct sequencing and DHPLC Wave analysis.
RESULTS: p53 mutations were detected in 2/14 (14%) of tumour samples at relapse or progression but not pre-treatment. One was a missense mutation at codon 270 (Phe-Leu) in a patient with high-risk (stage 4 >1yr) MYCN amplified NB. The same mutation was identified in DNA from tumour after less than 3 months of induction (rapid COJEC) chemotherapy, the patient died 1 month following relapse. A second p53 mutation was identified post progression at codon 157 (Val to Gly) in an infant with stage 4 non MYCN amplified disease. The incidence of p53 mutations in cases at relapse/progression in children who died from disease was 2/10 (20%).
CONCLUSIONS: These results show an increased incidence of p53 mutations in NB at relapse/progression, suggesting that clonal selection of p53 mutant cells by chemotherapy may occur in a subset of NBs. In such cases response to treatment may be improved by therapies which act independently of p53.

Ref ID: 299.

Cyclooxygenase-2 (COX-2) is abundantly expressed in neuroblastoma and its inhibition induces apoptosis and prevents tumour growth in vivo: implications for a novel non-toxic neuroblastoma therapy

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Cyclooxygenase-2 (COX-2), one of the two enzymes that catalyse the conversion of arachidonic acid to prostaglandins, is upregulated in several cancers and linked to proliferation and resistance to apoptosis. We investigated neuroblastoma tumours from different biological subsets and from all clinical stages for expression of COX-2. We detected specific cytoplasmic COX-2 expression in 27/28 neuroblastoma tumours and in all cell lines (8/8), but not in healthy adrenal medullas from children. Treatment of neuroblastoma cells with COX-inhibiting NSAIDs induced caspase-dependent apoptosis via the intrinsic mitochondrial pathway. Diclofenac added in the drinking water potently inhibited (200 mg/L), or at the higher dose (250 mg/L) abolished growth of established human neuroblastoma xenografts in nude rats ($p<0.001$). No toxicity was noted. Immunohistochemistry of diclofenac treated neuroblastoma xenografts showed elevated expression of cleaved caspase-3 as compared with untreated tumours, indicating induction of apoptosis by NSAIDs in vivo. Arachidonic acid and diclofenac synergistically induced neuroblastoma cell death. This effect was further pronounced when lipooxygenase was simultaneously inhibited. Exogenous PGE2 did not salvage neuroblastoma cells from apoptosis induced by COX-inhibition suggesting that forced accumulation of arachidonic acid by abrogation of its downstream metabolism is highly toxic to neuroblastoma cells in vitro. Proton MR-spectroscopy (1H MRS) showed accumulation of polyunsaturated fatty acids and choline depletion in neuroblastoma cells treated with COX-inhibitors. Using clinical MR-scanners 1H MRS is likely to provide pharmacodynamic markers of neuroblastoma treatment response to COX-inhibition. This could provide an early non-invasive response evaluation in children treated with NSAIDs Taken together, these data suggest NSAIDs as a novel adjunct therapy for children with neuroblastoma.

Ref ID: 310.1

[131I]meta-iodobenzylguanidine and topotecan: Experimental combination treatment of tumours expressing the noradrenaline transporter

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INTRODUCTION: The aim of this study was to determine the efficacy of [131I]MIBG in combination with topotecan in vitro and in vivo.
RESULTS: Two cell lines, expressing the noradrenaline transporter (NAT) were used in this study: SK-N-BE(2c) (neuroblastoma) and PN3 (NAT gene transfected glioma cell line). Three treatment schedules were assessed: topotecan administered 24h before (i), after (ii) or simultaneously with (iii) [131I]MIBG. DNA breakage was evaluated by comet assay and cytotoxicity by clonogenic survival. Efficacy was measured by growth delay of tumor xenografts. Supra-additivite clonogenic sterilisation was achieved by combination schedules (ii) and (iii) but not (i). The combination index values at the IC50 level were (i) 1.379 (± 0.025) (ii) 0.761 (± 0.014) and (iii) 0.880 (± 0.016). This order of effectiveness of the sequence of treatments was reflected in their generation of long-term DNA damage: in cells assayed 24h after treatment (to allow for DNA repair), significant damage was observed following schedules (ii) and (iii) (both $p < 0.005$), but not (i) (NS). The mean times required for a 10-fold increase in experimental tumor volume, were 18.6 days (untreated), 31.9 days ([131I]MIBG alone), 25.3 days (TPT alone), 37.1 days (combination schedule (i)) and 49.7 days (combination schedule (ii)) whereas combination schedule (iii) cured 100% of tumors. This treatment caused no myelotoxicity, according to platelet production or
stem cell clonogenic capacity.
CONCLUSIONS: Long-term DNA damage and supra-additive levels of toxicity to NAT expressing cells and xenografts may be achieved using a combination of TPT and [131I]mIBG. These effects are dependent on the scheduling of the two agents.

Ref ID: 192.1

In vivo resistance to CPT-11 in neuroblastoma: Does pleiotrophin play a role?

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To study resistance mechanisms to topoisomerase I inhibitors acquired in a therapeutic setting, we established a neuroblastoma xenograft model (IGR-NB8R) with in vivo resistance to CPT-11. Tumor resistance was achieved after 25 passages in nude mice treated with several cycles of 27 mg/kg/dx5 CPT-11 every 21 days and was revertible after 15 passages without treatment. Cross-resistance existed to the topoisomerase I inhibitor topotecan, but not to cyclophosphamide and cisplatin. Common mechanisms of resistance, such as topoisomerase I alteration, MDR1, MRP, or BCRP expression, were not involved in this revertible resistance. Using cDNA expression arrays (Atlas Human cDNA Expression Arrays, Clontech) on 3 sensitive, 5 resistant, and 3 reverted tumors, we determined 159 out of 588 cancer-related genes which displayed a significant change in expression (ratio >1.75), and were involved in a variety of cellular mechanisms. Moreover, we found Pleiotrophin (PTN), a heparin-binding growth factor with diverse function including angiogenesis and proliferation, as the only gene significantly affected in accordance with acquired resistance: PTN gene expression was dowregulated in all resistant tumors (11-16 fold) as compared to sensitive tumors, and increased (2.5-4 fold) in all reverted tumors as compared to the resistant tumors. Thus, PTN appears to be a likely candidate gene associated with this resistance to CPT-11. We determined PTN expression in 6 neuroblastoma cell lines using western blot and quantitative real-time PCR. PTN was highly expressed in IGR-NB8 and SK-N-AS compared to IGR-N91, CHP-212, NJB, and SH-SY5Y. To investigate the direct implication of pleiotrophin in neuroblastoma, we transfected IGR-NB8 and SK-N-AS with silencing RNA interferences to PTN and are currently evaluating their impact on the sensitivity of these cell lines to CPT-11. (Granted by Ligue).

Ref ID: 227.1

Interferon-mediated anti-angiogenic therapy for neuroblastoma

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AIMS: Type I interferons (IFN-a/b) have pleiotropic antitumour activities but have shown limited clinical efficacy and significant toxicity. We hypothesized that the anti-angiogenic and antitumor activity of IFN-a/b could be enhanced, while limiting its systemic toxicity, by chronic, low-dose (metronomic) delivery, and sought to test this in murine neuroblastoma models.
METHODOLOGY: Continuous delivery of hINF-b was established using a gene therapy-mediated approach in which adeno-associated virus vector encoding human interferon-b (AAV hINF-b) was administered by tail vein. Localized, orthotopic neuroblastoma was established by inoculation of NB-1691 cells into the retroperitoneal space of scid mice while disseminated disease was established by tail vein injection. Successful tumor engraftment was confirmed by ultrasonography.
RESULTS: No overt toxicities were observed. The development of both localized and disseminated disease was prevented in all mice expressing hINF-b (n=8 for each model). Established retroperitoneal tumors, treated with AAV hINF-b when an average of 280 mm3, grew to only 27% the size of control treated tumors ($p<0.0002$) after 12 additional days. All mice treated with AAV hINF-b once established disseminated disease was documented in the liver are still alive 35 days later whereas those treated with a control vector died within 13 days of vector administration.
CONCLUSIONS: Chronic, metronomic AAV-mediated delivery of interferon-b successfully prevented localized and disseminated neuroblastoma engraftment and significantly retarded growth of established retroperitoneal and disseminated disease. Consideration of this approach should be given for the treatment of patients with neuroblastoma, perhaps in combination with cytotoxic therapy, and in the setting of minimal residual disease.

Ref ID: 150.1

Anti-tumour effects of anti-GD2-targeted liposomal antisense oligonucleotides result from a CpG-mediated immune stimulatory effect synergising with an anti-c-myb effect

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Expression of the c-myb proto-oncogene in neuroblastoma (NB) is linked to cell proliferation and differentiation. We recently showed that coated cationic liposomes selectively targeted to the GD2 molecule expressed by NB cells (targeted-CCL) and entrapping an antisense oligonucleotide (asODN) against c-myb (myb-as), increased their anti-tumour activity in vitro down-modulating the c-myb protein. AsODN containing CpG motifs produce a potent immune response that could synergise with the direct inhibitory action of myb-as. Here we checked the anti-tumour effects of our myb-as-liposomal and their mechanisms of action in a metastatic mouse model of human neuroblastoma. Long-term survival was only obtained for animals treated with CCL entrapping CpG-enriched myb-as targeted via Fab' fragments of anti-GD2 (targeted-CCL-CpG-myb-as). Treatment with targeted-CCL containing scrambled CpG sequences or with non-targeted CCL containing CpG-myb-as resulted in reduced anti-tumour effects compared to targeted-CCL-CpG-myb-as; non-targeted CCL containing CpG-free ODN had no effect. Following ablation of NK cells, c-myb-mediated anti-tumour activity was only observed in mice treated with targeted-CCL-CpG-myb-as. Administration of CpG-containing liposomes produced elevated plasma concentrations of immunostimulatory cytokines.Splenocytes were able to lyse target NB cells following activation by targeted-CCL-CpG-myb-as. We have dissected out the contributions to anti-tumour activity of CpG-mediated immune effects from the effects due to c-myb down-modulation. We hypothesise that the therapeutic results derive from the selective targeting of CCL-CpG-myb-as to NB cells resulting in down-modulation of the c-myb protein, which synergizes with a systemic stimulation of an anti-tumour immune response by the CpG sequences.

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Oral Presentations

Plenary Session D
Molecular Biology

Ref ID: 322.1

Senescent F-cells - from oncogene amplification to cellular senescence

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Human somatic normal cells undergo cellular senescence either triggered by telomere shortening or induced by physiological stress including oncogene activation. Both processes, the replicative and the premature or stress-induced senescence, lead to irreversible growth arrest. Immortalization and malignant transformation must overcome the telomere-shortening problem, i.e. the senescence barrier, by expressing telomerase or an alternative lengthening and by inactivation/mutation of cell cycle inhibitory genes. High telomerase expression is known to occur in unfavorable neuroblastomas, especially in those with MYCN amplification in which the occurrence of senescence has not been described so far. In neuroblastoma cell lines, which are predominantly established from oncogene amplified tumors, the occurrence of two distinct cell types have been observed since decades. The small N-cells show a neuronal phenotype and expression pattern, the F-cells are enlarged, have a fibroblastoid appearance, lack neuronal traits and were believed to represent the equivalent of the Schwann cells occurring in maturing neuroblastic tumors. Based on previous work and current investigations, we provide evidence that the F-cell formation reflects the spontaneous revertance and senescence. The process is accompanied by a gradual reduction of the MYCN copy number up to a normal status which is due to expulsion of the double minutes via micronuclei. Furthermore, the cells exhibit an altered phenotype and expression pattern including upregulation of MHC I and downregulation of telomerase, a reduction of telomere lengths and expression of the SA-B-galactosidase. Thus, senescent F-cells (senF-cells) fulfil the criteria of replicative senescence. Moreover, we show that senF-cell formation can be induced and enhanced by exposure to hydroxyurea, a fact which could be used for therapy. Altogether, the results show that gene amplification and malignant transformation is principally reversible and allow the tumor cells to re-enter the program of cellular senescence.

Ref ID: 043.1

MDM2 is a Direct Target of the MYCN Oncogene in Neuroblastoma

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Improved targeted therapeutic approaches to neuroblastoma require a deeper understanding of the molecular pathogenesis of the disease. In order to identify novel MYCN targets we performed anti-MYCN chromatin immunoprecipitation (ChIP) with lysates from MYCN amplified cell lines. Subsequent linker-mediated PCR based cloning identified MDM2 as a putative MCYN transcriptional target. Quantitative real time PCR confirmed the interaction of MYCN at a consensus E-box sequence within the MDM2 promoter. We generated an MDM2-luciferase reporter construct (with and without mutated E-box) and demonstrated specific up-regulation of reporter activity upon MYCN induction in neuroblastoma cell lines. MDM2 is an essential negative regulator of p53 function. MDM2 amplification has been associated with multidrug resistance at relapse, inhibition of p53 activity and inhibition of p14ARF mediated cell cycle regulation. This is the first evidence of a direct link between MYCN expression and MDM2 / p53 regulation. We hypothesize that aberrant MYCN driven expression of this oncogene contributes to neuroblastoma tumorigenesis.

Ref ID: 354.1

Differential Effects of TrkA or TrkB Expression on DNA Repair Capacity Might Contribute to the Genomic Stability of SY5Y Neuroblastoma Cells

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The capacity to repair DNA double strand breaks (DSB) is crucial in maintaining genomic stability. The non-homologous end-joining pathway (NHEJ) for DNA repair has been suggested to be an important caretaker of the mammalian genome. High expression of TrkA is associated with favorable prognosis and a lack of structural chromosomal changes, whereas TrkB is mainly expressed on aggressive neuroblastomas demonstrating high genomic instability. We here examined the contribution of TrkA or TrkB expression to the regulation of the NHEJ-pathway in neuroblastoma. SY5Y cells stably transfected with the TrkA- or TrkB-cDNA served as a model. NHEJ activity was determined in cell-free extracts of SY5Y-TrkA, SY5Y-TrkB and parental cells by a plasmid rejoining assay. Consistent with higher resistance to irradiation, SY5Y-TrkA extracts demonstrated a high rejoining activity in comparison to parental cells. In contrast, NHEJ activity was significantly reduced in SY5Y-TrkB extracts. To screen for underlying mechanisms, gene expression data (Affymetrix U95Av2) obtained in the same SY5Y-TrkA/TrkB model were reanalyzed focussing on genes involved in DNA repair. While no changes were detected for most repair genes, expression of XRCC4, a central effector gene of NHEJ, was significantly up-regulated in SY5Y-TrkA compared to SY5Y-TrkB cells. Expression data were confirmed by quantitative PCR and western blot. XRCC4 expression was also up-regulated in primary neuroblastomas with high TrkA expression. These data suggest a contribution of Trk receptors to the genomic stability in SY5Y neuroblastoma cells by mediating DSB-repair capacity. Further functional analyses of the NHEJ pathway may shed light on DNA repair capacity and regulation of genomic stability in neuroblastoma and might partially explain current hypothetic models of neuroblastoma evolution.

Ref ID: 087.1

Nuclear IkB kinase-alpha (IKK-alpha) regulates the proapoptotic function of p73, but not p53, during the cisplatin-mediated apoptosis

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p73, mapped to chromosome 1p36.2, is a member of the gene family comprising tumor suppressor p53 and p63. We previously demonstrated that SH-SY5Y neuroblastoma cells undergo apoptosis in response to cisplatin in a p73-dependent manner, suggesting that p73 plays a critical role in regulating DNA damage response in neuroblastoma. In a sharp contrast to the p73-mediated apoptotic pathway, NF-kB signaling pathway plays a role in cellular protection against a wide variety of proapoptotic stimuli including DNA damage. Here we found that IKK-alpha, one of the cytoplasmic upstream regulators of NF-kB activation, accumulated in the nucleus during the cisplatin-induced apoptosis in association with a remarkable induction of p73 as well as p53. Consistent with the previous reports, cisplatin treatment had no significant impact on the NF-kB-dependent transcriptional activation. Ectopic expression of IKK-alpha increased the stability of p73 by inhibiting its ubiquitination, and thereby enhancing its transactivation and pro-apoptotic activities, whereas IKK-alpha did not affect the stability of p53. In vitro pull-down assay and Immunoprecipitation experiments demonstrated that p73 was directly associated with IKK-alpha through its DNA-binding domain. In contrast, p53 was unable to interact with IKK-alpha as examined by co-immunoprecipitation analysis. Although the kinase-deficient mutant form of IKK-alpha retained an ability to interact with p73, it failed to increase the expression level of p73. Of interest, expression of the kinase-deficient IKK-alpha abrogated the cisplatin-mediated stabilization of endogenous p73. Thus, our present
findings strongly suggest that the IKK-dependent positive regulation of p73 is a novel nuclear function of IKK to modulate DNA damage response, which could contribute to develop new therapeutic strategies against aggressive neuroblastoma by enhancing the efficiency of cisplatin and by overcoming acquisition of cisplatin resistance.

Ref ID: 075.1

Deregulated WNT/BCATENIN pathway in neuroblastoma without MYCN amplification

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High-risk neuroblastomas (NBs) without MYCN amplification frequently express cMyc at high levels. We hypothesized that aberrant Wnt/Bcatenin signaling might account for this as cMyc is a Bcatenin transcriptional target and many embryonal malignancies have alterations in this pathway (medulloblastoma, nephroblastoma, hepatoblastoma, pancreatoblastoma). We assessed expression and localization of Bcatenin in 8 NB cell lines (by immunoblot and immunocytology) and correlated findings with MYCN and cMYC expression. Bcatenin mutation screening (by immunoblot, RT-PCR and SSCP) and Bcat:TCF activity using Bcatenin reporter constructs were performed. In primary NBs, Bcatenin mutation analysis was performed as was expression profiling of 50 high-risk NBs (20 with MYCN amplification) using the Affymetrix U95Av2 genechip to assess Bcatenin pathway components and transcriptional targets. Cell lines without MYCN amplification expressed high levels of cMyc and higher Bcatenin with aberrant nuclear accumulation (similar to HepG2 with mutant Bcatenin) though no mutations were identified. Despite this, there was no increase in Bcat:TCF transcriptional activity by reporter assay in NB cell lines. Expression profiling of primary NBs showed the major Bcatenin interacting TCF, TCF7L2, to be near-absent in high-risk NBs (amplified or not), and decreased Axin1 in non-amplified NBs with no change in other components of the Bcatenin scaffolding/degradation complex. Finally, Bcatenin target genes (e.g., CCND1, PPARD, NRCAM, TCF7) were overexpressed in NBs without MYCN amplification further supporting pathway activation. Thus, NBs without MYCN amplification may deregulate cMyc via altered Bcatenin signaling. We demonstrate aberrant expression and localization in NB cell lines correlated with cMyc expression. Although no pathway mutations nor direct evidence of Bcat:TCF transactivation was found in cell lines, primary NBs evidenced pathway activation. Bcatenin may deregulate target genes through heterodimeric interactions with alternate partner genes in select high-risk NBs.

Ref ID: 323.1

The Delta-Notch pathway integrates the wnt pathway, the noradrenalin synthesis route and the neurotrophin pathway in neuroblastoma

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The Delta-Notch pathway plays an important role in embryonal development in species ranging from Drosophila to humans. It controls cell fate decisions during differentiation of cell lineages. The Dlk-1 gene is very highly expressed in normal adrenal medulla and in a subset of neuroblastoma cell lines. The expression in cell lines closely correlates with expression of Dopamine Beta Hydroxylase (DBH), one of the key enzymes in the noradrenalin synthesis route. Neuroblastomas with high Dlk-1 expression therefore probably represent tumors from a relatively late differentiation stage of the chromaffin cell lineage. In general, Notch genes function as receptors for Delta proteins on adjacent cells and this interaction can start a process of divergent differentiation between the two cells. Indeed, the Notch3 gene shows an inverse expression pattern with Dlk-1 in the neuroblastoma cell line panel. Notch3 positive cells therefore represent another differentiation stage or lineage than Dlk1 positive cells. The Delta-Notch pathway includes many different genes. We used SAGE and micro-arrays to analyze the changes in gene expression in neuroblastoma cell lines transfected with members of the Delta-Notch pathway. This showed that Dlk1 can control the expression of genes of the Wnt pathway. In addition, we found that activation of neurotrophin receptors of the Trk family can strongly regulate expression of members of the Delta-Notch route in neuroblastoma. The final result of these interactions is de-differentiation of neuroblastoma cells and abrogation of Dopamine Beta Hydroxylase expression. This identifies the Delta-Notch pathway as a central player in differentiation route of the adreno-sympathetic cell lineage. We are sequencing the key genes in a large neuroblastoma tumor series to establish whether defects in the pathway contribute to neuroblastoma pathogenesis.

Ref ID: 071.4

Cisplatin induces p53 target genes expression independently of p73 in human neuroblastoma cells

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As we have recently shown, TAp73 over-expression in neuroblastoma (NB) cells harboring endogenous wild-type p53 (wt-p53) triggers apoptosis more efficiently than in p53-mutated NB cells (Goldschneider et al., 2004). Moreover, cisplatin (CDDP), a DNA-interacting drug used in NB treatment, induces damage response involving p73 (Gong et al., 1999) and, whatever the p53 status, TA-p73 abolition leads to CDDP resistance in human cancer cells (Irwin et al., 2003). Here, to address the question of the biological effects of CDDP according to p53 status in human NB cells, we examined p53-target gene expression in SH-SY5Y cells (p53+/+, p73+/+) and LAN-1 cells (p53-/-, p73+/+). Our results showed that, in SH-SY5Y cells, CDDP induced an up-regulation of p53, p73, p21, GADD45, and MDM2. However, in LAN-1 cells, neither p21 nor GADD45 induction was observed, although p73 expression was induced. Dominant negative p53DD transfection of SH-SY5Y cells dramatically suppressed p21 gene expression induced by p53 but not by p73. To further assess the role of p73 isoforms in CDDP cytotoxicity, transient transfections of TA or DeltaNp73 in SH-SY5Y cells were performed: CDDP cytotoxicity did not change in either of the p73 isoform transfected cells in comparison with nontransfected cells; in parental cells, p53 expression was highly activated by both isoforms whereas p21 expression was induced only by TAp73. Overall, our data indicate that p53 seems to act alone in response to CDDP treatment of human NB cells. Thus, p53 status should be taken into account for CDDP-based chemotherapy of NB patients.

Oral Presentations

Parallel Session C
Biology - Genetics

Ref ID: 087.2

UFD2a, whose gene is localized in a 500 kb homozygously deleted region at chromosome 1p36.2 found in a neuroblastoma cell line, modulates p73 function through regulation of its stability

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We previously found for the first time a 500 kb homozygously deleted region at 1p36.2 in an NB1 neuroblastoma cell line, and subsequently identified six genes within this region including UFD2a (Oncogene, 2000). UFD2a belongs to a new family of E3/E4 ubiquitin protein ligases with the U box, but its function remains unknown. p73 which is a new member of the p53 family, has an important role in the cellular response to genotoxic damage, and has recently been reported that it functions during induction of programmed cell death of newborn mouse sympathetic neurons. The cellular level of p73 is mainly regulated posttranslationally. Here we found that UFD2a negatively regulates the stability of p73.
Results and Discussion: During the cisplatin-mediated apoptosis in SH-SY5Y neuroblastoma cells, p73a accumulated at a protein level, whereas the intracellular level of endogenous UFD2a was significantly decreased in response to cisplatin. Ectopic expression of UFD2a in COS7 cells inhibited the cisplatin-induced accumulation of p73a. In vitro pull-down assays and immunoprecipitation experiments revealed that p73a physically interacted with UFD2a, and this interaction was mediated by both the COOH-terminal SAM domain of p73a and the extreme NH2-terminal region of UFD2a. In contrast, p53 did not bind to UFD2a. Of note, degradation of p73 by UFD2a was independent of ubiquitination. More interestingly, mutant UFD2a, which lacks the U box, retained an ability to down-regulate the level of p73a. Thus, our results imply that UFD2a promotes the degradation of p73a through a mechanism distinct from the ubiquitination-dependent proteolysis. These suggest that, since p73a has both TAp73a and deltaNp73a isoforms, which function vice versa, UFD2a may regulate their balance at protein levels and contribute to the decision of life and death of neuroblastoma cells.

Ref ID: 214.1

Germline mutations of the paired-like homeobox 2B (PHOX2B) gene in hereditary neuroblastoma.

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Hereditary predisposition to neuroblastoma accounts for less than 5% of neuroblastomas and is probably heterogeneous. Recently, a predisposition gene has been mapped to 16p12-p13 but has not yet been identified. Occurrence of neuroblastoma in association with congenital central hypoventilation and Hirschsprung's disease suggests that genes, involved in the development of neural crest derived cells, may be altered in these different conditions. The recent identification of phox2B as the major causing gene in congenital central hypoventilation prompted us to test it as a candidate gene in familial neuroblastoma. We report a family with three first-degree relatives with neuroblastic tumours (namely two ganglioneuromas and one neuroblastoma) in one branch and two siblings with Hirschsprung's disease in an other branch. A R100L phox2B mutation was identified in all three patients affected with tumours but not in tumour-free members of this family.
We also report a germline phox2B mutation in one patient treated for Hirschsprung's disease who subsequently developed a multifocal neuroblastoma in infancy. Both mutations disrupt the homeodomain of phox2B protein. No loss of heterozygosity of phox2B was observed in the tumours suggesting that haplo-insufficiency or dominant negative effect may account for the oncogenic effects of these mutations. This observation identifies mutations in phox2B as the first germline predisposition to hereditary neuroblastic tumours.

Ref ID: 297.1

Identification of relevant MYCN downstream effectors by a combinatorial multimodel approach

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Amplification of the MYCN transcription factor is the hallmark of a subgroup of advanced stage neuroblastoma tumors and was one of the first genetic parameters used for therapy stratification. However, up till now, the molecular circuit governed by MYCN and its target genes is not fully understood. One of the problems that confound deeper insights is the time and cell specific context that influences the MYCN transcriptional program. To address this issue, we designed a multimodel strategy to identify consistent and relevant MYCN downstream effectors. cDNA subtractive cloning between an amplified and MYCN single copy neuroblastoma cell line generated a list of 300 differentially expressed transcripts (including some novel genes), that were spotted onto a custom cDNA array. Subsequent expression profiling of an extended series of both amplified and MYCN single copy cell lines, and a stably MYCN transfected cell line versus its parental non-amplified cells provided a list of putative MYCN transcriptional target genes. To discriminate between early (direct) and late (secondary) effectors, we profiled different time points in a cell line with tetracycline controllable MYCN expression. Genes that were found consistently differentially expressed in all tested model systems, were further scrutinized by in silico promoter analysis, and ongoing reporter gene assays. In general, MYCN appears to be a more potent transcriptional silencer, and our data provide possible insights into the way MYCN exerts its oncogenic effects. Further validation in primary tumor biopsies indicated that some MYCN target genes are significantly correlated with reduced patient survival, hence providing potential targets for molecularly oriented therapies for patients with this type of aggressive tumor.

Ref ID: 117.1

Gene expression profiling of neuroblastoma: analysis of nonmetastatic versus metastatic tumors

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BACKGROUND: Previously we reported a significant distinction in gene expression profile between neuroblastoma (NB) tumors and cell lines, and stroma-rich and stroma-poor NB tumors. Tumors clustered into 2 groups correlating with clinically defined prognostic groups of favorable and unfavorable tumors. In this study we analyzed tumors with and without clinically proved metastatic potential.
METHODOLOGY: we present the analysis of 25 locoregional and 27 stage 4 tumors all stroma poor and >1 year of age. Microarray analysis was carried out using Affymetrix Genechip Human Genome U95 SetTM with features for 63,175 gene/EST. To calculate the ability of individual genes to distinguish between sample groups we used 1. Calculation of maximal variability and >3 standard deviation between groups and 2. Step-down permutation and false discovery rate methods. For multivariate analysis, unsupervised methods like SOMS, PCA, K-nearest neighboring, and hierarchical clustering were applied.
RESULTS: By contrast analysis, 61 out of 12,000 known annotated genes from chip A were differentially expressed in LR cases compared to stage 4. By permutation analysis only 3 out of the 12000 annotated genes showed statistical significance. Multivariate analysis showed a poor distinction between LR and stage 4 tumors with a third of samples classifying in the wrong groups. Classification models build to distinguish groups using support vector machines, k-nearest neighboring and log regression showed a cross validation accuracy that ranged from 76 to 89%.
CONCLUSIONS: the gene expression profiles of tumors presenting as LR or disseminated disease suggest the existence of more than 2 groups of tumors. A \"metastatic\" gene expression profile could not be detected.

Ref ID: 223.4

High resolution detection of neuroblastoma hemi- and homo-zygous deletions with array-based comparative genomic hybridization (aCGH)

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Human neuroblastoma provides a prototypical example of the biological relevance and clinical utility of somatically acquired chromosomal aberrations. The pattern of genomic alterations present in neuroblastomas is strongly associated with tumor behavior and patient outcome. We have used aCGH (4300 BACs, 1 Mb resolution) to perform an unbiased survey of genomic aberrations, and for high resolution mapping of relevant chromosomal loci. We first used a panel of 43 neuroblastoma cell lines (1p36, 2p24, 11q14-23, and 17q23-25 allelic status determined by FISH/PCR) to validate detection of known aberrations. We showed outstanding sensitivity and specificity with all amplicons detected and high concordance with PCR-based hemizygous deletion detection methods. Using a variety of class discovery (eg hierarchical clustering) and prediction (eg k-fold cross validation) algorithms, we have identified frequent regions of gain on chromosomes 20 and 22, and loss on 7, 16, and 20. Deletions of 3p were present in 30% of the lines, and we have identified a 5 Mb 3p homozygous deletion, verified by FISH and PCR, that considerably narrows the published region of interest. Using global correlation analyses we confirmed associations such as concordant 3p and 11 deletions, as well as identified several new potential cooperating loci. In addition, we have precisely mapped the boundaries of a 40 Mb 11q constitutional deletion in a patient with multifocal neuroblastoma. We have also used this platform to map the critical regions of chromosome 11 required for tumor suppression in functional complementation studies, allowing us to define two critical 11q regions of 1.3 and 5.9 Mbs, respectively. These data demonstrate that aCGH can accurately measure copy number in the neuroblastoma genome, refine common regions of deletion not amenable to traditional positional cloning approaches, detect homozygous deletions, and be used to identify novel regions of genomic imbalance.

Ref ID: 135.1

Array comparative genomic hybridization (aCGH) defines genomic subgroups with a set of distinct aberrations which are strongly associated with the prognosis of patients with neuroblastoma

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To unveil DNA copy number aberrations (CNA) which characterize distinct subsets of neuroblastoma, we applied array CGH (2,464 human BAC clones) to 244 primary neuroblastomas (120 sporadic, and 124 mass screening) and 25 recurrent tumors. Hierarchical clustering showed the presence of at least five genetic subgroups. Group 1 (G1) (n=36; 5-year survival rate: 84%; and DNA ploidy: 73% diploidy) had few CNAs. G2 (n=6; 0% survival; and 100% diploidy) had a pattern similar to G1, except they had MYCN amplification. The prognosis of the patients in G2 was extremely poor. G3 (n=49; 54% survival; and 50% diploidy) typically had both 1p loss and 17q gain and moderate grades of whole chromosome gains and losses. MYCN amplification occurred in 57% of cases. G4 (n=55; 70% survival; and 47% diploidy) had both 11q loss and 17q gain and other chromosomal abnormalities. MYCN amplification occurred in 13%. G5 (n=98; 93% survival; and 8.5% diploidy) had whole chromosome gains and losses, and most were diagnosed by mass screening. Five-year survival rates of 120 sporadic cases were G1:74%(n=23), G2:0%(n=6), G3:38%(n=32), G4:46%(n=31) and G5:83%(n=28). Overall, G1 and G2 had little instability, except G2 had MYCN amplification. Both G3 and G4 had a pattern of genomic instability, and the abnormalities in G5 were more consistent with a pattern of mitotic dysfunction. The presence of G2 itself suggested that MYCN amplification might occur as an early event. More interestingly, the analysis of the paired primary and recurrent tumor samples suggested progression from G1 and from G2 to G3. These suggest that both 1p loss and MYCN amplification can occur independently in neuroblastoma, and that MYCN amplification might progress into 1p deletion.

Oral Presentations

Parallel Session D
Clinical

Ref ID: 157.1

Multivariate evaluation for heterogeneous neuroblastomas: the discrimination of progressing risk tumors detected clinically and through infantile mass-screening program

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OBJECTIVE: The aim is to discriminate biology of Neuroblastomas (NBs) with high sensitivity and specificity.
PATIENTS AND METHODS: NBs were enrolled into two groups; 226 NBs were diagnosed clinically (non-mass NBs) and 301 NBs were detected through Japanese infantile mass-screening program (mass NBs). The prognostic indicators were MYCN amplification, International Neuroblastoma Pathology Classification (INPC), Ha-ras/Trk A expression.
RESULTS: (I) Discreminating biology in the non-mass 226 NBs: 1) Specificity for poor clinical outcome was confirmed in "MYCN amplification", "INPC Unfavorable Histology" and "Low Ha-ras/trk A expression", respectively. However, the sensitivity was not enough to use singly. They were 42%, 56% and 60% among the patients with tumor progression, respectively. 2) The sensitivity to the progressing events could increased up-to 84%, when we picked up NBs with any of three factors mentioned above (HIGH RISK NBs). 3) 94% of the patients were disease-free survivors, of whom NBs had "no MYCN amplification" and "INPC Favorable Histology" and "High Ha-ras/trk A expression" (LOW RISK NBs). 4) Multivariate analysis showed three NB categories defined with "MYCN" and "Ha-ras/trk A " and "INPC". The hazard ratio of three groups were 1.00, 2.66 and 7.74. (II) Predicting biology of mass-NBs according to MYCN, INPC, and Ha-ras/trk A; Of 248 mass-NBs, 40% showed low risk biology, 25% were in high risk group and 35% were in intermediate risk group.
CONCLUSION: This study presents an useful risk discrimination of NBs with high specificity and sensitivity and the heterogeneity is also shown in the mass-NBs with variety of progression risk.

Ref ID: 123.1

From genotype to phenotype in neuroblastoma: The derivation of clinically different entities

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BACKGROUND: From high throughput genetic studies several characteristic signatures have been proposed. We asked whether or not the suggested genetic categorization is associated with particular clinical phenotypes.
METHODS: The clinical characteristics of 176 patients from three neuroblastoma categories were retrospectively analyzed. Group 1 was defined by no MYCN amplification, no 1p, no 11q aberration and stage 1 (n=91). Group 2 patients comprised tumors without MYCN amplification, without 1p aberration, with 11q aberration and stage 4 (n=47). Group 3 consisted of stage 4 patients with MYCN amplification and without 11q aberrations (n=38).
RESULTS: Marked differences were found between the groups in several aspects. Age: Group 2 patients were older (40 months) compared to group 3 (23 months) and group 1 (10 months) ($p < 0.001$). Site: In group 3 the adrenals were more frequently involved (95%, 2: 60%, 1: 44%; $p < 0.001$). No differences were detected in the metastatic pattern between groups 2 and 3. Histology: The tumors of group 3 were more frequently neuronal undifferentiated (3: 89%; 2: 50%, 1: 41% ($p = 0.006$)). Unfavorable Shimada histology was similar in groups 2 and 3 (1: 7%; 2: 67%; 3: 69%). Tumor markers: VMA was frequently elevated in group 2 (96%), but only half in group 1 (53%) and 3 (50%) ($p < 0.001$). Outcome: The 3 year overall survival rate was 100%, 73% and 49% ($p <0.001$) for the groups 1, 2 and 3, resp.. The recurrences of group 2 presented later that those of group 3.
CONCLUSION: The genetically defined neuroblastoma categories demonstrated distinct clinical differences even within the stage 4 subgroups.

Ref ID: 370.1

131-I-MIBG double infusion with autologous stem cell transplant (ASCT) for neuroblastoma: A New Approaches to Neuroblastoma Therapy (NANT) study

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131-I-Metaiodobenzylguanidine (131-I-MIBG) elicits 30% response in refractory neuroblastoma, but the activity infused is limited by radiation safety and hematologic toxicity. The goal of this study is to determine the maximum dose of 131-I-MIBG that can be administered in two consecutive infusions at a 2-week interval, supported by ASCT 2 weeks after the second dose. The dose of 131-I-MIBG was escalated in a 3+3 Phase I trial design, with levels calculated by delivered red marrow radiation index (RMI) from the double infusion. The target levels of RMI were 400, 600, and 800 cGy. Using detailed dosimetry, the second infusion was adjusted to achieve the target RMI. Seven patients have been enrolled; 6 are evaluable for toxicity. Median age was 8 years, all were heavily pretreated, including 6/7 with prior high dose therapy and ASCT. Mean total activity delivered was 23 mCi/kg for patients at Level 1, with mean measured RMI of 425 cGy; Level 2, total activity 30 mCi/kg, with a mean measured RMI of 571 cGy; Level 3 begins in 3/04. No dose-limiting toxicity occurred to date. Hematologic toxicity has been acceptable, with median time to ANC>500 after PBSC of 13 days. All patients required platelet transfusion, with median time to platelet independence of 17 days after PBSC. There have been no infections, hemorrhage, fever with neutropenia, or other non-hematologic toxicity above grade 1. Of 6 evaluable patients, there has been 1 MR and 3 SD. Four patients are alive with stable disease at 20-215 days. The lack of toxicity with this approach will allow dose intensification of 131-I-MIBG, with the possibility of improved response in refractory disease.

Ref ID: 136.1

High risk stage 3 neuroblastoma: favorable outcome with myeloablative therapy and 13-cis-retinoic acid

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AIMS: We assessed whether myeloablative chemotherapy with purged autologous bone marrow rescue (ABMT) compared to chemotherapy (CC) alone and whether subsequent treatment with 13-cis-retinoic acid (RA) compared to no RA therapy improved survival (S) for patients with high risk (HR) stage 3 neuroblastoma (NB) defined as greater than 1 year of age with amplified MYCN copy number (MYCN-A), unfavorable Shimada histopathology or elevated serum ferritin level.
METHODS: Seventy-three of 539 patients enrolled onto CCG study 3891 had HR stage 3 NB. Patients were analyzed using a log-rank test.
RESULTS: The 5-yr EFS and S was 54 +/- 6% and 58% +/- 6%, respectively (n=73). MYCN-A (n=24) adversely effected prognosis with 5-yr EFS of 25% +/- 9% compared to 75% +/- 7% for patients with single copy MYCN (n=38), p<0.0001. Patients randomized to ABMT (n=20) had 5-yr EFS of 65% +/- 11% and S of 65% +/- 11% compared to 41% +/- 11 (p=0.21) and 46% +/- 11% (p=0.34) for patients randomized to CC (n=23), respectively. Patients randomized to RA (n= 23) had 5-yr EFS of 70% +/- 10% and S of 78% +/- 9% compared to 59% +/- 12% (p=0.52) and 62% +/- 12% (p=0.30) for patients randomized to no RA, respectively. Patients randomized to both ABMT and RA (n=6) had a 5-yr EFS and S of 100%.
CONCLUSION: Although there was insufficient statistical power to detect treatment differences within this small subset, the data suggest an improved outcome for HR Stage 3 NB randomized to receive ABMT and/or RA. Additional tumor molecular and minimal residual disease analyses are currently ongoing to better define subsets of stage 3 patients that require aggressive therapy.

Ref ID: 278.1

Prediction of MYCN amplification in neuroblastoma using serum DNA and real-time quantitative PCR

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BACKGROUND: Serum DNA of cancer patients includes a significant amount of tumor-released DNA. Several groups reported that tumor-related aberrations (e.g., loss of heterozygosity) could have been detected using serum DNA.
AIMS: To facilitate evaluation of MYCN status of tumors, we developed a new method to predict MYCN status using serum DNA of patients with neuroblastoma (NB).
METHODS: Using real-time PCR, we simultaneously quantified MYCN (2p24) and a reference gene, NAGK (2p12, NM 017567), and evaluated MYCN copy number as a MYCN/NAGK (M/N) ratio in 57 NB patients. The patients included 11 MYCN-amplified (1 each in stages 1 and 3, and 9 in stage 4) and 46 MYCN-non-amplified cases (13 each in stages 1 and 2A+2B, 3 in stage 4S, 5 in stage 3 and 12 in stage 4), as determined by Southern blotting.
RESULTS: The serum M/N ratio in MYCN-amplified patients (300.1Å}68.3, meanÅ}SE, n=11) was significantly higher than that in non-amplified patients (1.1Å}0.1, n=46, p=0.0014, by Welch's t-test). In MYCN-amplified cases, the M/N ratios in serum DNA and tumor DNA were significantly correlated (r2=0.55, p=0.0092).
CONCLUSIONS: The serum MYCN/NAGK assay can be a non-invasive predictor of MYCN status in NB. Moreover, in cases with relapse, the serum M/N ratios had risen prior to relapse. The serum M/N ratio appears to be a promising indicator of therapeutic efficacy and relapse for MYCN-amplified cases.

Ref ID: 115.1

Association Of High-Level MRP1 Expression With Poor Clinical Outcome In A Large Prospective Study Of Primary Neuroblastoma

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We have previously shown in a retrospective analysis that high expression of the multidrug transporter gene MRP1 is strongly predictive of poor outcome in neuroblastoma (NEJM, 334:231-8, 1996). We have now undertaken a prospective analysis of MRP1 expression in a large cohort (n=209) of primary untreated neuroblastomas from patients enrolled on POG biology protocol 9047. Real-time PCR was used to determine expression of MRP1, MDR1, MYCN and TrkA. Older age, advanced stage, MYCN amplification and low TrkA expression were all predictive of poor outcome in this cohort. MRP1 expression was significantly higher in MYCN-amplified tumors (p=0.0025). Although dichotomising MRP1 expression around the median failed to predict outcome, using higher cutpoints MRP1 expression became a significant predictor of outcome. Thus, high levels of MRP1 (upper decile) were highly predictive of both event-free-survival (p<0.0001) and overall survival (p=0.0003). This cut-off closely approximated the level of MRP1 expression in SK-N-SH cells, which we previously recommended as a reference standard for MRP1. Following adjustment for the effect of MYCN amplification and of other prognostic indicators by multivariate analysis, MRP1 expression retained significant prognostic value for both event-free survival (hazard ratio=3.0; p=0.0011) and survival (hazard ratio=2.6; p=0.0095), whereas MYCN amplification lost prognostic significance. MDR1 expression demonstrated no prognostic significance. The results of this prospective study confirm our earlier findings and support a clinically relevant role for MRP1 gene expression in drug refractory neuroblastoma.

Ref ID: 025.1

Id2 is Sufficient and Necessary for Growth and Angiogenesis in Neuroblastoma

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The inhibitor of differentiation Id2 is a target of the Retinoblastoma protein (Rb) during embryogenesis. Expression of Id2 is directly activated by Myc oncoproteins (c-Myc and N-Myc) and Id2 accumulates in human neuroblastoma cells with amplification and/or overexpression of N-myc. Recent data from our lab showed that Id2 is essential for tumor progression and angiogenesis in the embryonal tumor model from Rb+/- mice. Through gain- and loss-of-function experiments in human neuroblastoma cell lines, we now show that Id2 is necessary and sufficient for tumor growth and for the angiogenic switch in neuroblastoma. This conclusion is supported by: 1) the enhanced growth, angiogenesis and production of Vascular Endothelial Growth Factor (VEGF) in vitro and in vivo by neuroblastoma cells lacking amplification of N-myc but engineered to express ectopic Id2. 2) the coregulation of Id2 and VEGF in the antimitogenic response of neuroblastoma cells to Retinoic Acid and the rescue of VEGF inhibition by expression of Id2. 3) the reduced synthesis of VEGF in N-myc amplified/Id2-overexpressing neuroblastoma in which endogenous Id2 has been suppressed by siRNA. These findings explain why Id2 overexpression is selected in neuroblastoma carrying activation of the N-myc oncogene, as tumor cells expressing Id2 have a considerable proliferative and survival advantage. Given the broad role attributed to Id proteins for tumor progression and angiogenesis, we suggest that aggressive neuroblastoma is an excellent candidate for therapeutic intervention with novel anti-Id drugs.

Ref ID: 204.1

H-prune, nm23-H1 and nm23-H2: new markers of Neuroblastoma progression

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In Neuroblastoma (NB) an enhanced effort is dedicated to identify genes concurring for aggressiveness and tumor progression. We investigated the role of a protein complex including h-prune, nm23-H1 (NDPK-A) and nm23-H2 (NDPK-B) present in the nuclei of neuroblastoma cell lines. We have shown that h-prune and nm23-H1 are expressed during mouse embryonal development (neural crest and dorsal root ganglia) and we have demonstrated the capability of h-prune to bind nm23-H1, interaction impaired with nm23H1-S120G, a mutation occurring in stages 4 of NB. Furthermore h-prune is able to bind nm23-H2 and its binding is impaired with mutants affecting the NDPK activity. The overexpression of h-prune in SH-SY5Y genome cell-line correlates with an increase of nm23-H1 and H2 expression. A new function of h-prune in the nucleus has been found as transcriptional activator of nm23-H2 expression. We present data related to the capability of h-prune to bind the nm23-H2 and transactivate its expression while nm23-H1 is activated from the binding of nm23-H2 to its promoter. An in vivo analysis on 57 NB cohorts (from stage 1 to 4S) at both mRNA (Real Time) and protein (ISH) levels, we observed an increased level of h-prune and of nm23-H1 and nm23-H2 expression, predominantly in stage 4 (highly aggressive and metastatic tumors). All together our finding indicate that increased levels of nm23-H2 and nm23-H1 are dependent from h-prune activation, this phenomenon lead to an increased metastatic potential, as found in NB stage 4.

Ref ID: 078.1

KIF1B is a variant-dependent tumor suppressor gene mapped to a 500-kb homozygously deleted region at chromosome 1p36.2 in neuroblastoma

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KIF1B encodes the kinesin superfamily motor protein and has recently been identified as one of the candidate genes of Charcot-Marie-Tooth neurodegenerative disease. We have previously reported that KIF1B is located in the 500-kb homozygously deleted region at chromosome 1p36.2 in a human neuroblastoma cell line. We here report that KIF1B is a tumor suppressor gene whose function is dependent on its splicing variants.
RESULTS AND DISCUSSION: The expression of KIF1B was significantly down-regulated in aggressive neuroblastomas. However, our extensive search failed to detect any particular mutation in both the coding as well as the promoter region that we identified by luciferase reporter assay. The latter region was also examined for possible inactivation by methylation using methylation-specific PCR, resulting in the absence of such mechanism. Surprisingly, however, inoculation of the NMuMG cells stably expressing retroviral antisense-KIF1B gene into nude mice induced remarkable tumor formation with lung metastasis.
Furthermore, expression of KIF1B mRNA was down-regulated during NGF- and retinoic acid-induced differentiation of neuroblastoma cells, whereas it was up-regulated during NGF depletion- and retinoic acid-induced apoptosis in newborn mice SCG neurons and CHP134 neuroblastoma cells, respectively. These suggested that KIF1B may possess tumor-suppressive as well as proapoptotic function. We further found that the KIF1B gene had at least four splicing variants, and that the variants III and IV, but not I, were up-regulated during the cellular apoptotic process. More interestingly, overexpression of KIF1B variants III and IV, but not I, in NB1 and SH-SY5Y cells induced growth suppression by inducing apoptosis. Thus, our results have demonstrated that KIF1B is a splicing variant-dependent tumor suppressor gene mapped to the critical region of chromosome 1p36 in cancers including neuroblastoma.

Ref ID: 301.1

Response of neuroblastoma to the hypoxic environment

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We have studied the changes in gene expression profile of neuroblastoma cell lines caused by exposure to hypoxia. Cells were exposed to hypoxia (1% oxygen) or normoxia (20% oxygen) for variable lengths of time, and total RNA was extracted and analyzed by microarray using Affymetrix chips. The data were analyzed using Gene Spring software. Hypoxia caused a significant modulation of gene expression, which suggested a profound change in the biology of the cells. Functional clustering and Gene Ontology allowed the identification of groups of genes defining the hypoxic signature of neuroblastoma cell lines, which is being verified in in vivo experiments in animal models. Of particular relevance was the upregulation of genes connected to the angiogenic process and potential targets of antiangiogenic therapy. Furthermore, we found the upregulation of important genes coding for secretory products or for integral proteins not previously associated to neuroblastoma, whose expression can be associated to the transformed phenotype. In parallel experiments, we studied the gene expression profile of neuroblastoma cells exposed to picolinic acid, a tryptophan metabolite which shares with hypoxia the ability to activate the Hypoxia Responsive Element (HRE) responsible for the transcriptional response to hypoxia. Comparison of gene expression profiles in response to the two signals, together with the analysis of the presence of HRE in the promoter region of modulated genes, allowed us to propose the existence of HRE-independent pathways characterizing the response to hypoxia or PA and to narrow down the role of HRE in the response of neuroblastoma cells to hypoxia. The results obtained studying the gene expression profile of neuroblastoma cells treated with drugs with anti-hypoxia properties will be discussed.

Ref ID: 309.1

Identification of MYCN transcriptional activity inhibitors yields compounds which preferentially inhibit the growth of neuroblastoma cells

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Amplification of the MYCN oncogene is associated with rapid tumour progression and poor outcome in human neuroblastoma. We have explored MYCN as a potential cancer therapeutic target by developing a MYCN reporter gene assay, using a luciferase gene construct under control of the ornithine decarboxylase (ODC) gene promoter, to screen for specific inhibitors of MYCN transcriptional function. This luciferase gene construct has been stably transfected into MYCN amplified neuroblastoma cells (NGP, clone19) and MYC-overexpressing neuroepithelioma cells (NB/CHP100, clone11). A pilot screen of 2800 compounds identified two (NUMYCNA1, IC50=7±2µm and NUMYCNA2, IC50=6±1µm) that reduced MYCN-dependent luciferase activity in NGP19, but not NB11, suggesting these compounds preferentially inhibit MYCN function. Western blot analysis showed that MYCN protein levels did not change, whereas its transcriptional target, ODC, was reduced to 50% in NGP19 after 24hrs exposure to these compounds. Electrophoretic mobility shift assays indicated that the inhibitory mechanism did not involve direct disruption of MYCN/MAX complex formation and binding to its E-box DNA recognition sequence. Growth inhibition was examined in a panel of eight MYCN amplified and non-amplified neuroblastoma cell lines, and compared with six non-neuroblastoma tumour cell lines, all chosen to be wild-type for p53. There was no clear relationship between MYCN amplification and the sensitivity of neuroblastoma cell lines to these compounds, however the neuroblastoma cell lines were markedly more sensitive (median IC50=6.8µM and 7.0µM respectively for NUMYCNA1 and NUMYCNA2) to the growth inhibitory effects of these compounds compared with the non-neuroblastoma cell lines (median IC50=56.1µM and 58.2µM respectively for NUMYCNA1 and NUMYCNA2), indicating a potential class of neuroblastoma specific agents.

Ref ID: 053.1

Methylation Patterns in Ganglioneuroma and neuroblastoma

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The hypermethylation of CpG islands within Gene promoter regions is an epigenetic phenomenon that is often associated with the transcriptional silencing of downstream genes and contributes to cell transformation. We have determined the pattern of methylation of several genes involved in distinct biological pathways, including cell proliferation and apoptosis, in neuroblastoma (NB) and in the non-malignant ganglioneuroma (GN). Tha purpose of this work was to search for a "methylator phenotype" in NB; i.e.: of a cluster of genes whose frequent methylation in this tumor could be associated with defined clinical and biological parameters. We have analyzed 9 NB cell lines, 31 NB at different stages with or without MYCN amplification and 13 GN. We have observed dramatic differences in the methylation pattern of 5 genes (CASP8, 14.3.3s, DN-p73, RASSF1A and DCR2) between NB and GN indicating that this phenomenon is not tissue-specific and can be considered as cancer-dependent aberrant methylation. Importantly, the oncogenic isoform of p73 was fully methylated in GN and partially methylated in NB confirming our previous observation on the role of this gene in this tumor. Furthermore, in MYCN-amplified tumors the methylation level of CASP8, 14.3.3s, RASSF1A and DCR2 was higher than that observed in MYCN-single copy samples. Our data suggest the existence of a methylator phenotype associated with MYCN-amplified tumors.

Ref ID: 374.1

Expression of TrkA in SY5Y Neuroblastoma Cells Sensitizes for Chemotherapy-Induced Apoptosis by Up-Regulation of Caspase-8

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High expression of TrkA is associated with favorable prognosis of neuroblastoma, whereas TrkB is mainly expressed on aggressive, unfavourable neuroblastomas. TrkB/BDNF signaling has recently been shown to mediate chemotherapy resistance in neuroblastoma cells.To investigate the effect of TrkA expression on the susceptibility of neuroblastoma cells to cytotoxic drugs, SY5Y neuroblastoma cells and their stable TrkA transfectants were treated with the chemotherapeutic drugs doxorubicin, etoposide and cisplatin in clinically achievable concentrations. Cell death in response to chemotherapy was measured by FACS analysis with Nicoletti-assay and annexin-V-staining. Interestingly, expression of TrkA efficiently enhanced drug-induced apoptosis in comparison to mock-transfected parental cells (SY5Yvec). After 48 h of treatment, the dose required for 50% specific apoptosis of the cells was 27-fold reduced for doxorubicin, 20-fold reduced for etoposide and 10-fold reduced for cisplatin in SY5Y-TrkA cells compared to SY5Yvec. To identify the underlying molecular mechanisms, we analysed expression profiles of SY5Y-TrkA and SY5Yvec cells on a genome-wide scale using Affymetrix U95Av2 arrays. Several differentially regulated genes involved in apoptotic signaling were identified including caspase-8, previously shown to be silenced by gene hypermethylation in aggressive neuroblastomas. RT-PCR and western blot analysis confirmed up-regulation of caspase-8 in SY5Y-TrkA cells in comparison to caspase-8 negative SY5Yvec cells. TrkA-mediated apoptosis was inhibited by the caspase-8 specific inhibitor zIETD. The effects of caspase-8 silencing by RNA interference on drug-induced apoptosis of SY5Y-TrkA cells is currently investigated. Expression of TrkA might enhance the response of low-stage neuroblastomas to chemotherapy by up-regulation of caspase-8. This effect may contribute to the favorable prognosis of TrkA expressing neuroblastomas.

Ref ID: 338.1

p53/MEK1/NF-kappaB Signaling Pathway Mediates Anticancer Drugs Induced Cell Death in N-type Neuroblastoma Cells

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Neuroblastoma (NB) is the most common extracranial solid tumor of childhood. N-type NB cells are characterized by a neuronal phenotype, they are tumorigenic in mice, MYCN amplified, express wild-type p53. In previous work, we determined NF-kappaB activation is required for doxorubicin (Dox) and etoposide (VP16)-induced cell death of N-type NB (JBC 2001, 276:48921-9). The present study was designed to determine the upstream signaling pathway(s) that mediate Dox and VP16-induced death of N-type NB. p53 and MAPK are both known to mediate NF-kappaB activation in other cells expressing p53 (Nature 2000; 404:892-7). p53 expression was determined by immunoblotting following Dox or VP16 treatment. p53 was detected within 1h of treatment with Dox or VP16. This induction occurred prior to a decrease in I-kappaBalpha protein expression level and NF-kappaB activation. Importantly, Dox and VP16 failed to induce NF-kappaB activation in cells expressing a dominant negative mutant form of p53 (DNp53/SH-SY5Y) and these cells were resistant to killing by Dox or VP16. In complimentary experiments, p53 was inactivated in SH-SY5Y cells by expressing human papillomavirus E6. E6/SH-SY5Y cells were also resistant to Dox and VP16-induced cell death. Furthermore, when SH-SY5Y or IMR32 cells were pre-treated with PD98059, a MEK1-specific inhibitor, Dox and VP16-induced I-kappaBalpha degradation and NF-kappaB-dependent luciferase activity were blocked. PD98059 also inhibited Dox and VP16 induced cell death in a dose-dependent manner. These data suggest NF-kappaB activation is required for Dox or VP16 to kill N-type NB. The response involves MEK1 activity, which depends on p53 induction. Agents that directly target these signalling molecules should be studied to determine their therapeutic potential in the treatment of NB.

Oral Presentations

Selected Posters 2

Ref ID: 008.1

Enhancement of targeted radiotherapy in neuroblastoma: a novel gene therapy approach

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The radiopharmaceutical 131I-MIBG used as a single agent, has obtained long term remissions and palliation in neuroblastoma patients. A curative effect of 131I-MIBG-treatment could be attained by increased concentration of the radiopharmaceutical in malignant cells. We previously reported that transfection of the NAT gene into human neuroblastoma cells induced the expression of a functional transporter which improved the active uptake of 131I-MIBG and resulted in dose-dependent toxicity to the host cells. A transgenic construct with radiation-inducible WAF1 promoter driving NAT should be able to upregulate the synthesis of the NAT in neuroblastoma cells. This strategy involves WAF1/NAT transfection followed by an initial dose of radiation in the form of 131I-MIBG. This will facilitate the tumour-specific overexpression of NAT. A second administration of 131I-MIBG should be avidly concentrated by target tumour cells, leading to their sterilisation. We transfected a neuroblastoma cell line (SK-N-BE) with a plasmid construct which contains the GFP cDNA transcriptionally controlled by X-ray inducible promoter of WAF1 (p21). A 6 Gy external-beam radiation dose increased the GFP protein level up to 1.4 times the unirradiated cells protein level. In the same conditions preliminary studies showed that 4 MBq doses of 123I-MIBG and 5 MBq doses of 131I-MIBG were able to increase GFP protein level to respectively 1.6 and 1.7 times the unirradiated cells protein level. In similar experiments, even low doses (50 nCui) of 211At-MABG (an analogue of MIBG) were able to increase GFP protein level to 3.5 times the unirradiated cells protein level. These encouraging results suggest that the radiation-inducible promoter of WAF1 could be a valid candidate to drive the over-expression of the NAT gene in neuroblastoma cells in a radiopharmaceutical-dependant manner.

Ref ID: 349.1

Genes associated with multi-drug resistance in neuroblastoma cell lines identified by gene expression profiling

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Loss-of-function of p53 (p53-LOF) is one mechanism of acquired multi-drug resistance (MDR) in neuroblastoma. We examined 17 neuroblastoma cell lines (10 drug-sensitive, 7 MDR) to identify other mechanisms of MDR. Higher intracellular glutathione levels correlated with MDR [median glutathione (nmol/mg protein): 403 vs. 589] ($P < 0.001$). RNA expression (quantified by TaqMan RT-PCR) of glutathione synthetic (GCSc, GCSR) and utilization (GPX, GSTμ, GST-p, and GGT) enzymes showed increased expression of GCSR (median 1.0 vs. 2.5) and GSTμ (median 7.9 vs. 30) ($P = 0.001$) correlated with MDR. To identify additional drug resistance genes, we compared RNA expression in 2 drug-sensitive and 3 MDR cell lines using the human U133 Affymetrix gene-chips. This included a pair of cell lines established from a patient before treatment and at disease progression after ABMT. Expression of mdr-1, FN1, hspB1, HDAC1, Gal1, and SMUG was >2-fold higher in MDR cell lines relative to drug-sensitive. TaqMan RT-PCR in our panel of 9 drug-sensitive and 11 drug-resistant (6 with p53-LOF) neuroblastoma cell lines showed the median RNA expression in MDR cell lines vs. drug-sensitive was higher for all the genes tested: mdr1 (0.6 vs. 0.02), FN1 (1.1 vs. 0.2), hspB1 (1.1 vs. 0.3), HDAC1 (0.7 vs. 0.4), Gal1 (0.4 vs. 0.2), SMUG (1.5 vs. 0.9), however statistical significance ($P < 0.05$) was demonstrated only for mdr1 and HDAC1. In p53 functional vs. p53-LOF MDR cell lines, only HDAC1 ($P = 0.02$) was significantly associated with p53-LOF. Our data indicate that these genes associated with drug resistance in neuroblastoma cell lines should be studied in patient samples and may provide new therapeutic targets.

REF ID: 002.1

IMPROVED OUTCOME IN HIGH-RISK NEUROBLASTOMA AFTER APPLICATION OF INTENSIFIED MULTIMODAL THERAPY

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AIMS: Improve outcome in high-risk neuroblastoma.
MATERIAL: An unselected population-based consecutive series of high-risk neuroblastoma at a single institution. Forty-five not previously treated children were diagnosed 1984-2003 with high-risk neuroblastoma (stage 4 >1year or stage 4 <1year with amplified MYCN, or stage 3 >1year with MYCN-amplification).
TREATMENT: Therapy was step-wise intensified over time during three distinct periods. Participation in collaborative trials was given priority. 1984-1989 nine children received OPEC-chemotherapy, surgery, and in some cases high-dose Melphalan and autologous bone-marrow rescue (ABMT).
1990-1995 12 children were randomised (ENSG5) to OPEC/OJEC or COJEC followed by surgery, Mel-ABMT and, daily low-dose retinoic acid (RA).
1996-2003 24 children of which the first 8 were randomised in ENSG5, the rest received COJEC. Surgery was aiming at complete removal. Local irradiation was applied as intraoperative radiotherapy (IORT) and/or external irradiation to the pre-operative primary tumour volume. High-dose chemotherapy (Melphalan alone, or combined with etoposide-carboplatin or liposomal busulphan) was followed by stem-cell rescue, local irradiation and six months high-dose RA in two-week pulses. Selected patients received MIBG-therapy from 1990.
OUTCOME: Event-free survival probability was 0%, 17% and 71% at 36 months for the three periods 1984-1989, 1990-1995 and 1996-2003 resp ($p<0.001$). EFS at 60 months for children treated 1996-2003 is 63%. Only 2/21 children from 1984-1989 are alive whereas 17/24 from 1996-2003 are alive in CR (4-86 months from diagnosis, median 39, $p<0.001$). Nine-teen of 24 during 1996-2003 was MYCN-amplified, 14 of these are alive and relapse-free.
CONCLUSIONS: Survival was significantly improved in high-risk neuroblastoma over time with intensified multimodal therapy. Dose-intensive induction (COJEC), high-dose busulphan followed by biological MRD-treatment (RA) is feasible in a single-institution setting and seems promising to improve survival and cure for children with high-risk neuroblastoma. We conclude that today the majority of children with high-risk neuroblastoma can be long-term survivors.

Ref ID: 222.1

Intensive targeted chemo-radiotherapy of metastatic neuroblastoma with high-dose iodine-131 labelled meta-iodobenzylguanidine, topotecan and haemopoietic support

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BACKGROUND AND AIMS: Iodine-131 labelled meta-iodobenzylguanidine (131-I-mIBG) is an established palliative treatment for metastatic neuroblastoma. The topoisomerase 1 inhibitor topotecan has cytotoxic activity against neuroblastoma and is a radiosensitiser. Laboratory data show that the combination of topotecan and 131-I-mIBG is synergistic. Theoretically the clinical effectiveness of 131-I-mIBG therapy could be improved by dose escalation and combination with topotecan. The dose limiting toxicity of this approach is myeloablation, which can be circumvented by haemopoietic support. The aim of this pilot study is to show that this combination is a feasible treatment.
METHODOLOGY: 12 mCi/kg 131-I-mIBG is given on day 1, with topotecan 0.7mg/m2 from day 1 to day 5. In vivo dosimetry is used to calculate a second quantity of 131-I-mIBG which when given on day 15 will result in a total whole body absorbed radiation dose of 4 Gy. Further topotecan 0.7 mg/m2 is given from day 15 to day 19. This is followed by peripheral blood stem cell or bone marrow reinfusion.
RESULTS: Four boys and four girls with relapsed stage 4 neuroblastoma have been treated. Peripheral blood stem cell support was used in six, and bone marrow in two. The measured total whole body radiation absorbed dose ranged from 3.7 to 4.7 Gy (mean 4.2 Gy). Satisfactory haematological reconstitution was observed in all patients. There was no unacceptable toxicity.
CONCLUSIONS: Intensification of 131-I-mIBG therapy by dose escalation and combination with a radiosensitiser with a haemopoietic autograft appears safe and practicable. This approach should be tested for efficacy in a Phase II study in patients with refractory stage 4 neuroblastoma.

Ref ID: 029.2

A clustering of gene hypermethylation distinguishes high and low-risk neuroblastoma patients

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BACKGROUND: Although neuroblastoma is the most common solid malignancy in infancy, the biological factors involved in its development and progression are still unclear. Promoter hypermethylation and transcriptional silencing of tumor suppressor genes is a hallmark of human tumors.
METHODS: We examined 45 candidate genes representative of diverse cellular pathways in 10 neuroblastoma cell lines and 45 tumors.
RESULTS: Clustering of neuroblastoma cell lines on the basis of hypermethylation distinguished highly proliferative and/or MYCN-amplified vs. low-malignant tumors ($P=0.0124$, Fisher exact test). Univariate analysis of ten commonly hypermethylated genes in primary tumors identified three relationships: promoter hypermethylation of the developmental gene HOXA9 correlated with mortality in non-infant patients ($P=0.0404$, Kaplan-Meier analysis) and non-MYCN amplified tumors ($P=0.0229$); the aberrant methylation of the pro-apoptotic gene TMS1 and the cell cycle gene CCND2 was associated with stage 4 progressing tumors, but uniformly absent in stage 4S tumors undergoing spontaneous regression ($P<0.0001$); and hypermethylation of the differentiating gene RARß2 was restricted to patients that survived the disease ($P=0.0323$). The unsupervised hierarchical cluster analysis of all tumors revealed a comprehensive picture of hypermethylation events that classified tumors according to their high and low-risk behavior.
CONCLUSIONS: Our exploratory study in neuroblastoma provides evidence that profiling of the status of CpG island hypermethylation in human primary tumors may have clinicopathological value.

Ref ID: 305.1

Antigen specific immunity in neuroblastoma patients: antibody and T-cell recognition of NY-ESO-1 tumor antigen

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BACKGROUND AND AIMS: Neuroblastoma cells have been shown to express molecularly defined tumor-associated antigens and to be induced to express HLA by cytokines, thus becoming potential targets for T cell immunity. However, the immune response against tumor antigens has not been shown in NB patients. We have evaluated the expression of of NY-ESO-1 tumor antigen in NB tumors and we evaluated the immunogenicity of this antigen in a set of HLA typed NB patients.
RESULTS: The analysis of antigen expression by RT-PCR and IHC showed that a large fraction of NB tumors at different stages express NY-ESO-1. In some patients NY-ESO-1 induced a humoral and T cell-mediated immune response. The antibody response was a late event in the disease course: in fact NY-ESO-1 antibodies were present in 10% sera from stage 3 and 4 NB patients at diagnosis, while were not detected in stage 1 and 2 patients as well as in sera obtained during remission. A T cell-mediated response against NY-ESO-1 could be induced by stimulating patients’ PBL with NY-ESO-1 recombinant protein or with the HLA-A2 restricted peptide 157-167. HLA-class I and class II restricted specific T cells were detectable after in vitro sensitization by tetramer staining and by Elispot analysis in PBL from patients at different disease stages and in seronegative patients. In addition, NB cells expressing NY-ESO-1 antigen were recognized by activated T lymphocytes.
CONCLUSION: These data provide evidence to the in vivo immunogenicity of NB and indicate that NY-ESO-1 is a potential target antigen for immune–based intervention strategies in NB patients.

Ref ID: 072.1

Results of NB 97 SFOP Protocol in children > 1 year with a stage 4 neuroblastoma

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To improve the prognosis of children > 1 year with a stage 4 NB, we developed a strategy with an induction chemotherapy (IC) according to N7 protocol, a surgical excision of the tumor and a consolidation with high-dose chemotherapy (Busulfan 600 mg/m2 - Melphalan 140 mg/m2) (Bu-Mel) followed by stem cell transplantation when complete or very good partial remission of metastases was obtained. Radiotherapy of the primary tumor site was performed in patients whose tumor had an MYC N amplification. From June 1998 to April 1999, 47 patients were treated according to this strategy. 43 of them, with positive metastatic sites on MIBG scan were included in the phase II study evaluating the response rate after IC. Hematological toxicity was the main problem observed after IC. Treatment was stopped in 4 cases (1 PD, 1 hematological toxicity, 1 hemorrhagic cystitis, 1 severe hearing loss), 1 post surgical toxic death occurred. CR of metastases was obtained in 17/40 evaluable patients (43%, (27-59%)). 34 received Bu-Mel. As of November 2003, with a median follow-up of 60 months, the 4-year EFS and OS are 40% (27-54%) and 51% (37-64%) respectively. In conclusion, following this IC, the CR rate was not superior to those observed with other less toxic IC. In this strategy, post-transplant retinoic acid was not used. However, despite these facts, the long term survival appears particularly encouraging.

Ref ID: 303.2

The SIOPEN-R-NET Project: Building a European network for neuroblastoma treatment (HR-NBL-1/ESIOP) and research

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The SIOPEN-R-NET project (EC grant No. QLRI-CT-2002-01768) aims to build a European Neuroblastoma Research Network to optimise the use of preexisting infrastructures, to improve consistency and complementarity through harmonised standard operating procedures and to build material resources and repositories for current and future research tasks. A Web based centralised databank and communication system is developed allowing clinical trial management with remote data entry, electronic data capture, remote randomisation, image transfer, information on trial progress and offers communication tools and links between clinical data and research tasks. The current European high-risk neuroblastoma treatment protocol, the HR-NBL-1/ESIOP study serves as backbone to build these structures. This is a randomised study of the European SIOP Neuroblastoma group for children over one year with stage 4 disease or stages 2 and 3 with MycN-amplified neuroblastoma and was activated on 02/02/2002. At least 1000 patients will be recruited over 5 years. Currently it is activated in 14 European countries. The protocol consists of a rapid, dose intensive induction chemotherapy (COJEC) adopted from the UK-ENSG5 protocol with randomised use of G-CSF [R0] to rapidly reduce bulky disease. It aims to reduce the incidence of local relapse and hence encourages extensive surgical removal of the primary tumour at the end of induction and adds local irradiation to all patients after megatherapy/PSCR. It compares the therapeutic benefit and toxicity of two megatherapy regimens (CEM and BuMel) through randomisation [R1] and attempts to eradicate minimal residual disease with differentiation therapy (13-cis retinoic acid). To date 274 patients are registered on study via the WEB based study tool, 114 patients participate in R0 and 91 patients have been randomised in R1. Study safety is monitored continuously and surveyed by a data monitoring committee.

Oral Presentations
Parallel Session E
Molecular Biology-Translational

Ref ID: 231.1

Cyclophosphamide, but not melphalan or carboplatin, synergistically enhanced topotecan activity against neuroblastoma cell lines in hypoxia

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The role of hypoxia (2% O2) in neuroblastoma drug resistance is largely unknown. We determined the effect of hypoxia on the cytotoxicity of cyclophosphamide (as 4-hydroperoxycyclophosphamide = 4-HC), melphalan, or carboplatin with topotecan against 2 sensitive and 6 multi-drug resistant neuroblastoma cell lines. Cytotoxicity was measured by DIMSCAN digital imaging fluorescence assay, synergy calculated as Combination Index (CI). Synergistic cytotoxicty of melphalan or carboplatin with topotecan observed in normoxia (20% O2) did not occur in hypoxia. However, 4-HC significantly (P < 0.04) and synergistically (CI < 1) enhanced topotecan cytotoxicity in both conditions, with greater synergism in hypoxia (CI<0.1) than in normoxia (CI<1). The increased cytotoxicity of 4-HC + topotecan in hypoxia (compared to melphalan or carboplatin + topotecan) was associated with increased DNA damage (antibody to single strand breaks by flow cytometry) in the multi-drug resistant (post-ABMT) CHLA-136 cell line (78% relative to 2% control, 25% and 24% single agents). Treated/Control (T/C) survival ratios in days for athymic mouse neuroblastoma xenografts treated daily x 5 with cyclophosphamide, topotecan, or both, paralleled in vitro results, where high-dose cyclophosphamide + topotecan (T/C = 5.1; complete responses durable for a median of 51 days) was more effective than cyclophosphamide (T/C = 3.9) or topotecan (T/C = 2.1). Thus, only cyclophosphamide + topotecan retains activity in hypoxia. The steep dose-response curve for cyclophosphamide + topotecan suggests that dose escalation with stem cell support may significantly increase the clinical response rate and magnitude for this active combination.

Ref 198.1

The protoncogene HMGA1 is a molecular target for MYCN in neuroblastoma

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BACKGROUND AND AIMS: HMGA1 is an architectural transcription factor and a putative protoncogene. Deregulation of its expression has been demonstrated in most human cancer. We had previously shown that the expression of the HMGA family members is deregulated in neuroblastoma (NB) cell lines and primary tumors. Upon retinoic acid (RA) treatment of MYCN amplified NB cell lines, HMGA1 decreases with a kinetic that strictly follows repression of MYCN. In addition MYCN constitutive expression abolished HMGA1 repression by RA. Thus we explored the possibility that HMGA1 expression might be sustained by MYCN in amplified cells.
METHODOLOGY: We used transient transfection assays to shown MYCN effects on HMGA1 expression and HMGA1 promoter activation; quantitative PCR to monitor HMGA1 expression in primary human NBs an in tumors arisen in MYCN transgenic mice; in vitro DNA binding assays to demonstrate MYCN binding to HMGA1 promoter.
Results. We observed HMGA1 overexpression in a transgene dose-dependent fashion in NBs developing in MYCN transgenic mice. Although we could detect HMGA1 expression in many human primary NBs, it was much higher in MYCN amplified compared to MYCN single copy NBs. MYCN overexpression increased HMGA1 expression in SK-N-AS and HEK 293 cells. Luciferase activity from a HMGA1 1600bp-promoter fragment/luciferase gene reporter significantly increased upon MYCN cotransfection, indicating that HMGA1 is a transcriptional target for MYCN. MYCN/MAX heterodimers could bind three different HMGA1 promoter fragments containing putative MYCN binding sequences, in vitro. Luciferase activity strongly decreased in 5' deletion variants of the 1600bp promoter lacking the first three putative E-boxes. Point mutation of the third E-box did not significantly reduce luciferase activity suggesting that, more than the activity of a single site, the cooperative function of the multipe cis-acting elements mediates HMGA1
transactivation by MYCN.
CONCLUSIONS: We propose that HMGA1 is a novel MYCN target gene relevant for NB tumorigenesis.

Ref ID: 401.1

Inhibiting the Cyclin D1-pRb pathway in neuroblastoma

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INTRODUCTION: Cyclin D1 regulates G1 cell cycle progression by controlling the phosphorylation of the retinoblastoma protein (pRb). mRNA expression analysis by SAGE and Northern blot revealed a high expression of Cyclin D1 in a subset of neuroblastoma. Subsequent immunohistochemical staining of neuroblastoma tissue arrays containing 183 neuroblastoma showed high expression of Cyclin D1 in 75% of the tumours. We screened by using Southern blot analysis for amplifications and rearrangements of the Cyclin D1 gene. Five amplifications and one rearrangement were found in 202 neuroblastoma
tumours.
AIM: To study the biological relevance of Cyclin D1 and the functional dependency on Cyclin D1 of neuroblastoma cell lines.
RESULTS: We developed five small interfering RNA's of which one showed a good transient silencing of Cyclin D1 after 48 hours. We could achieve an effective knock down in the neuroblastoma cell lines SK-NB-E, SK-N-FI, SJ-NB-6 and SJ-NB-10 on RNA as well as on protein level. Western blot analysis showed an almost complete disappearance of CDK4 specific pRb phosphorylation and a decrease of Cyclin A expression as downstream target of the Cyclin D1-pRb pathway. The Cyclin D1 knock down resulted in a significant cell growth reduction after 72 hours. By FACS analysis we showed a G1 specific cell cycle arrest in the Cyclin D1 siRNA treated cells. No increase in apoptosis was detected in these cells but a decrease in Cyclin D1 expression caused neural differentiation.
CONCLUSION: Cyclin D1 is required for unlimited cell proliferation in neuroblastoma cell lines. This is of specific interest because Cyclin D1 functions through activation of Cyclin Dependent Kinases. Small molecule inhibitors against these kinases are developed as therapeutic drugs. We are now testing several new small molecules in neuroblastoma cell lines.

Ref ID: 116.1

Establishment and characterisation of cell lines from MYCN transgenic murine tumors

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Overexpression of the human MYCN oncogene driven by a tyrosine hydroxylase promoter causes tumors in transgenic mice that recapitulate human neuroblastoma (EMBO J. 16:2985-95, 1997). To further investigate the role of MYCN in neuroblastoma pathogenesis, we have developed a series of cell lines from these MYCN-driven murine tumors. Lines were established from tumors arising in two homozygous and two hemizygous MYCN transgenic mice. Hemizygous tumors gave rise to cell lines growing only in suspension. Homozygous tumors gave rise to similar suspension lines as well as morphologically distinct substrate-adherent lines. FISH analysis demonstrated selective MYCN transgene amplification in cell lines derived from hemizygous mice, but not from homozygous mice. CGH and FISH analysis confirmed a range of neuroblastoma-associated genetic changes in the various lines, in particular, gain of regions syntenic with human 17q. Adherent lines displayed lower levels of MYCN expression than the suspension lines. Expression of Tyrosine Hydroxylase (TH), and MYCN targets Ornithine Decarboxylase (ODC) and Multidrug Resistance-associated Protein (MRP1) closely paralleled MYCN expression in the panel of lines at both RNA and protein levels. The relatively low expression of MYCN, TH, ODC, and MRP1 in the adherent lines, together with their non-neuronal morphology suggested that these lines represent the murine equivalent of S-type human neuroblastoma cells. High expression of the S-type marker S100A6 supported this hypothesis. These unique murine cell lines thus provide a valuable model for studying neuroblastoma tumorigenesis.

Ref ID: 202.2

Association of HLA class I antigen downregulation with multiple defects in antigen processing machinery components in human neuroblastoma

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Most of human tumors display defects in the expression of the antigen processing machinery components that may be associated with low expression of human leukocyte antigen (HLA) class I. Neuroblastoma (NB) is a neural crest derived pediatric tumor virtually HLA class I negative. Expression of the antigen processing machinery components in primary NB tumors has never been investigated. We have addressed this issue by staining tissue section from twenty four NB tumors with a set of newly developed monoclonal antibodies, and compared the results with those obtained from the analysis of six NB cell lines. The proteasomal components zeta, delta, MB-1, the immunoproteasomal components LMP2 and LMP10, the chaperons calreticulin and calnexin, and ERp57 were expressed in most tumors. In contrast LMP7, the peptide transporter TAP1, tapasin, HLA class I heavy chain, _-2 microglobulin and complete HLA class I or II molecules were never detected in primary tumors. No correlation was found between antigen processing machinery component expression and clinical tumor presentation. Antigen processing machinery component expression in NB cell lines resembled that detected in tumors. Upon incubation of cell lines with human interferon-_;, some components were up-regulated together with HLA class I molecules, indicating that the defects found in NB reflect dysfunctional and non structural mechanisms. In conclusion, the present results suggest that NB tumor cells cannot present tumor associated antigen-derived peptides to cytotoxic T cells, but may represent an appropriate target for NK cell based immunotherapy.

Ref ID: 012.1

Predicted MHC class I Epitopes deriving from Mouse Tyrosine Hydroxylase Are as Effective in DNA Vaccination Against Murine Neuroblastoma as MHC class I Ligands Naturally Expressed on Neuroblastoma Cell Surface

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The disruption of self-tolerance against neuroblastoma is the ultimate goal of an effective DNA vaccine. Here, we tested the hypothesis that for successful DNA vaccine design epitope prediction by MHC class I binding algorithms is equivalent to identification of epitopes by isolation from MHC class I presented on tumor cell surface. This hypothesis was investigated in a syngeneic murine NXS2 neuroblastoma model using peptide epitope predictions of murine tyrosine hydroxylase (mTH) and peptide epitopes isolated from NXS2 cells. For this purpose, MHC class I were isolated from NXS2 neuroblastoma cells which lead to the identification of three novel natural MHC class I ligands: TEALPVKLI, NEYIMSLI and FEMVSTLI. Epitope prediction was carried out with the complete protein sequence of mouse tyrosine hydroxylase (mTH) from which we selected three epitopes with high predicted binding affinity to MHC class I (FETFEAKI, EERDGNAV, VEYTKEEI). Subsequently, we constructed three different minigenes by overlapping RT-PCR and cloning into expression vector pCMV-F3Ub: one encoding for the isolated, the second for the mTH derived epitopes. As a negative control we designed a minigene consisting of three epitopes with no predicted binding affinity to MHC class I. The design of the final pCMV constructs involved an ubiquitin tag upstream the epitope sequences which should facilitate proteasomal degradation in vivo. Finally, we demonstrate the induction of protective immunity against neuroblastoma in vivo using an attenuated strain of Salmonella typhimurium as a carrier harboring the minigene-vector vaccines. Interestingly, the mTH3-based and the alternative minigenes were equally efficient in inducing suppression of subcutanous tumor growth. These findings establish proof of concept that a DNA vaccine encoding for predicted MHC class I binders is as effective in inducing anti-tumor immune response as a vaccine encoding for peptides isolated from MHC class I molecules on tumor cell surface.

Ref ID: 055.1

Single chain Fv-Streptavidin substantially improved therapeutic index in multi-step targeting directed at disialoganglioside GD2

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BACKGROUND/AIMS: The multistep targeting (MST) approach separates administration of antibody from cytotoxic ligand, thus decoupling "slow" blood clearance of the antibody from the potentially "fast" kinetics of ligands. GD2 is ideal for MST of neuroblastoma because of its cell surface retention following antibody binding and its high density.
METHODS: Anti-GD2 5F11scFv ligated to full-length streptavidin cDNA was expressed in E.Coli. Purified 5F11scFv-streptavidin (5F11SA), a homotetramer, showed comparable avidity to antibody 5F11 and 30-fold improvement over monomeric scFv. Biodistribution of 5F11SA was studied in nude mice xenografted with neuroblastoma LAN-1. 24h after IV injection of 5F11SA, a thiogalactoside-containing clearing agent was administered IV, followed by 111In-DOTA-biotin IV 4h later and clocked as time 0.
RESULTS: Optimal doses of 5F11SA, clearing agent and DOTA-biotin determined using the xenograft model were 900ug, 450ug, and 2.5ug, respectively. Tumor uptake at 2h was 7%ID/g, decaying with a T1/2 of 72h, while blood %ID/g rapidly decreased to <1/500 of tumor at 24h. Tumor-nontumor ratio at 72h was high (median 106, ranging from 3.4 [kidney] to 1660 [blood]). Area under the radioactivity curve (AUC) computation demonstrated favorable tumor to normal organ ratios (median 66, ranging from 3.6 [kidney] to 66 [blood] to 189 [muscle]), compared to anti-GD2 IgG3 3F8 (tumor to blood AUC ratio 2.8). In a panel of GD2-bearing xenografts, average tumor-tissue ratios correlated with antigen density. Selective tumor targeting was achieved when MST was performed using 125I-labeled biotinylated peptides or bovine serum albumin, instead of 111In-DOTA-biotin, as ligands.
CONCLUSIONS: This improvement in therapeutic index using a pretargeting strategy may improve the therapeutic index of antibody-based targeted approaches in cancer as well as render biotinylated polypeptides tumor-selective.

Ref ID: 059.1

T-Cell mediated suppression of neuroblastoma following Fractalkine gene therapy is amplified by targeted IL-2

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Fractalkine is the sole member of the CX3C chemokine subfamily, known to induce adhesion and migration of T-cells through its membrane-bound version and soluble form. We tested the hypothesis that chemokine gene therapy with fractalkine (FKN) induces an effective T-cell mediated anti-neuroblastoma immune response amplified by an anti-ganglioside GD2-IL2 fusion protein (ch14.18-IL2).
For this purpose, mouse FK was cloned and NXS2 neuroblastoma cells were genetically engineered to produce it (NXS2-FKN). Both versions of fractalkine protein production and its bioactivity were demonstrated in vitro.
The antitumor effect of fractalkine was determined by injection of NXS2-FKN cells into syngeneic A/J mice and a non-curative amounts of ch14.18-IL-2 was administrated simultaneously. In FK and ch14.18-IL-2 combination group (groupC), tumor growth was further reduced and 30% of mice presented with complete tumor rejection. Challenge of these mice with NXS2 wild type cells revealed a complete absence of experimental liver metastasis only in groupC indicating the induction of an immunological memory.
In tumor tissue explanted from the groups injected with NXS2-FK cells, we demonstrated continuous transcription and expression of fractalkine and T-cell was the predominant phenotype of tumor infiltrating leukocytes. We also proved the upregulation of T-cell activation markers and proinflammatory cytokine TNF-alpha by factor 3.2 and 4, respectively, in groupC. Finally, the highest specific target cell lysis was also found in this group. Importantly, the same effector cells were unable to lyse NK-sensitive YAC-1 target cells further supporting the T-cell mediated mechanism.
In summary, we demonstrate that gene therapy with fractalkine is effective against neuroblastoma and the T-cell mediated anti-tumor effect is amplified by targeted IL-2 which provides a new approach for immunotherapy in neuroblastoma.

Oral Presentations

Parallel Session F
Clinical

Ref ID: 250.1

Neuroblastomas that might benefit from mass screening (MS)

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The results of MS programs for infants with neuroblastoma in Germany and Canada showed the increased incidence, but no decreased mortality, although some Japanese studies showed the modest decrease in the mortality. We studied 401 neuroblastomas (all of them by FISH using 1pter and 1qcen probes, 109 by flowcytometry, and 58 by CGH) and classified them into either diploidy or triploidy. Diploidy was shown in 13% of 285 tumors which were found in infants by MS (MS+) and in 74% of 116 tumors which were found clinically in older children (>12m, MS-). The incidence of stage 4 was lower in MS+ diploid tumors than in MS- diploid tumors (23% and 73%). MS+ triploid tumors also had the lower incidence of stage 4 than MS- triploid tumors (6% and 46%). While the incidences of 11q- and +17q were similarly high in MS+ and MS- diploid tumors, those of 1p- and MYCN amplification were lower in MS+ diploid tumors than in MS- diploid tumors (1p-, 38% and 64%; MYCN, 14% and 45%). MS+ triploid tumors had the lower incidences than MS- triploid tumors (1p-, 10% and 35%; MYCN, 0% and 6%). Overall survival +/- SE at 5 years was best for MS+ triploid tumors (99+/-0.4%), and worst for MS- diploid tumors (49+/-6%), and intermediate for MS+ diploid tumors (83+/-7%) and MS- triploid tumors (65+/-9%). These findings suggest that most MS+ diploid tumors and a tiny portion of MS+ triploid tumors could have evolved to more malignant diploid and triploid tumors, respectively, if they had not undergone MS. Thus, 10% of MS+ infants might benefit from MS, and a new trial that avoids overdiagnosis should be initiated.

Ref ID: 138.1

Whole-body and tumour dosimetry for I-131 mIBG for neuroblastoma

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BACKGROUND AND AIMS: I-131 mIBG therapy is a systemic therapy option for neuroblastoma. Treatment schedules vary, and absorbed dose calculations are seldom carried out, which has prevented quantitative comparisons between centres. However a recent European directive stipulates that treatment planning should be applied to all targeted therapies. We present here our experience, methodology and results for both whole-body and tumour dosimetry.
METHODOLOGY: Patients were treated according to a predicted whole-body dose (usually 2 Gy) rather than with standard activities. Dosimetry was carried out using measurements obtained with a ceiling-mounted Geiger counter. Effective decay phases were determined interactively and absorbed doses obtained using standard methodology with interpolated S values. Tumour dosimetry was determined using successive SPECT scans. Both 1D (maximum and mean) and 3D dosimetry was performed. In the majority of cases pre-therapy tracer studies were carried out to predict whole-body and tumour absorbed doses.
RESULTS: In 25 patient studies, the whole-body dose delivered during therapy was predicted by a previous therapy with an accuracy of 5%. Previous tracer studies predicted the therapy dose to within 14%. The activities required to deliver a 2Gy whole-body dose ranged from <2GBq to >32GBq. Dosimetry predictions for tumour doses tended to overestimate the therapy dose by up to 50%.
CONCLUSIONS: The large range of activities required to deliver a 2 Gy whole-body dose and the accuracy of the whole-body dosimetry underlines the necessity and feasibility of conducting a dosimetry-based patient-specific approach to treatment. Further work is required for tumour dosimetry. Quantitative analysis will form a strong basis for multi-centre trials.

Ref ID: 217.1

Early prediction of resistance or response to chemotherapy in experimental neuroblastoma in vivo using magnetic resonance spectroscopy

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Proton magnetic resonance spectroscopy (1H-MRS) enables biochemical assessment of tissues and is increasingly used in cancer patients. We investigated the possibility of 1H-MRS to provide markers of response or resistance to chemotherapy in neuroblastoma. Suspensions of SH-SY5Y (p53wt) or SK-N-BE(2) (p53-/-) cells treated with etoposide, cisplatinum or irinotecan were examined by 1H-MRS and cell viability was assessed. In SH-SY5Y cells, cytotoxic treatment was associated with increased resonances from lipid methylene groups and polyunsaturated fatty acids, whereas total choline decreased in intensity, compared to other metabolites. The methylene/choline ratio correlated significantly with cell death (r=0.84, p<0.001). SK-N-BE(2) cells were resistant to all three drugs and treatment did not induce significant changes on 1H-MRS. In order to validate these findings, nude rats carrying SH-SY5Y or SK-N-BE(2) xenografts were treated for 4 days with irinotecan (5 mg/kg) or saline, and examined with 1H-MRS before and after 1, 2 and 3 days of treatment. In rats with SH-SY5Y tumors receiving irinotecan the methylene/choline ratio increased significantly after 2 days (5-fold increase, p<0.01), preceding tumor shrinkage (in mean -66 % on day 10, p<0.01). Contrary, irinotecan had no effect on the mean methylene/choline ratio in the SK-N-BE(2) group, and these tumors did not decrease in volume. Changes on 1H-MRS were absent in saline-treated tumors, which grew exponentially.
CONCLUSIONS: Response or resistance to chemotherapy of experimental neuroblastoma is accurately predicted with 1H-MRS in vivo. We suggest that 1H MRS, which can be carried out with most clinical MR-scanners, should be investigated for its potential to non-invasively provide dynamical information about neuroblastoma patient response to cytotoxic treatment.

Ref ID: 116.2

Expression of multidrug transporter MRP4/ABCC4 is a marker of poor prognosis in neuroblastoma and confers resistance to Irinotecan in vitro

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Members of the Multidrug Resistance-associated Protein (MRP) family of transporters are believed to contribute to cytotoxic drug resistance and chemotherapy failure. Although MRP4 is known to transport some nucleoside analogues, which are useful in the treatment of haematological malignancies, this transporter has not previously been thought to mediate resistance to cytotoxic drugs relevant to treatment of neuroblastoma or other solid tumours. Irinotecan/CPT-11, a novel topoisomerase I poison, is currently in clinical trials and shows promising activity against a number of cancers. We now demonstrate for the first time that MRP4 mediates substantial resistance, in vitro, to irinotecan and its active metabolite SN-38. Resistance is relatively specific to these compounds, as we did not observe any substantial resistance to the related drugs camptothecin and topotecan. In addition, we observed frequent MRP4 over-expression in aggressive primary neuroblastoma, a disease for which we have previously shown MRP1 to be a prognostic indicator (New Engl. J. Med. 334:231-8, 1996). In a cohort of 52 primary neuroblastoma tumours, high-level MRP4 expression correlated with MYCN oncogene amplification and was significantly associated with poor clinical outcome (p<0.001). These results suggest that irinotecan may not provide a therapeutic advantage for neuroblastoma patients whose tumors display high levels of MRP4.

Ref ID: 031.1

The importance of Surgical Risk Factors (SRF) in primary surgery for localized neuroblastoma (NBL): results of LNESG I Study

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AIM: To analyze the role of SRF in the surgical approach and in the outcome of the patients enrolled in the study.
MATERIALS AND METHODS: The main objective of the study was to evaluate the efficacy of surgery as the only treatment. The surgical objectives were to develop criteria which would predict safe resection of the mass. SRF related to site, size and fragility of primary tumour were defined to minimize postoperative complications and the risk of leaving macroscopic residual disease (MRD). The surgical data of 466 patients aged 2d-176m (med.12m), operated at diagnosis from January 1995 to October 1999 in 9 Countries, were evaluated.
RESULTS:
1) Stage (INSS). St.1:305, St.2:131, St.3:30.
2) Complications. The complication rate of operations performed in the presence or absence of SRF were 10.8% and 5% in St.1, 21.1 and 6.3 in St.2, 31.6 and 0 in St.3. Complications were significantly more frequent in cervico-mediastinal and abdominal NBL operated in the presence of SRF.
3) Incomplete excision. A significant higher incidence of MRD was detected in 92/320 abdominal and 35/119 thoracic NBL when operation was performed in spite of SRF; the difference was not significant for other sites.
4) Outcome. Relapse free survival was lower in patients with MRD, but the difference was significant only for abdominal NBL. The amount of MRD, evaluated in 50 cases, did not influence the outcome.
CONCLUSIONS: SRF were validated as predictors of adverse outcome: patients operated on in spite of SRF had more complications, a higher incidence of MRD for thoracic and abdominal sites and MRD was an un favourable prognostic factor for abdominal NBL.

Ref ID: 223.3

Chromosome 11q deletions are an independent marker for decreased survival probability in patients with neuroblastoma: a Children's Oncology Group Study

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The clinical heterogeneity of neuroblastoma provides a therapeutic challenge. Current stratification schemas use clinical and biological variables to assign risk groups, but imprecision may exist. Deletions of 11q occur frequently in neuroblastoma, but target genes and clinical relevance are not known. We analyzed 1008 primary neuroblastoma samples for chromosome 11 LOH with a panel of microsatellite markers. The only inclusion criterion was availability of a constitutional DNA sample. LOH at 11q and unbalanced 11q LOH (unb[11q]LOH; retention of 11p material) were present in 328 (33%) and 159 (16%) cases. Most 11q deletions extended to the telomere, but 25 interstitial deletions were identified that confirm and refine the existence of a minimum of two common regions of deletion. The vast majority of 11q deletions (98%) overlap a constitutional deletion at 11q13.4-q23.3 recently mapped in a neuroblastoma patient, and all but one involved 11q23. Clinical correlative studies confirmed a strong inverse correlation of 11q LOH and unb[11q]LOH with MYCN amplification (11q LOH p<0.0001; unb[11q]LOH p=0.0059). Despite this, both 11q LOH and unb[11q]LOH remained tightly associated with high-risk disease (p<0.0001). Patients whose tumors showed unb[11q]LOH had 3-year EFS and OS of 47±7% and 65±6% (n=146), compared to 70±3% (p=0.0005) and 81±3% (p=0.0003) in those cases that did not (n=746). Unb[11q]LOH was independently prognostic of EFS (p=0.0247) and OS (p=0.0391) in multivariable analyses. These data strongly suggest that 11q deletions are an independent marker for aggressive tumor behavior and that this may be especially relevant for patients with low- and intermediate-risk disease. Identification of the gene(s) targeted by 11q deletions should provide insight into the acquisition of a more malignant neuroblastoma clinical phenotype.

Ref ID: 114.1

Early use of anti-GD2 antibody 3F8 plus reduction from 7 to 5 cycles of dose-intensive induction chemotherapy can improve molecular response in high-risk neuroblastoma (NB)

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BACKGROUND AND AIMS: We previously reported a high response rate with the N6 regimen for NB (JCO 12:2607, 1994). We test if using 5 instead of 7 chemotherapy cycles while adding monoclonal antibody 3F8 can improve molecular remission.
METHODOLOGY: 86 consecutive newly-diagnosed patients were treated with induction that initially had 7 cycles of chemotherapy (57 patients) but was later limited to 5 cycles (29 patients). Surgery to resect the primary tumor followed a minimum of 3 cycles. 3F8 (50 mg/m2) was initiated after each of the last 3 cycles of chemotherapy (37 patients). Marrow molecular remission was studied by quantitative RT-PCR of GD2 synthase mRNA.
RESULTS: Complete histologic marrow remission was achieved in 91% of 76 patients with marrow disease at diagnosis. Following cycle 3 or 4, 86% of primary tumors assessable for response showed >50% shrinkage. After induction, 84 patients had MIBG scans with normalization of metastatic sites in 73 (87%). Overall results were as follows: 67 (78%) CR/VGPR; 14 (16%) PR; 3 (4%) <PR; 1 (1%) death from infection; and 1 nonevaluable. 5-cycle chemotherapy yielded a CR/VGPR rate of 82%, compared to 77% in the 7-cycle group. Molecular remission achieved after 5-cycle induction with 3F8 versus without 3F8 was 61% versus 52%, and after 7-cycle induction, 68% versus 49%, respectively. Four patients (all received 7 cycles) developed myelodysplasia/leukemia.
CONCLUSIONS: Five cycles of this induction regimen plus surgery suffice to achieve CR/VGPR in the majority of children with high-risk NB. Early use of 3F8 may improve molecular remission.

Ref ID: 234.1

Natural Killer T Cells Infiltrate Metastatic Neuroblastomas Expressing The Chemokine CCL2/MCP-1

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CD1d-restricted V-alpha24/J-alpha18-invariant Natural Killer T cells (iNKT) are potentially important in tumor immunity. However, little is known about their localization to tumors. We analyzed seventy-nine untreated primary neuroblastomas from patients with stage 4 disease for tumor infiltrating iNKT cells using TaqMan PCR and immunofluorescent microscopy. Thirty-three tumors (42%) contained iNKT cells, and oligonucleotide microarray analysis of the iNKT-positive and iNKT-negative tumors revealed that the former expressed higher levels of chemokines CCL2/MCP-1, CXCL12/SDF-1, CCL5/RANTES, and CCL21/SLC. Eight tested neuroblastoma cell lines secreted a range of CCL2/MCP-1 (0 - 21.6 ng/ml), little CXCL12/SDF-1 (<0.1 ng/ml), and no detectable CCL5/RANTES or CCL21/SLC. CCR2, the receptor for CCL2/MCP-1, was frequently expressed by iNKT cells from blood of normal adults and patients (median, 78%; range, 40%-98%) compared to NK cells (5%; 1%-11%) and T cells (21%; 10%-37%) (P<0.001). Supernatants of neuroblastoma cell lines induced in vitro migration of iNKT cells from blood of patients and normal adults, and this was abrogated by an anti-CCL2/MCP-1 neutralizing mAb. iNKT cell infiltration and CCL2/MCP-1 expression by tumors was inversely correlated with MYCN proto-oncogene amplification and expression. A multivariate logistic regression model using categorized values of MYCN and CCL2/MCP-1 expression demonstrated that both genes were independently significant (P≤0.01) so that low MYCN/high MCP-1 and high MYCN/low MCP-1 had 76% and 100% accuracy in predicting presence or absence of iNKT cells in tumors, respectively. Thus, iNKT cells migrate toward neuroblastoma cells in a CCL2/MCP-1-dependent manner, preferentially infiltrating MYCN-non-amplified tumors that express CCL2/MCP-1.

Oral Presentations

Plenary Session E
Translational - Clinical

Ref ID: 426.1

Histoprognostic value of INPC classification in localised resectable neuroblastoma

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Pathology and Clinical Sub-Committees LNESG1,

BACKGROUND: In order to assess the prognostic value of clinical, biological and morphological data, a multinational protocol including a phase II Trial of surgery as the only treatment for INSS stages 2A and 2B Neuroblastoma was initiated in 1995 (LNESG1: Localised Neuroblastoma Study Group 94.01). We present the results of the morphological features following INPC classification as prognostic indicators of relapse in those patients included in this protocol.
MATERIAL AND METHODS: Paraffin sections of 124 neuroblastic tumors from the patients included in the trial were reviewed. The pathology review was a step-wise evaluation of morphologic features without knowledge of clinical information, following the guidelines of the INPC classification. The cases were classified into favorable and unfavorable categories.
Overall survival (OS), and Event free survival (EFS) were analysed by the Kaplan-Meier test while correlations were assessed with Cox regression.
RESULTS: A total of 115 cases were evaluated following INPC criteria. 4 cases (3.2%) were not evaluable and 5 cases (4%) remain unreviewed. The 115 evaluated cases were classified in favorable (91 cases-79.1%) and unfavorable (24 cases-20.9%) categories.
Regarding OS after 60 months of follow-up, 97.8% [94.7-100] of favorable cases were alive compared with 72.6% [53.2-91.9] of unfavorable cases ($p=0.0001$).
EFS analysis showed that a relapse rate of 13.2% [6.3-20.2] and 29.2% [11-47.4] was observed in favorable and unfavorable cases ($p<0.05$) respectively. Statistical analysis demonstrated a significant association between INPC and LDH ($p<0.004$).
On the contrary, no significant correlation was found between INPC categories and 1p deletion ($p>0.2$)
CONCLUSION: Histopathological classification therefore has greater prognostic impact than other factors in predicting relapse in localised neuroblastoma.

Ref ID: 028.1

TRAIL-induced apoptosis is fully restored in a caspase 8-complemented neuroblastoma cell line and is further enhanced by low doses of drugs via activation of intrinsic and extrinsic pathways

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Neuroblastoma (NB) is a childhood neoplasm which heterogeneous behavior can be explained by differential regulation of apoptosis. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) selectively induces rapid apoptosis in most tumor cells and thus represents a promising anti-cancer agent. We have reported silencing of caspase-8 expression in highly malignant NB cells as a possible mechanism of resistance to TRAIL-induced apoptosis. To explore the particular contribution of caspase-8 in such resistance, retroviral-mediated stable caspase-8 expression was induced in the IGR-N91 neuroblastoma cell line. As a result, sensitivity to TRAIL was fully restored in the caspase-8 complemented cells. TRAIL-induced cell death could be further enhanced by co-treatment of IGR-N91-C8 and SH-EP cells with cycloheximide or sub-toxic concentrations of chemotherapeutic drugs in a caspase-dependent manner. Sensitisation to TRAIL involved enhanced death receptor DR5 expression, activation of Bid and the complete caspases cascade. Interestingly, combined treatments also enhanced the cleavage-mediated inactivation of anti-apoptotic molecules, XIAP, Bcl-xL and RIP. Our results show that restoration of active caspase-8 expression in caspase-8-deficient NB cells is necessary and sufficient to fully restore TRAIL-sensitivity. Moreover, the synergistic effect of drugs and TRAIL results from activation of the caspase cascade via a mitochondrial pathway-mediated amplification loop and from the inactivation of apoptosis inhibitors.

Ref ID: 127.1

Effect of R116010 on retinoic acid metabolism and action in neuroblastoma

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Treatment with 13-cis retinoic acid (13cisRA) significantly improves the clinical outcome of children with high-risk neuroblastoma. Retinoic acid (RA) isomerisation is thought to play a key role in drug efficacy, whereas oxidative metabolism has been connected with retinoid resistance. R116010, a novel inhibitor of RA metabolism, has been shown to enhance the biological activity of all-trans RA (ATRA) and exhibit anti-tumour activity. We have evaluated the effect of R116010 on RA metabolism and action in neuroblastoma cells in vitro. SH-SY5Y cells were incubated with R116010 (0-10μM) in the presence of either ATRA or 13cisRA (0-10μM) for 24 h. Intracellular retinoid concentrations were determined by HPLC and retinoid response measured using a cell-based reporter assay (SEAP). RARbeta and CYP26A1 expression were determined by real-time PCR and RT-PCR respectively. Incubation with 10μM R116010 resulted in 2.7- and 6.5-fold selective increases in ATRA concentrations after incubation with ATRA and 13cisRA respectively (95.2±16.9 to 254.7±31 μM, $p<0.005$; 5.4±0.7 to 35.6±6.2 μM, $p<0.005$), with no significant difference in 13cisRA levels. The relative increase in induction of SEAP expression in the presence of R116010 (1μM) was 2.3-fold with ATRA (11.8±1.2 to 27.2±2.3, $p<0.005$) and 1.8-fold with 13cisRA (11.1±1 to 19.8±1.8, $p<0.005$), compared to either retinoid alone. Co-incubation of R116010 with either ATRA or 13cisRA resulted in increased CYP26 and RARbeta expression compared to either retinoid alone. These data indicate that inhibitors of RA metabolism can increase the effectiveness of 13cisRA in neuroblastoma cells in vitro.

Ref ID: 035.1

Targeting VEGF expression: Serum and/or IGF via the PI3kinase/mTor path stimulate HIF-1a and VEGF expression in Neuroblastoma (NB)

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The extent of angiogenesis and/or VEGF expression in NB tumors correlates with metastases, N-myc amplification and poor clinical outcome. VEGF-targeted therapy may be effective for NB patients since antibody blockade of VEGF function in pre-clinical models inhibits NB tumor growth. Understanding the mechanisms regulating VEGF expression in NB cells provides additional therapeutic targets. VEGF mRNA is controlled by growth factors and hypoxia via the transcription factor, hypoxia inducible factor (HIF-1a). HIF-1a protein levels are regulated by the Von-Hippel Lindau tumor suppressor gene, VHL, which targets HIF-1a degradation. To determine whether the levels of VEGF in NBs are due to mutations in VHL, we evaluated genomic DNA from15 NB cell lines using PCR. We found no mutations in Exons 1, 2, or 3 of the VHL gene. VEGF mRNA levels in NB cells cultured in serum-free media increased after 8-16 hrs in serum (8-fold), IGF (8-fold), EGF (4-fold), BDNF (4-fold) and PDGF (2-fold). Serum/IGF induced increases in HIF-1a protein that temporally paralleled increases in VEGF mRNA and both were blocked by inhibitors of PI3K and mTOR but not MAPK or PLCg paths. IGF induced increases in Conditioned media (CM) from IGF-NB cells caused a 6-fold increase in endothelial cell proliferation in vitro compared to IGF alone. CM from NB cells treated with IGFs in the presence of PI3K and mTOR inhibitors failed to stimulate endothelial cell proliferation. These data indicate that growth factors in an autocrine or paracrine manner play a major role in regulating VEGF levels. Targeted therapies to PI3K, mTOR and/or HIF-1a may provide additional agents to target VEGF function and inhibit NB tumor growth.

Ref ID: 350.1

Coamplification of DDX1 Correlates with an Improved Survival Probability in Children with MYCN Amplified Human Neuroblastoma

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BACKROUND AND AIMS: Amplification of the MYCN oncogene at chromosome 2p24-25 identifies an aggressive subtype of human neuroblastoma with a poor clinical outcome. Differences in amplicon structure and coamplification of genes telomeric and centromeric to the MYCN oncogene have previously been described. A relevant role of gene coamplification for neuroblastoma pathogenesis remains elusive.
METHODOLOGY: We analysed 98 primary neuroblastoma tumors with MYCN amplification for coamplification of 7 additional genes at chromosome 2p24-25 (DDX1, NAG, NSE1, LPIN1, EST-AA581763, SMC6 and SDC1). Two semiquantitative multiplex PCR were used to obtain the amplification status of the target genes in relation to a reference gene on chromosome 2q (Inhibin-beta-b). Furthermore, mRNA expression pattern of coamplified genes in a subset of tumors were analyzed.
RESULTS: Our results show that the frequency of gene coamplification on 2p24-25 in neuroblastoma is correlated directly to the physical distance to MYCN. Coamplification is correlated to an upregulated gene expression for DDX1 and NAG. Coamplification of the DDX1 gene within 400kb telomeric to MYCN identifies a subgroup of advanced stage neuroblastoma tumors with a more favourable outcome ($p=0.027$, Log-Rank test for all investigated patients n=98; $p<0.0005$, Log-Rank test for patients that survived the first two years after diagnosis n=46). A high expression level of DDX1 is associated with a trend towards a better survival probability ($p=0.058$, log-rank test).
CONCLUSIONS: Our results indicate that DDX1 coamplification correlates with a better prognosis and improved patients survival in MYCN amplified neurobastoma. Our findings are of largely clinical interest for patients with MYCN amplified neuroblastoma for evaluation of survival probability and reevaluation after initial treatment.

Ref ID: 009.1

Promising results of a pilot trial of a GD2 directed anti-idiotypic antibody as a vaccine for high risk neuroblastoma

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We conducted the first pediatric trial of mAb1A7, an anti-id antibody directed against anti-GD2, 14G2a, as a vaccine for high risk neuroblastoma. Thirty one patients with high risk neuroblastoma who achieved a first or subsequent complete response, or a very good partial response were entered into this trial. Patients received SQ injection of mAb1A7 (Titan Pharmaceutical Inc.) + QS-21 (Aquila Pharmaceutical Inc.) q2 weeks x 4, q m x 11. The treatment was well tolerated with only transient local reactions, transient fever and chills in 4 patients, and serum sickness in 1. Neuropathic pain, often seen with infusion of anti-GD2, was not observed. Despite prior intensive treatment including stem cell transplantation, all 31 patients generated anti-mAb1A7. The mean peak anti-mAb1A7 was 665 ± 390 ng /ml (83-1467). Anti-mAb1A7 inhibited the binding of mAb1A7 (Ab2) to 14G2A (Ab1) in 31/31. Immunoaffinity purified Ab3 from 5/5 patients bound purified GD2 and was predominantly IgG. More importantly, immune sera from 9/13 patients tested after 37 weeks of treatment, displayed CDC activity. Furthermore, purified Ab3 from 6/8 patients mediated ADCC against neuroblastoma. To date, 17 of 20 patients who enrolled during first remission have no evidence of disease progression at 32+ to 52+ months (median + 47 m) from study entry, while only 1 of 11 patients who enrolled during 2nd remission remains progression free. In conclusion, mAb1A7 + QS21 is safe and effective in inducing biologically active anti-GD2 in heavily pre-treated neuroblastoma patients and may be useful in controlling minimal residual disease.

Abstracts

Poster Display

Ref ID: 362.1 #1

Characteristics of Stem Cell Clones from Human Neuroblastoma Cell Lines

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Within neuroblastoma cell lines, multiple cellular phenotypes analogous to those of the embryonic neural crest exist: neuroblasts (N cells), nonneuronal progenitors (S cells), and malignant stem cells (I cells). Since recent evidence suggests that cancer stem cells may play an important role in tumor incidence and progression, we have compared the morphological, biochemical, differentiative, and tumorigenic properties of seven (five new) malignant I-type stem cell lines with those of five N-type and four S-type cell lines. I-type stem cells are distinguishable from the other two cell types in that they (1) express properties of both N [extending short neuritic processes, expressing neurofilaments, granins, and neurotransmitter enzymes] and S cells [substrate adhesivity, expressing vimentin and CD44] and (2) show bi-directional differentiation potentials, converting to either N or S cells with exposure to specific agents. Of interest, all I-type cell lines examined are significantly more malignant than either N- or S-type cells; they have 4- to 5-fold greater plating efficiencies in soft agar and a 6-fold higher tumorigenicity in athymic mice. Differences in N-myc amplification/expression do not account for the differences in malignant potential. Microarray analyses are in progress to identify genes differentially expressed in I cells compared to either N or S cells and have identified approximately 15 potential candidates. Thus, we propose that, in human neuroblastoma cells, the I-type stem cell may be a determinative factor in human neuroblastoma malignancy and progression.

Ref ID: 359.1 #2

I-like stem cells are indicators of malignancy of human neuroblastoma tumors

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Human neuroblastoma cell lines comprise a mixture of neural crest-derived cell phenotypes, including neuroblastic/neuroendocrine (N) cells, nonneuronal S cells, and I-type stem cells capable of giving rise to both lineages as well as of self-renewal. These three phenotypes differ markedly in malignant potential. Nonneuronal cells are weakly or non-tumorigenic, neuroblastic/neuroendocrine cells are moderately tumorigenic, and stem cells are highly malignant. We and others have identified these three cell types in primary tumors. We have postulated that a similar variation in malignancy exists in tumors, with the stem cell being the most malignant, and are quantifying I-like frequency in tumor sections to test this hypothesis. Potential stem cells have been identified in tumors of all stages through sequential labeling with antibodies for N (neurofilament) and S cell (S100A6) marker proteins; tumor cells that express both N and S cell-specific proteins appear to be analogous to I-type cells in tissue culture. Preliminary analysis of 16 tumors had suggested that higher frequencies of I-like cells correlate with disease progression, metastatic stage 4 disease, and patient age of >1.5 years. Evaluation of 50 additional tumors by H&E as well as immunocytochemical procedures has confirmed these general findings. Of interest, the incidence of doubly labeled cells appears greater at the interface between areas of neuroblasts and stroma, strengthening the identification of I-like cells as stem cells. A better understanding of the differentiation and malignant properties of I-like tumor cells and of their participation in the clinical evolution of this disease is most definitely warranted.

Ref ID: 108.1 #3

On the search of neuroblastoma stem cell compartment: electrophysiological and immunocytochemical studies

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BACKGROUND: Neuroblastoma is an aggressive tumor of childhood, characterized by a wide spectrum of clinical behavior, probably reflecting the persistence, in the tumor, of multipotent progenitors, derived from the neural crest stem cells (NCSCs).
METHODOLOGY AND RESULTS: Two neoplastic cellular components have been identified in an adrenergic clone of human neuroblastoma, SH-SY5Y, by limiting dilution procedure: neuronal-like (N-type) cells and substrate-adherent (S-type) cells. An immunocytochemical and electrophysiological study showed that S cells were characterized by a spontaneous evolution, with three successive phases. Soon after isolation, S clones were characterized by the lack of ion currents, except a scanty expression of the HERG current (IHERG). This "nude profile", named S0, differs unequivocally from that of the N cells constituting the bulk of the tumor. The S0 electrical phenotype is associated with the expression of markers of NCSCs, such as nestin and vimentin. After several months in culture, S0 cells progressively changed their phenotype, displaying an high expression of IHERG, lack of IKDR and a sufficient INa to evoke a typical action potential. These cells, named S1, maintained vimentin and nestin expression throughout about two months, while, successively they converted into another cell-type (S2), expressing smooth-muscle actin and desmin. This latter subpopulation was easily identifiable by the scanty expression of IHERG and by a substantial expression of INa and IRK current. This evolution pattern was renewed substantially identical for each S subpopulation obtained by limiting dilution.
CONCLUSIONS: On the whole, our results suggest that S0 cells represent a residual stem cell compartment, maintaining some of central features of the NCSCs, including the low growth rate and low sensitivity to antiblastic agents.

Ref ID: 115.2 #4

The TH-MYCN Transgenic Mouse As A Preclinical Testing Model For Neuroblastoma

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The lack of good animal models for neuroblastoma has impaired testing of urgently needed new clinical approaches. Although neuroblastoma cell lines and in vivo xenograft models are useful, they do not reflect clinical tumor biology. The recently developed transgenic mouse model in which expression of the MYCN oncogene is targeted to neural crest cells with the use of a tyrosine hydroxylase promoter (EMBO J. 16:2985-95,1997) closely recapitulates human neuroblastoma with respect to major molecular, biologic and cytogenetic features, and we have recently shown that down-regulation of MYCN expression using antisense oligonucleotides reduces tumor formation in this model (JNCI 95:1394-1403,2003). We have previously demonstrated that MYCN regulates expression of the multidrug transporter gene, MRP1, and that high expression of MRP1 is associated with poor response to chemotherapy (NEJM 334:231-8,1996). We have now determined the in vivo response of transgenic murine neuroblastomas to a range of antitumour agents commonly used in neuroblastoma therapy. Responses to individual chemotherapeutic agents including cisplatin, doxorubicin, cyclophosphamide, teniposide and vincristine, were highly consistent and closely mirrored the clinical efficacy of the drugs tested. To determine whether response to chemotherapy could be improved by modulating expression of MRP1, we analysed the effect of combining MRP1-modulating agents with well-characterised chemotherapeutic agents. Our results show prolonged tumour-free survival in transgenic mice receiving vincristine, an MRP1 substrate, in combination with these agents, but no effect with cisplatin, a non-MRP1 substrate. Our data show that the TH-MYCN mouse is an ideal preclinical testing model for aggressive neuroblastoma and demonstrate the potential clinical importance of modulating MYCN and MRP1 in the treatment of this disease.

Ref ID: 210.1 #5

ALK kinase oncogenic potential in neuroblastoma cells

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ALK (Anaplastic Lymphoma Kinase) is a transmembrane receptor tyrosine kinase of 200 kDa normally expressed at low levels in few scattered cells of central (peicytes, glia cells) and peripheral (ganglia) nervous system. ALK catalytic domain is in 60% of Anaplastic Large Cell Lymphomas as consequence of a chromosomal translocation. Data from the literature suggest that ALK is expressed in neuroblastomas. While the causative role of activated ALK kinase function has been extensively demonstrated in the pathogenesis of the ALK+ lymphomas, nothing is known about the activation status of ALK in neuroblastoma.
Immunohistochemistry data indicate the presence of ALK in about 50% of neuroblastoma biopsies analyzed with an unexpected subcellular localization. We examined 2 cell lines (IMR-32, UKF-NB3) with high protein level, one with low level (SK-N-SH) and one negative (NB5). In addition to the ALK receptor, an isoform of 140 kDa lacking a consistent extracellular portion was detected possibly accounting for the intracellular localization. Both ALK variants resulted phosphorylated and showed kinase activity suggesting the presence of a constitutive activation mechanism. To determine whether ALK activation was due to an autocrine loop, the production of pleiotrophin (PTN), the ALK natural ligand, was investigated. In all the cell lines analyzed PTN transcripts were evident by RT-PCR. Interestingly, PTN was readily detected by ELISA in the supernatant of 2X105 NB5 (4,2 ng/ml) and SK-N-SH (2,2 ng/ml) cells cultured for 48 hours. Low level (0,5 ng/ml) or no PTN was found in the supernatants of IMR-32 and UKF-NB3 cells respectively inversely correlating with ALK level of expression. We suggest that the ALK-pleiotrophin network can play a role in the pathogenesis of ALK positive neuroblastoma.

Ref ID: 347.1 #6

Fluorescence-based Models for Imaging Alterations in the Growth, Metastasis, Angiogenesis and Apoptosis of Neuroblastoma Tumors

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To provide clinically relevant systems for the investigation of novel strategies for the treatment of neuroblastoma, we have developed a transplantable orthotopic model of intraadrenal murine neuroblastoma, as well as induced metastasis models that incorporate the use of fluorescence-based technologies to enable imaging and/or quantitation of tumor growth, neovascularization and metastasis. TBJ neuroblastoma cells transfected to overexpress the red fluorescent protein (RFP) gene display intense expression in vitro as well as in vivo after orthotopic implantation, or in occult metastatic sites in the liver, lung and/or bone marrow. Gross metastases are more readily visualized in visceral organ sites, and tumors as small as a few cells in size can be imaged, allowing for efficient detection of even microscopic residual disease. To facilitate imaging of tumor neovascularization, we have established a model system using C57BL/6-TgN (ACTbEGFP)10sb trangenic mice that are engineered to ubiquitously overexpress the green fluorescent protein (GFP). Orthotopic implantation of transplantable UN0092 neuroblastoma cells (newly-derived from C57BL6/J N-myc transgenic mice) into actin-GFP mice leads to the formation of tumors that are vascularized by green fluorescent blood vessels. Concurrent staining with DAPI provides tumor sections in which vascular density (green) can be readily imaged and/or quantitated, and nuclear morphology (blue) can be assessed for apotosis in both endothelial and tumor cell populations. We are now using these model systems to help develop novel biologic approaches for the treatment of neuroblastoma and have validated their ability to document potent inhibition of tumor growth, neovascularization and metastasis and the induction of apoptosis in mice treated with systemic cytokines and/or chemotherapeutic agents.

Ref ID: 391.1 #7

Morphological cell variants in the SH-SY5Y cell populations

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The trice-cloned N-type SH-SY5Y cell line has been extensively used to study neuroblastoma biology and cell death. We aimed to: (1)Test that SH-SY5Y cells obtained from different sources are homogeneous and composed of N-type cells; (2)Compare the expression of the S-type marker Vimentin in cells isolated by a differential adhesion method and by cytotoxic antibiotic selection; (3)Quantify the impact of SH-SY5Y cell variants on the response to apoptosis-inducing agents fenretinide and cisplatin and cytotoxic antibiotics. Measurement of Vimentin by flow cytometry showed: (1)Heterogeneity within and between SH-SY5Y cell populations; (2)That the differential adhesion method allowed enrichment of cells of different morphologies. Apoptosis-inducing agents were less effective in eliminating cells with high Vimentin levels and with I-/S-type morphology. These results further expand on our knowledge of the spectrum of cellular morphology within the N/I/ S-cell type categorisation and raise important questions regarding the use of neuroblastoma cell lines for research relying on specific cell types and cell population heterogeneity.

Ref ID: 372.1 #8

Possible viral etiology in human neuroblastoma

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BACKGROUND AND AIMS: In spite of having being intensely studied both molecularly and clinically for over twenty years, neuroblastoma's aetiology still appears to be enigmatic. We have isolated a new virus, called Micro-foci, inducing Virus or MFV- from a space-time association of NB cases with MYCN amplification (approx. 1000 fold amplification).
METHODOLOGY: Classical methods of cellular and molecular biology have been employed.
RESULTS: SK-N-SH cells, which survive the initial infection by MFV at low MOI are characterized by 1. changed /transformed morphology and 2. very rapid proliferation rates in comparison with the uninfected parental cells. Transformed cells reach saturation densities, which are typically 20x higher than uninfected ones. Very large tumoral masses were detected 7-9 weeks after injection of transformed cells into one flank of nude-mice, while no tumor appeared with control cells on the contra-lateral flank. Additional experiments by directly injecting MFV directly into adult Fisher 344 rats, just before mating them, show that carcinogenesis could be induced during pregnancy. In cells undergoing transformation, viral structural genes are totally shut off, thus explaining apoptosis-repression, while some expression of viral regulatory genes is detectable. Different levels of MYCN amplification are detected: SK-N-SH show MYCN amplification of 10-20 folds (Southern Blotting, semi-quantitative PCR, quantitative PCR) while SK-N-AS, levels in the order of 100 folds (SB).
CONCLUSIONS: A virus previously isolated from a cancer-cluster of NB cases, MFV, changes neuroblastic cells from slowly growing/non-tumorigenic into transformed and tumour inducing cells, with typical markers of aggressive neuroblastoma, thus suggesting viral aetiology in the cluster situation. Additional studies should address the issue of frequency of this phenomenon and whether it may also occur congenitally.

Ref ID: 389.1 #9

Regulation of Mitogenic Signaling by Nerve Growth Factor (NGF) and Brain-derived Neurotrophic Factor (BDNF) in Neuroblastoma cells

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INTRODUCTION: Neuroblastoma has been shown to have distinct subtypes characterized by differences in Trk receptor expression, which may affect aggressiveness and prognosis. Although TrkA and TrkB receptors have been shown to activate the same signaling pathways (MAPK and AKT), we hypothesized that regulation and downstream action of these pathways by the Trk receptors are distinct.
OBJECTIVE: To elucidate differences in mitogenic signaling downstream of NGF-activated TrkA versus BDNF-activated TrkB in the cell lines SMS-KCN or IMR-32.
Methods: Cells were stimulated with 100 ng/ml NGF or BDNF, and total cell lysates were analyzed by western blot for phosphorylated and total MAPK and AKT. NGF or BDNF-stimulated SMS-KCN proliferation was analyzed by immunohistochemistry for BrDU incorporation.
RESULTS: NGF activation of MAPK peaked at 5 minutes and was back to baseline at 30 minutes, whereas BDNF activated MAPK at 5 minutes and was still active at 90 minutes. Activation of AKT by NGF peaked at 5 minutes and rapidly diminished, but activation by BDNF peaked at 5 minutes and was at baseline at 90 minutes. BrDU incorporation increased ~3-4 fold with NGF but ~8 fold with BDNF. The MEK inhibitor PD098059 and the PI3K inhibitor LY294002 each inhibited NGF and BDNF-stimulated BrDU incorporation to basal levels.
CONCLUSION: MAPK and AKT are stimulated by NGF and BDNF. However, BDNF activation of both pathways is sustained, and BDNF appears to be a more potent activator of cell proliferation. Both MAPK and AKT pathways have roles in mitogenesis. These results indicate that regulation of mitogenic signaling by NGF and BDNF is different, suggesting distinct roles for NGF and BDNF in neuroblastoma cell growth.

Ref ID: 225.1 #10

Neuroblastoma Differentiation is Regulated by the Cooperation of the RET and TRKA Signaling Pathways

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The histopathology of neuroblastoma (NB) ranges from well-differentiated tumors to undifferentiated malignancies and stage IVs tumors may even spontaneously regress. Lack of TRKA, the receptor for nerve growth factor (NGF), in NB tumors correlates with poor prognosis and ectopic reconstitution of this signal pathway induces NB differentiation. Hence, we hypothesized that signaling pathway(s) that regulate normal neuronal maturation also control NB cell differentiation and therefore may open new treatment modalities for patients with high-risk NB. Here, we demonstrate that activation of the RET receptor by its ligand glial cell line derived neurotrophic factor (GDNF) leads to an increase in RET receptor expression in a panel of NB cell lines. Sustained activation of the RET receptor pathway causes growth cessation and arrest in the G0-G1 phase of the cell cycle. Ciliary neurotrophic factor (CNTF) induces low-level of trkA gene expression while GDNF synergizes with CNTF to enhance TRKA protein levels. Additional presence of NGF leads to extensive neuronal differentiation manifested by neurite outgrowth. NB differentiation is accompanied by cessation of cell proliferation, expression of neuron specific genes and silencing of the over expressed N-myc gene. Notably, differentiated NB cells are dependent on functional TRKA signaling, since removal of NGF induces extensive cell death, consistent with the trophic dependence of normal sympathetic neurons. Subcutaneous NB tumors in nude mice exhibited a substantial decreased in the level of N-MYC protein following short-term treatment with these trophic factors suggestive of a functional in vivo response. Taken together, the RET and TRKA signal pathways collaborate to promote neuronal maturation of malignant NB in vitro, thus recapitulating the differentiation of normal sympathetic precursor cells.

Ref ID: 133.1 #11

TrkA Expression in Peripheral Neuroblastic Tumors: Prognostic Significance and Biological Relevance

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AIMS: To determine prognostic significance and biological relevance of trkA (high-affinity NGF-receptor) in peripheral neuroblastic tumors (neuroblastoma, ganglioneuroblastoma, ganglioneuroma).
MATERIALS & METHODS: We examined the expression levels from 263 tumors by quantitative PCR. The results were analyzed with histopathology (favorable histology-FH vs. unfavorable histology-UH according to the International Neuroblastoma Pathology Classification) and MYCN status (amplified-A vs. non-amplified-NA) of the tumors and with clinical stage and outcome of the patients.
RESULTS: The levels of trkA expression were significantly different between “alive and well” group (N=170) and “progressed or deceased” group (N=93), and between “alive” group (N=188) and “deceased” group (N=75). When adjusted by clinical stage, histopathology, and MYCN status (N=194), however, the prognostic significance of trkA levels diminished. Among the cases in the neuroblastoma category (N=171), tumors in the [FH&NA] subset (N=112) expressed higher trkA levels, and showed neuroblastic differentiation age-dependently: they were classified into either poorly differentiated subtype (N=91, all patients <1.5 years) or differentiating subtype (N=21, 57% of patients between 1.5 and 5 years). Tumors in the [UH&A] subset (N=28) expressed significantly lower trkA levels, and a very limited neuroblastic differentiation. Tumors in the [FH&A] subtype were very rare (N=3) and expressed higher trkA levels. Tumors in the [UH&NA] subtype (N=28) had trkA levels in a wide range, and showed a limited neuroblastic differentiaton.
CONCLUSION: It does not seem useful to add trkA expression levels to the other prognostic factors (age, clinical stage, histopathology, MYCN status, DNA index) of the current COG risk-grouping system.

Ref ID: 018.1 #12

Association of Epigenetic Inactivation of RAS Association Domain Family Protein 1 (RASSF1A) with Poor Outcome in Human Neuroblastoma (NB)

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BACKGROUND: Epigenetic silencing of tumor suppressor genes plays an important role in the pathogenesis of cancer. In NB, we and others have demonstrated that a number of genes are methylated. The tumor suppressor gene, RASSF1A, is one of the most frequently methylated genes in NB. In this study, we examined the DNA status of RASSFIA in NB tumors and correlated the epigenetic changes with clinico-pathologic parameters.
METHODOLOGY: The methylation status of the RASSF1A promoter was assayed in 13 NB cell lines, 56 NBs, and 5 ganglioneuromas (GNs) by methylation-specific PCR. RASSF1A expression studies were performed by RT-PCR. The Kaplan-Meier method was used to estimate survival probabilities and survival functions were compared by the log-rank test. Multivariate analysis was based on Cox's regression analysis. Statistical analysis was also carried out using the X2 or Fisher's exact test.
RESULTS: Aberrant methylation and silencing of the RASSF1A CpG island promoter region was detected in 100% of the NB cell lines. Methylation was also detected in the 70% NB tumors, whereas this gene was unmethylated in all 5 GNs. Significantly worse survival (S) was observed in patients with methylated RASSF1A compared to those without methylation, with 10-year S rates of 50% + 8% versus 95% + 5%, respectively ($p<0.013$). As expected, high-risk disease was predictive of poor outcome in this cohort [10-year S: 23% + 10% (high-risk) vs 86% + 6% (low- and intermediate-risk), $p<0.0001$]. Methylation of RASSF1A was statistically associated with MYCN amplification and advanced stage disease.
CONCLUSIONS: Our findings indicate that RASSF1A is methylated in a large subset of NB tumors, and that this epigenetic alteration is strongly associated with poor outcome. Aberrant promoter methylation and silencing of RASSF1A may contribute to the pathogenesis of high-risk NB.

Ref ID: 010.1 #13

Inhibition of apoptosis in Neuroblastoma cells by the bifunctional apoptosis regulator (BAR)

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BACKGROUND: Neuroblastoma (NB) is the most common extracranial solid pediatric tumor. Because treatment failures are due to recurrent chemotherapy-resistant tumors, understanding the mechanisms that lead to apoptosis in NB cells will help to understand the development of drug-resistance. We have previously shown that NB cells are type II cells, because Fas-mediated apoptosis proceeds through a mitochondria-dependent pathway, that Bcl-2 plays an important inhibitory role in NB and a novel inhibitory mechanism for Bcl-2 through binding with caspase 8, which prevents its activation at the death inducing signaling complex.
AIMS: Determine whether the newly discovered protein BAR, which have been reported to bridge Bcl-2 and caspase 8 in co-transfection experiments, is responsible for the formation of Bcl-2/caspase8 complexes and whether it plays an important role in apoptosis signalling in NB cells.
RESULTS: We found that 4 NB cell lines express BAR .In immunoprecipitation experiments of whole cell lysates, the Bcl-2 immunoprecipitates contained BAR and the BAR immunoprecipitates contained caspase 8 in all 4 NB cell lines. We also showed colocalization of BAR with caspase 8 and Bcl-2 in NB cells by confocal microscopy. Downregulation of BAR with a specific antisense oligonucleotide led to the disruption of caspase 8/Bcl-2 complexes and reversed the resistance of NB cells to the Fas agonistic antibody CH11 and doxorubicin. Sub-cellular fractionation of NB cell lines showed that pro-caspase 8 is localized in the mitochondrial fraction with lower levels in the cytosol and increases in the cytoplasmic fraction after downregulation of BAR.
CONCLUSION: These studies suggest that BAR plays an important inhibitory role in NB cell apoptosis functioning as a scaffold protein to facilitate caspase8/Bcl-2 complexes and thus lowering the available levels of free caspase 8 in the cytoplasm.

Ref ID: 394.2 #14

Resveratrol, a novel survivin antagonist: sensitization of neuroblastoma cells for TRAIL- or anticancer drug-induced apoptosis through cell cycle-mediated survivin depletion

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Resistance to current treatment protocols remains a major concern in neuroblastoma therapy and may be caused by defects in apoptosis programs. We discovered a novel function of the dietary compounds resveratrol: resveratrol depletes survivin levels through p21-mediated cell cycle arrest, thereby sensitizing neuroblastoma cells for TRAIL- or anticancer drugs-induced apoptosis. Accordingly, cell cycle inhibitors or transfection-enforced overexpression of p21 induced G1 arrest, resulting in survivin depletion and sensitization for TRAIL-induced apoptosis. Consequently, cell cycle arrest, survivin depletion and sensitization for TRAIL-induced apoptosis by resveratrol were all impaired in p21-deficient cells. Importantly, resveratrol-mediated cell cycle arrest, survivin depletion and sensitization for TRAIL occurred independently of wildtype p53 even in p53-deficient or p53 null cells. Interestingly, resveratrol depleted survivin levels through both transcriptional and posttranscriptional mechanisms, as resveratrol blocked survivin promoter activity and survivin mRNA expression and also enhanced survivin protein degradation. Similarly, downregulation of survivin using survivin antisense oligonucleotides sensitized cells for TRAIL-induced apoptosis. Interestingly, overexpression of Bcl-2 or FADD-DN did not interfere with resveratrol-mediated cell cycle arrest or survivin depletion, but blocked apoptosis upon combined treatment with resveratrol and TRAIL. This indicates that Bcl-2 or FADD-DN decoupled the effect of resveratrol on cell cycle and apoptosis. Importantly, resveratrol sensitized various tumor cell lines and primary neuroblastoma cells, but not normal human fibroblasts, for death receptor- and also for cytotoxic drug-induced apoptosis and even inhibited neuroblastoma growth in a mouse model. Thus, this combined sensitizer (resveratrol)/ inducer (e.g. TRAIL or anticancer drugs) strategy may be a novel approach to enhance the efficacy of anticancer therapy in neuroblastoma.

Ref ID: 005.1 #15

Multiple Isoforms of PCNA in Neuroblastoma: Changes in structure, Changes in function?

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BACKGROUND/AIMS: Clinical outcome in neuroblastoma (NB) is associated with the proliferation activity of the tumor. The role of proliferating cell nuclear antigen (PCNA) as a proliferation marker in NB is controversial. PCNA has roles in human cell DNA replication/repair. Since genomic damage is a hallmark in cancer, we hypothesized that the structure and/or function of this protein may be altered in NB. We therefore performed structural analysis of PCNA in NB.
METHODOLGIES: Subcellular fractionation, two-dimensional electrophoresis, and immunoblotting from 4 human NB lines (IMR-32, SK-N-AS, SK-N-DZ, and SK-N-FI) were utilized to identify structural alterations in PCNA.
RESULTS: The cDNA coding for PCNA predicts that the protein has an acidic pI (approx. 4.5). Structural analysis of PCNA in NB showed two isoforms of the protein, one with a pI of 4.5, the other with a basic pI (approx. 8.0). The two isoforms of PCNA were present in all NB cell lines. While the PCNA gene encodes for an acidic protein, we have determined that the two forms do not arise via genetic mutation, but through an epigenetic mechanism. Previously, we observed two forms of PCNA are associated with the human breast cancer DNA replication apparatus. We are currently determining the post-translation modification(s) responsible for the two PCNA isoforms in NB and whether their co-expression in NB plays a role in accumulation of genomic damage.
Conclusions: Two variants of PCNA exist in NB. These data imply that altered cellular function may be present in NB with different PCNA isomers. The role that these PCNA isomers play in the expression of its biological actions is not completely understood. Studies evaluating the structure-activity relationships of these 2 PCNA forms in NB are needed.

Ref ID: 069.2 #16

Metastasis-associated genes in an experimental model of MYCN-amplified neuroblastoma

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MYCN amplification is a common feature of disseminated neuroblastoma (NB) with bone marrow as the main metastatic site. To understand the metastatic process in disseminated NB, we used a human MYCN-amplified NB experimental model which is capable of disseminating in the bone marrow (BM) and myocardium (Myoc) of mice. The model was derived from a primary tumor xenograft (PTX), as previously described (Blanc et al., AJP, 2003). Agilent oligo-microarray analysis was performed to compare gene expression profiles in BM and Myoc metastatic sites versus PTX neuroblasts. We identified a differentially expressed 107 gene cluster in metastatic neuroblasts including up-regulated genes involved in chemoresistance/detoxication (ABCB1/MDR1, CDO1), cell motility (ENPP2), neuronal structure/signalling (RELN, PRPH, SYCP2) while down-regulated genes involved in cell adhesion/cell-cell interaction (EMP2, DCN). Expression of the top eight overexpressed genes and the top seven underexpressed genes was validated by real time RT-PCR in the metastatic neuroblasts. Thus this experimental model defines NB metastatic dissemination by alterations of the genes mainly involved in i) in adhesion and extracellular matrix remodulling and ii) in specific neuronal pathways including neural signalling, synaptogenesis and steroid biosynthesis. We focused our interest on the « top » up-regulated gene of the set, a member of the glycosyltransferase family. As it has been known for over 10 years that glycosylation processes are involved in the progression of primary tumor to metastatic disease, we are conducting ongoing research to establish the diagnosis and prognostic potentials of this gene expression for the NB disease.

Poster Display

BIOLOGY

Ref ID: 202.1 **#17**

Functional expression and release of ligands for the activating immunoreceptor NKG2D in human neuroblastoma

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NKG2D is an activating immunoreceptor involved in NK-cell-mediated cytotoxicity. The target cell ligands for NKG2D (NKG2Dl) are MHC class I-related chain MICA and MICB glycoproteins, and the family of UL-16-binding proteins (ULBPs). Neuroblastoma (NB) is a pediatric extracranial tumor characterized by multiple defects of the antigen processing machinery components and downregulation of HLA class I molecules. This observation suggests that NB cells could represent an appropriate target for NK-mediated lysis. In this study, the expression of NKG2Dl was evaluated in human NB cell lines and primary tumors. MICA mRNA was expressed both in NB cell lines and primary tumors, however, the protein was never detected on the cell surface or intracellularly. Supernatants of a few NB cell lines and sera from NB patients, but not from healthy donors, contained elevated levels of soluble MICA (sMICA). Functional experiments are currently being investigated in order to clarify if sMICA is able to downregulate NKG2D and/or partially prevent killing of MICA+ tumor cells by NKG2D. MICB protein is strictly confined in the cytoplasm of NB cell lines and primary tumours, but the mechanism of this phenomenon is yet undefined. ULBPs mRNA and proteins are expressed in most human NB cell lines. ULBPs' expression is under investigation in primary NB tumours. Absence of surface NKGD2l on NB cells may represent a mechanism of tumor escape from the control of NKG2D+ cytotoxic lymphocytes.

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Ref ID: 344.1 **#18**

Mismatch repair protein expression in pre and post treatment neuroblastoma

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BACKGROUND AND AIMS: Mechanisms of drug resistance in neuroblastoma are incompletely understood. Loss of mismatch repair protein expression particularly loss of hMLH1 and hMSH2 and high levels of MGMT have been associated with resistance to platinum agents in adult cancers. We investigated expression of mismatch repair proteins in neuroblastoma
METHODS: Immunocytochemistry for MLH1, MSH2 and MGMT was performed in 40 primary unmatched neuroblastomas 26 pre-treatment and 13 post-treatment. Positivity was scored by intensity and Labelling Index (LI). From 8 tumours DNA was analysed for MLH1 and MGMT methylation by methylation specific PCR of CpG-islands associated with the respective gene promoters.
RESULTS: Overall there was high expression of MLH1 median LI 50%(0, 95), MSH2 median LI 40%(0, 99) and low expression of MGMT median LI 0%(0, 70). Nuclear expression of MLH1 and MSH2 was strongly positive in neuroblastoma and ganglion cells, weakly positive in stroma and adrenal cortex and negative in lymphoid cells. There was no difference in MLH1 or MGMT expression between tumours pre and post treatment, but MSH2 expression was lower post treatment median difference in LI 65% ($p < 0.001$). MGMT expression was associated with stage 3 and 4 neuroblastoma ($p < 0.05$). MLH1, MGMT and MSH2 expression were not related to patient survival. None of the DNAs were methylated for MLH1 or MGMT at the region examined. These cases all expressed MLH1 but 2 cases were negative for MGMT expression.
CONCLUSIONS: MLH1 and MSH2 are expressed at high levels and MGMT at low levels in neuroblastoma. The reduction in MSH2 expression post treatment is being further investigated in paired tumours.

Ref ID: 080.1 **#21**

Hypoxia induced dedifferentiation of neuroblastoma cells: phenotypic persistency after reoxygenation

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In solid fast growing tumours neovascularization is usually insufficient and leads to areas with poorly oxygenated tumour cells. The stabilization of the hypoxia inducible transcription factors HIF-1a and HIF-2a, has been considered one functional definition of hypoxia and in neuroblastoma cells this occurs between 1 and 5 % O2. We have earlier shown that the expression of marker genes of the sympathetic nervous system were down-regulated whereas markers of neural crest cells were up-regulated in hypoxic (1% O2) neuroblastoma cells in comparison to cells cultivated at 21 % O2. These findings suggest that the hypoxic tumour cells adopted a less mature phenotype, which in the clinical setting could result in more aggressive tumour cells with increased metastatic potential. Here we have compared changes in gene expression in cells grown at 1 % O2 (hypoxia) and a more physiological oxygen level of 5% O2. We have also examined the stability of the hypoxia-induced dedifferentiated phenotype when cells are reoxygenated at 21 or 5% O2, respectively. The dedifferentiated phenotype persisted for at least 24 h of reoxygenation at both 21 and 5 % O2. Genes like NPY and chromogranin A and B were still down-regulated, and hypoxia-induced genes like tyrosine hydroxylase and Id2 remained up-regulated. Thus, in the case of metastasizing hypoxic tumour cells that have entered the bloodstream, the aggressive phenotype might persist long enough for the cells to be able to home to a secondary site and metastasize, in part due to their immature characteristics.

Ref ID: 333.1 **#22**

Normalization to averaged expression levels of four control genes results in reliable transcript quantification by real-time RT-PCR in primary neuroblastoma

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Real-time RT-PCR represents a sensitive and efficient technique to determine expression levels of target genes in multiple samples. Normalization of raw data is required to obtain comparable results between different specimens and is usually achieved by correlating transcript abundances of target genes with those of a single control gene with putatively stable expression levels. However, transcript levels of frequently used control genes such as beta-ACTIN or GAPDH have been demonstrated to exhibit strong variations depending on tissue type or experimental conditions. To establish a suitable normalization factor for real-time RT-PCR experiments in neuroblastoma, the expression stability of putative reference genes HPRT1, LMNB1, PBGD, PGK1, PPIA and SDHA were evaluated in 64 tumor samples obtained from primary neuroblastoma of varying biological and clinical behavior. Variations of control gene mRNA levels observed among the samples considerably decreased when expression values were normalized to the geometrical mean of multiple reference genes instead of a single control gene. The geometrical mean of control genes SDHA, HPRT1, PPIA and PBGD was demonstrated to represent a reliable normalization factor and in addition was shown to be not associated with stage of disease or MYCN-amplification status of the tumor. In summary, these data indicate that normalization to the geometrical mean of more than one control gene can increase reliability of gene expression data determined by real-time RT-PCR significantly and that the geometrical mean of PPIA, HPRT1, PBGD and SDHA mRNA levels represents a suitable internal control for studies analyzing gene expression in primary neuroblastoma.

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Ref ID: 245.1 **#19**

GD2 loss variants in neuroblastoma

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BACKGROUND: Immunocytological bone marrow assessment for contamination with neuroblastoma cells is mainly based on their characteristic GD2 surface staining. So far, neuroblastomas without GD2 expression have not been reported.
METHODS: Conventional cytology was performed using Pappenheim staining. For immunocytology, the APAAP method was utilized with the 14 G2a GD2 mouse monoclonal antibody. 7 x 105 cells on cytospin prepara-tions were investigated.
RESULTS: In 2003, 288 bone marrow samples from 191 neuroblastoma patients were investigated by cytology and immunocytology. Of them, three cases demonstrated GD2 negativity on cytologically unambiguous neuroblastoma cells. Two female cases (94 and 37 months of age) with stage 4 neuroblastoma had GD2 expressing neuroblastoma cells in bone marrow at diagnosis. At 2nd relapse 25 and 23 months after diag-nosis and 8 months and 12 months after anti GD2-antibody treatment (ch14.18), the bone marrow infil-trating neuroblastoma cells lacked GD2 staining. The third patient, a 63 months old girl with bone marrow replacement by neuroblastoma cells showed at diagnosis a mixture of GD2-unstained tumor clumps and very weakly, atypical stained neuroblastoma cells.
CONCLUSION: Neuroblastoma cells may lack GD2 expression at diagnosis and at recurrence. This observation has di-agnostic and therapeutic implications.

Ref ID: 316.1 **#20**

Biological modifiers of RANKL activity prevents osteoclastogenesis induced by neuroblastoma cells

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Bone is one of the target organs of metastasis in advanced neuroblastoma. Metastatic osteolysis depends on osteoclast proliferation and differentiation, which are mediated by a cytokine system including receptor activator of NFkB ligand\" (RANKL) and its receptors: RANK, expressed in osteoclasts, and osteoprotegerin (OPG), a soluble decoy receptor. RANKL binding to RANK activates the signaling for osteoclast differentiation. RANKL binding to OPG limits its biologic actions. We investigated the role of OPG/RANKL/RANK network in the pathogenesis of bone metastasis in neuroblastoma. RANKL and OPG expression was investigated in neuroblastoma cell-lines, namely LAN1 and SH-SY5Y. Both cell-lines had a large amount of OPG and RANKL transcripts, but OPG protein was not released in culture medium. The paracrine activity of neuroblastoma in inducing the osteoclast differentiation from PBMCs was tested. SH-SY5Y and LAN1 up-regulated markers of osteoclast differentiation, including RANK, c-src, c-fos, cathepsin K, and tartrate-resistant acid phosphatase (TRAP). Biological modifiers of RANKL activity were evalutated for their ability to inhibit osteoclast differentiation. A neutralizing anti-RANKL antibody inhibited the expression of RANKL-dependent genes and the generation of multinucleated TRAP giant cells; antisense oligonucleotides and small interfering RNAs were able to decrease RANKL transcripts and protein, as well as a strong inhibition of osteoclastogenesis was obtained. Our findings confirm that neuroblastoma cells are able to induce the osteoclastogenesis via RANKL, and suggest that the RANKL expression, associated with the lack of the decoy receptor OPG could be the key mechanism by which
neuroblastoma cells are able to colonize bone and to induce bone metastases.

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Ref ID: 353.1 **#23**

NDSP, A Novel Secreted Protein in Neuroblastoma

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BACKGROUND: Secreted proteins such as growth factors, cytokines, and chemokines have important roles in tumor development. Using cDNA microarray analysis, we identified a gene encoding a novel secreted protein highly expressed in stage 4 neuroblastoma. We have named this protein neuroblastoma derived secretory protein (NDSP).
METHODOLOGY/RESULTS: The NDSP gene is found on chromosome 1q24.3 and encodes a 167 amino acid protein in its 501 base pair open reading frame. NDSP has a 30 amino acid signal peptide with cleavage site suggesting a secreted protein. NDSP contains 8 cysteine residues including a C-C motif associated with many chemokines. Northern blot analysis showed no evidence of NDSP expression in normal human tissues. Through RT-PCR, NDSP expression was confirmed in 7 neuroblastoma cell lines, 1 of 2 melanoma cell lines, and 1 retinoblastoma cell line but not in leukemia, lymphoma, glioma, glioblastoma, breast, colon, pancreatic, or lung cancer cell lines. NDSP is also expressed in 9 of 9 neuroblastoma tissue samples representing stages 1-4 and 4S. In order to show that NDSP is secreted, we constructed a mammalian expression vector containing a V5-tag fused to the C-terminus of the NDSP protein, NDSP-V5. Anti-V5 immunoblot analysis confirmed the presence of NDSP-V5 (20 kDa) in the supernatant of transfected 293 cells. In addition, we generated a rabbit polyclonal antibody against NDSP. Western blot analysis on culture supernatants from SH-SY5Y, a cell line that expresses the NDSP transcript, confirmed that NDSP was secreted from neuroblastoma cells. However, the secreted form exists as 40 kDa homodimer that separates into the 20 kDa monomer under reducing conditions.
CONCLUSION: NDSP is a novel protein actively secreted by neuroblastoma cells. This data also suggests that NDSP may have importance as a new diagnostic/prognostic marker for neuroblastoma.

Ref ID: 321.1 **#24**

Unequivocal identification of disseminated tumor cells in the bone marrow by immunofluorescence and FISH reveal essential functional and prognostic information

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The detection and quantification of disseminated tumor cells (DTC) present in the bone marrow (BM), peripheral blood (PB) and apheresis products (AP) is becoming increasingly significant in the treatment of neuroblastoma (NB) patients. So far only the identification of tumor cells in the BM and PB at diagnosis is implemented in the clinical practice of NB patients. However the response of occult tumor cells to high dose chemotherapy, the presence of tumor cells in the autograft and functional insights into the biological make up of DTC are becoming important too. In solid tumors the clinical significance of DTCs at diagnosis or during the course of the disease, usually termed minimal residual disease (MRD) testing, are still under debate. These indistinct results are mainly due to methodical reasons. Therefore, a fully automated fluorescence based microscopical system (RCDetect, MetaSystems, Germany) combining the detection of 'tumor-specific' immunological features (GD2) together with 'tumor-typical' DNA aberrations was developed allowing the unambiguous visualization of tumor cells in a hematopoietic surrounding. The system was validated by spinning experiments and is now in clinical use where more than 800 BM samples and 70 apheresis products from NB patients were analyzed. Furthermore, this automatic fluorescence approach allows insights into the expression profile and the apoptotic state of DTCs. We therefore conclude that the fluorescence based automatic system is an ideal device to unambiguously identify and quantify DTCs and to define their biological state.

Ref ID: 042.3 **#26**

Development and standardization of an immunocytochemical assay to detect residual neuroblastoma cells

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For the E-SIOP Neuroblastoma Bone Marrow Studies Sub-Committee.>

Background: Since bone marrow (BM) involvement influences staging and evaluation of therapeutic response in neuroblastoma patients, reliable detection of residual neuroblastoma cells is critical. Cytomorphological examination of BM aspirates appears not sensitive enough to detect single tumor cells. Therefore, the SIOPEN Immunocytology/Genetics group decided to standardize performance and evaluation of a new and sensitive immunocytochemical assay based on of GD2 disialoganglioside detection. This technique will be used to study the significance of minimal residual disease in BM samples from stage 4 neuroblastoma patients entered into the SIOPEN High Risk study. Standardized method: Technical performance: Standardization of cell preparation, paraformaldehyde fixation, anti-GD2 monoclonal antibody (clone 14G2a), detection system (DAKO APAAP complex, Fuchsin+TM Substrate Chromogene System), hematoxylin counterstaining and controls. Evaluation: Cells are called positive according to defined minimal criteria based on cytomorphology (cell size, shape, nucleo-cytoplasmic ratio, chromatin structure) and quality and subcellular distribution of the immunocytochemical staining product. Number of positive cells and evaluated mononuclear cells are reported. The sensitivity of this assay is practically unlimited and depends on the number of investigated cells (ideally 3x10e6). In non-conclusive cases, GD2 positive cells are checked by FISH for genetic aberrations. Quality controls: To test the validity of our method, quality control rounds were organized among members of the Immunocytology/Genetics group. After application of the standardized staining and evaluation procedure, two main improvements were achieved: (1) in discordant cases, the range between lowest and highest reported result was reduced 5 times and (2) discordant results were only found in samples with <10 positive cells per 1x10e6.
Conclusions: Our method is an appropriate tool to assure reliable detection of residual tumor cells in BM from neuroblastoma patients included in the SIOPEN High Risk Protocol.

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Ref ID: 241.2 **#25**

Immunocytochemical detection of GD2-positive cells in bone marrow on diagnosis of localised neuroblastoma: clinical implications

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INTRODUCTION: The staging of neuroblastoma patients according to the INSS is based on the detection of tumour cells in the bone marrow, by means of both cytomorphological examination of bone marrow aspirates and histological analysis of trephine biopsies. A new and sensitive immunocytochemical assay using a monoclonal antibody has recently been developed and standardised in order to detect single tumour cells in bone marrow samples.
METHODS.A comparison between the new technique and the traditional methods of bone marrow examination was made in a series of 303 patients, in order to assess the concordance among these three methods and to investigate the possibility that the increased sensitivity of the new technique may be of value in predicting clinical outcome.
RESULTS. Out of a series of 303 neuroblastoma patients diagnosed in Italy in the period 1997-2002 , 150 were staged as localised according to INSS criteria (including absence of tumour cells in both bone marrow aspirates and bone marrow biopsies). Of these, nine cases were excluded from the study because complete data from the three methods were not available. Of the remaining 141 cases, in 26 patients (18%), immunocytochemical assay with anti-GD2 monoclonal antibody (3F8) showed immunostained cells, in numbers varying from 1 to 1000 out of 1x106 cells analysed. Of these 26 patients, 7 (26.9%) relapsed, versus 7 (6.1%) in the group of 150 negative cases.
CONCLUSIONS. Our preliminary data suggest that immunocytochemical examination with anti-GD2 monoclonal antibody may be a sensitive and useful tool in identifying patients at high risk of relapse. While immunocytochemical assay of bone marrow aspirates is not a substitute for conventional methods for staging, it can provide additional useful information for patients with localized disease.

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Ref ID: 042.1 **#27**

Detection of residual neuroblastoma cells using a new four-color flow cytometric assay

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BACKGROUND: Detection of bone marrow (BM) involvement is critical for accurate staging and risk assessment in neuroblastoma. Since the therapeutic consequences of the BM findings may be far-reaching, the need for reliable and sensitive detection methods becomes evident. Therefore, two four-color flow cytometric (FC) assays using different combinations of CD9, CD81, CD56, CD45 and anti-GD2 were developed.
METHODS: Thirty-eight BM samples, 17 biopsies and 5 peripheral blood stem cell (PBSC) preparations from 28 neuroblastoma patients were analyzed and the number of CD9+/CD81+/CD45-/CD56+ or anti-GD2+/CD81+/CD45-/CD56+ cells was determined. The results were compared with those of an anti-GD2 immunocytochemical assay.
RESULTS: All tumor samples were CD9+/CD81+/CD45-/CD56+. All except one showed GD2 expression. The results between the FC and the immunocytochemical assay were concordant in 34 out of 43 BM samples and PBSC preparations. Twenty-seven BM samples and 3 PBSC preparation were double negative and four BM samples were double positive. A strong correlation between both assays was found (p=0.006). Seven BM and 2 PBSC samples scored positive for the immunocytochemical assay but were negative for the FC tests. These samples were taken during therapy and after immunocytochemical analysis only a few anti-GD2 positive cells considered as neuroblastoma cells were found.
CONCLUSIONS: FC can be used to detect neuroblastoma cells in a simple, quick and cost-effective way. The sensitivity of the FC assays was lower than that of the immunocytochemical test but it is still possible to screen for residual cells in a reliable way. Further investigations are necessary to determine the clinically relevant detection limit.

Ref ID: 125.1 **#28**

New advances in neuroblastoma minimal residual research: Five colour flow cytometry

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BACKGROUND: Despite high-dose myeloblative chemotherapy and autologous peripheral blood stem cell (PBSC) transplantation the prognosis for patients with advanced neuroblastoma (NB) is poor. The immunomagnetic CD34+/CD133+ selection of stem cells is a well-established strategy to remove contaminating tumor cells from autografts. By this treatment high purity (93-97%) of stem cell transplant is achieved, nevertheless remaining 3-7% including common leukocytes and cells from unknown cell type like residual neuroblasts. For both, detection of residual neuroblastoma cells in autografts and in bone marrow (BM) of patients prior to and after transplantation is necessary with regard to set timely therapeutic intervention.
METHODS: For detection of minimal residual disease we developed a new five colour flow cytometry panel. In a cell screening 85 monoclonal antibodies were tested for neuroblastoma binding. Four different NB cell lines, BM, PBSC and PB from neuroblastoma patients were examined. CD45 negative cell
gating strategy was applied. For the detection of residual neuroblasts on a molecular level, we established RT-PCR and a real-time PCR assay for tyrosine hydroxylase (TH) mRNA.
RESULTS: A range of six monoclonal antibodies, CD9, ch14.18, CD56, CD57, CD81 and CD138 were discovered to detect residual neuroblastoma cells. Antigen properties differed between the investigated samples. This requested an establishment of various antibody panels. Neuroblastoma samples of two patients lost antigen properties, CD56, during the treatment.
CONCLUSION: The five colour flow cytometry is a sensitive and specific method to detect occult neuroblastoma cells. The combination of real-time respectively RT-PCR for TH and the five colour flow cytometry is an effective proceeding to monitor residual disease of neuroblastoma patients.

Ref ID: 153.1 **#29**

Bone marrow infiltration in neuroblastoma: a flow cytometric tri-colour assay

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BACKGROUND: Bone marrow (BM) involvement is an adverse clinical prognostic factor in neuroblastoma (NB). For this reason, in addition to morphological evaluation, several methods have been developed for the detection of BM metastases. Immunostaining with antiGD2 antibody and molecular detection of tiroxine hidroxilase, are the two "high-sensibility" methods employed for BM micro-metastatic disease detection. However, carry-over contamination and illegittimate transcription for molecular study, and the endogenous enzymatic activities for immunocytological study, can lead to some false positive results.
AIMS AND METHODOLOGY: In the present study, we measured the extension of BM involvement at diagnosis in 25 consecutively patients with stage 4 NB at onset (by a flow cytometric whole blood assay), combining two well-known leukocytes antigens (CD45 and CD56) with anti-human anti-NB NB84 FITC conjugated antibody. The prognostic impact of BM infiltration was evaluated by the quantitative detection of NB cells with cytometry in BM aspirates, dividing the patients in two groups (group 1: 0-1% positive cells, group 2: >1% positive cells).
RESULTS AND CONCLUSION: BM involvement was detectable by both morphological and cytometric analysis in 14/25 patients with stage 4 NB. Two additionally patients with a minimal BM involvement were identified only by cytometry. The group 1 (13 patients) had a 3-yrs OS probability significantly higher than the group 2 (n= 12 patients): 0.7 vs 0.1, respectively; p<.05. This result could be taken into account to stratify patients in different risk groups, but has to be confirmed on a wider number of cases.

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Ref ID: 428.1 **#30**

Immunocytological evaluation of bone marrow disease in peripheral neuroblastic tumours

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The assessment of bone marrow (BM) involvement is crucial to staging and to the evaluation of response to therapy in neuroblastoma patients.
The Immunocytology/Cytogenetic Group of the ESIOP Bone Marrow Subcommittee has developed and standardised an immunocytochemical assay to detect neuroblastoma cells in BM aspirates by means of anti-GD2 monoclonal antibody.
Previous studies (Seeger et al, 2000) pointed out the prognostic relevance of "in vivo purging" of BM after the first chemotherapy cycles. This could help in identifying subsets of patients with high-risk disease who should be considered for innovative therapy.
Immunocytochemical detection of GD2 positive cells has proved to be a sensitive tool which can add useful quantitative information to that yielded by standard cytomorphological examination of BM smears and histopathological examination of BM trephine biopsies. A comparison between this latter and the anti-GD2 technique was carried out on a series of 250 cases, in which both BM trephine biopsies and BM aspirates were available. The study included patients with localized or disseminated disease, on diagnosis and at various intervals during the therapy.
Overall, a low concordance was observed between the two methods (37%) while the concordance was much higher (77%) when comparison was restricted to the evaluation at the time of diagnosis, or if a quantitative cut-off of fewer than 100 versus => 100 positive cells per 106 BM cells was applied to the anti-GD2 assessment.
On the basis of higher sensitivity (1+ cell out of 10 5 - 10 6 mononuclear cells) and lower invasiveness, anti-GD2 immunocytochemical assay may be considered a good substitute for the standard BM trephine biopsy, especially in children below the age of one year.

Ref ID: 320.1 **#31**

GD2 synthase mRNA is less specific for detection of neuroblastoma in blood and bone marrow than tyrosine hydroxylase mRNA and dopadecarboxylase mRNA

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BACKGROUND AND AIMS: Sensitive detection of tumour cells in blood and bone marrow (BM) seems useful to detect minimal residual disease and monitor therapy in neuroblastoma. We therefore compared the mRNA concentrations of tyrosine hydroxylase (TH), dopa decarboxylase (DDC) and GD2 synthase (GD2S) for these purposes.
METHODOLOGY: Quantitative RT-PCR was developed for TH, DDC and GD2S. 224 blood samples from 46 children and 106 bone marrow (BM) samples from 37 children with neuroblastoma were analyzed. Cord blood from 52 newborns and blood from 26 healthy children served as controls together with 22 blood samples and 28 BM samples from children with other diseases.
RESULTS: In nine children with stage 1-3 neuroblastoma, TH, DDC and GD2S were increased at diagnosis in 2, 0, and 2 blood samples. Corresponding numbers for BM samples in eight children were 1, 0 and 4. In stage 4 neuroblastoma 14, 9 and 12 blood samples from 16 patients were positive, and in 17 BM samples corresponding values were 16, 14 and 15. For all blood samples TH and DDC mRNA showed high correlation whereas GD2S and TH did not. Blood samples from healthy controls were below detection limits. BM samples (n=28) from patients with other diseases were increased in 3, 2 and 21 samples for TH, DDC and GD2S. Corresponding numbers for blood samples (n=22) were 1, 1 and 9.
CONCLUSIONS: Sensitivity for detection of stage 4 neuroblastoma was similar with the three transcripts. GD2S was less useful in discriminating stage 4 from stage 1-3 in BM and was less specific for neuroblastoma.

Ref ID: 383.1 **#32**

Pitfalls in detection of contaminating neuroblastoma cells by tyrosine hydroxylase RT-PCR due to catecholamine-producing hematopoietic (stem) cells

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RT-PCR analysis of compounds of the catecholamine metabolism (especially tyrosine hydroxylase, TH) is considered to be suitable for detection of contaminating neuroblastoma cells in hematopoietic stem cell preparations. Because of the heterogeneity of neuroblastoma cells, we used for this analysis not only primers for TH, but also for DOPA-decarboxylase, dopamine-ß-hydroxylase, and the noradrenaline transporter. Additionally, primers for tyrosinase were included because in some neuroblastoma cells DOPA production is catalysed preferentially by this enzyme instead of TH. Using this panel of primers, a moderate sensitive detection of the heterogeneous neuroblastoma cells is possible with single RT-PCR, that enables a clear discrimination from hematopoietic (stem) cells[1:1000]. In order to detect a smaller number of contaminating neuroblastoma cells we used nested RT-PCR. Under our experimental conditions, all the respective "neuroblastoma" markers were also positive in mononuclear blood cells, in apheresis preparations (G-CSF mobilized peripheral blood cells) and in highly purified CD34+ and CD133+ stem cells. Our results are generally in line with reports demonstrating both production and uptake of catecholamines by hematopoietic cells [e.g. Marino et al, Exp. Hematology 27 (1999)]. This raises the question whether the RT-PCR analysis of catecholamine metabolism is principally suitable and selective enough to detect a contamination of hematopoietic stem cell preparations with a small number of neuroblastoma cells. Interestingly, by using the TH primers for nested PCR described by Lode [Eur.J.Cancer, 33 (1997)] we found in 15 out of 19 investigated samples one TH signal that appears to be an alternative splice variant in hematopoietic cells but not in neuroblastoma cells. This enables a better discrimination between both cell types.

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Ref ID: 106.1 **#34**

Quality assurance of reverse transcriptase polymerase chain reaction (RT-PCR) to detect neuroblastoma: ESIOP Neuroblastoma Group

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RT-PCR for tyrosine hydroxylase mRNA to detect neuroblastoma cells will be employed to evaluate low level disease of bone marrow and peripheral blood from children entered into the ESIOP High risk study. To ensure robust and reliable information is acquired from this multi-centre, prospective clinical outcome study a task force has been established to

a. Create standard operating procedures (SOPs) for optimal clinical sample collection, storage and processing.
b. Determine the common causes of variability in RT-PCR analyses between laboratories and from these develop SOPs for RT-PCR.

a. Sample collection, storage and processing.
Sample volume collected will be assessed by weight. PAX gene blood RNA tubes are practical for collection, storage and transportation of clinical samples across multiple centres. The PAX gene blood RNA kit is useful for the isolation of RNA from 2ml blood samples, however the capacity of the columns is insufficient to isolate all the RNA from 0.5ml of diagnostic bone marrow. A standard method is under development.

b. RT and PCR sensitivity and specificity.
There have been six quality control rounds. Analysis of cDNA across all seven laboratories demonstrated good sensitivity and specificity, confirming that the PCR reaction is robust in all participating laboratories. Although the specificity of RNA analyses was good, there was unacceptable variability in sensitivity. Standardisation of the amount of RNA, RT conditions and source of RT enzyme increased the sensitivity of tyrosine hydroxylase mRNA detection to 10pg or 1 cell.

International quality assurance programmes are important to ensure accuracy of molecular assays.

Ref ID: 109.1 **#33**

Tyrosine Hydroxylase expression in blood of patients with Neuroblastoma: Analysis by a real time RT-PCR quantitative assay

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Molecular detection of minimal residual disease by a sensitive methodology could contribute to a better treatment in children with neuroblastoma. To detect circulating neuroblastoma cells we developed a quantitative assay for the analysis of Tyrosine Hydroxylase (TH).
METHODS: We analyzed 70 samples of peripheral blood (PB) and 13 leucapheresis products (PBSC) from 25 patients with neuroblastoma in advanced stages (8 stage 3, and 17 stage 4). TH mRNA was analyzed by a RT-PCR assay using TaqMan technology. For each test sample the amount of TH, and its endogenous reference gene 18S, were determined. Normalized TH value was obtained by dividing TH/18S. Twenty samples of PB from donors were used for normalizing TH, and values <37,05 were considered negative.
RESULTS: With a median follow-up of 47,3 months(range 15-96 months), 9 patients (36%) relapsed. TH expression was detected in all but one patient in PB at diagnosis. During treatment 6 patients cleared tumor cells, while at the end of treatment or during follow-up 18 patients were positive for circulating tumor cells. Nine of these patients relapsed while none of the negative patients did. Actuarial 5-year event-free survival was 100% for TH-negative patients and 40% for TH-positive (p<0.01). Patients with TH-positive PBSC had also a worse prognosis than patients negative for TH. Actuarial 5-year event-free survival was 60% for PBSC negative patients and 32% for positive patients (p=0.05).
CONCLUSIONS: TH-positive patients after treatment seem to have a worse prognosis compared with patients with undetectable TH. Further investigation into the detection of circulating tumor cells during follow up of patients with neuroblastoma is warranted.

Ref ID: 042.2 **#35**

Detection of residual neuroblastoma cells using quantitative real-time RT-PCR

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BACKGROUND: The presence of neuroblastoma cells in bone marrow (BM) is associated with relapse and unfavorable outcome. Consequently, detection of tumor cells in BM is important for risk assessment and evaluation of response to therapy. We developed real-time quantitative RT-PCR assays for three different genes (tyrosine hydroxylase (TH), GD2 synthetase and ELAVL4) in order to detect neuroblastoma cells in BM.
MATERIALS AND METHODS: The expression of the genes was evaluated using TaqMan technology. The amount of target mRNA was quantified using a relative standard curve. Three housekeeping genes were used to normalize the data. Twenty-eight BM and 11 tumor samples, obtained from 18 neuroblastoma patients, taken at diagnosis or during treatment were examined using the RT-PCR assay.
RESULTS: The three genetic markers were overexpressed in all tumor samples. The RT-PCR results were concordant in 21 out of 28 BM samples. Seven BM samples were positive for at least one marker. The RT-PCR results from 22 BM samples were compared with those of an anti-GD2 immunocytochemical assay. The results were concordant in 20 out of 22 samples. Eight BM samples, positive for the immunocytochemical test, scored also positive for at least one molecular marker. Twelve samples were double negative. All samples positive for the immunocytochemical test, overexpressed at least one marker. Finally, the RT-PCR results from 23 BM samples were compared with those of conventional cytomorphological evaluation. The results were concordant in 18 samples. In five BM samples at least one marker was overexpressed although no neuroblastoma cells were found by cytomorphological examination.
CONCLUSION: From these preliminary results, we conclude that real-time quantitative RT-PCR detecting TH, GD2 synthetase and ELAVL4 mRNA can be used to screen for NB cells. Further investigations are necessary to determine the clinical relevant detection limit.

Ref ID: 311.1 **#36**

Tumour cell clearing in the bone marrow of stage 4 neuroblastoma - Can it predict outcome?

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PURPOSE: This study was conducted to determine whether accurate monitoring of dynamics of bone marrow (BM) clearing could identify subgroups of patients with different prognoses.
PATIENTS AND METHODS: BM specimens of 44 stage 4 patients treated according to Austrian multi-centre protocols were tested at diagnosis and given time points during treatment by computer-assisted fluorescence scanning and repositioning system. The automatic immunofluorescence plus FISH (AIPF) device combining GD2-based immunocytology and subsequent molecular-cytogenetic analysis of the same cells enables unambiguous detection and exact quantification of the tumour cell infiltrate.
RESULTS: The median age of patients was 2,4 years. Median observation time was 5 years, the 5-year overall survival (OS) was 59%±8%. When the 5-year OS of patients with tumours presenting either MYCN amplification and/or del1p36 was compared to patients without these two features no difference was observed (58%±12% vs. 63%±10%). However, when the kinetics of tumour cell clearing in the bone marrow of patients with tumours showing either MYCN amplification and/or del1p36 (n=29) was evaluated, a correlation was observed between the speed of clearance and the outcome. The 5-year OS was 75±10% in patients showing rapid BM-clearing within 4 cycles of induction treatment and 25%±15% in those with delayed clearing (p=0.013). However, in patients with tumours not showing MYCN amplification and/or del1p36 (n=15) the BM-clearing time did not have the same clinical implications.
CONCLUSION: Although only a limited number of patients was analysed, the results allowed a distinct correlation of rapid BM-clearing with a favourable outcome in the group of patients with unfavourable genetic features expressing the response to chemotherapy and thus providing an excellent prognostic marker.

Ref ID: 137.1 **#37**

Tumor cell detection in apheresis products in pediatric patients with advanced neuroblastoma. Where to go?

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BACKGROUND: The detection of tumor cells (TCs) in apheresis products (AP) remains a crucial issue. The sensitivity and specificity of the methods used and the number of analyzed cells are variable. To address this question, we prospectively analyzed the APs from patients with neuroblastoma by an automatic immunofluorescence plus FISH (AIPF) device.
METHODOLOGY: We analyzed 55 APs from 21 patients (median age 2.01 years, 0.49-19.46; 9xfemale, 6xmale, 18xstage 4, 2xstage 3, 1xrecurrency). Apheresis procedures were performed at median 97d (20-346d) after diagnosis. Before cryoconservation a sample was taken from the APs for TC analysis [Ambros et al. Cancer Lett. 2003, 197: 29-34.]. We calculated the maximum amount of non detected TCs by the 95% limit of the binomial distribution.
RESULTS: The APs contained in median 3.787x10E9 MNCs (1.49-24.8). The analyzed samples contained in median 2.6x10E6 MNCs (0.45-6.25). In 6/55 APs from 4 patients (3xstage 4, 1xrecurrency) we found TCs by the AIPF method corresponding to a detection limit of 1 TC in 1,733,333 MNCs (450,000-3,120,000). This figure corresponds to a total contamination of 4,638 (1,085-17,600) TCs in the AP. In 49/55 AP we found no TCs (detection limit <1 TC out of 2,600,000 MNCs). This results in a possible contamination rate of 1,000-24,000 not detected TCs in the AP.
CONCLUSION: We found only a low number of TC contaminated APs by the AIPF method, we have to keep in mind that up to 24.000 TCs could be present in the AP which were not detectable with the number of cells available for analysis. This possible contamination on outcome should be addressed in prospective trials.

Ref ID: 138.2 **#38**

Standardised reporting of I-123 mIBG scans for determination of response to induction chemotherapy

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BACKGROUND AND AIMS: The NMPSC within the SIOPEN-R-NET EU grant has three major aims; (i) to develop a network for Nuclear Medicine & Physics within Europe, (ii) to implement a Europe-wide scoring system for the evaluation of mIBG scans in paediatrics with high-risk neuroblastoma and (iii) to implement a system of electronic image transfer within the participating countries. We present here initial results of this study.
METHODOLOGY: Hardcopy and softcopy scan data were acquired and transferred in a number of formats between participating centres in each of the countries involved in the grant. A scoring system previously used successfully by the SFOP was implemented by 25 members and associates of the NMPSC. This system takes into account both the number and intensity of lesions.
Results: To date approximately 800 images have been reported and scored, each by at least one team consisting of 3 reviewers. This represented 52% of the scans that should have been available. 21% of the scan sets were considered to be of poor quality and only 13% were of excellent quality. Following initial training, reviewers agreed consistently on scan scoring.
CONCLUSIONS: This study has effectively acted as a snapshot of the state of I-123 mIBG image scanning throughout Europe. It is evident that there is room for improvement in the quality and quantity of data acquisition and this is now being addressed. This study has demonstrated that it is possible to develop standard and quantitative Nuclear Medicine reporting procedures within Europe.

Ref ID: 405.1 **#39**

Assessment of chemotherapy response by MIBG scan: a blind quality control

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BACKGROUND: In European protocol of high risk neuroblastoma, the randomization after the first induction phase of chemotherapy is related to the response evaluation performed by MIBG results: only patients with not more than three residual bone lesions are eligible. Consequently, it is mandatory that MIBG scans must be technically optimized and that the evaluation of bone response assessed by a specific form is homogeneous between nuclear medicine specialist(NM) of several hospitals and countries.
AIMS: To investigate the diagnostic variability of NM in 123I-mIBG baseline and post therapy scans and to recognize in the evaluation form, if present, which parameters identify the patients with equivocal response to be discussed by a team of experts.
METHODOLOGY: Hard copies of two series of 24 hours scintigrams performed in ten patients from 4 Italian hospitals were digitalized by a scanner and sent by E-mail in JPEG format to six Italian NM. Most pictures were reproduced with two intensity normalization. Each specialist compiled the evaluation protocol form (with bone lesions divided in 7 regions, scored with 3 intensity and 2 extension levels) knowing only for every patients the sequence of examinations and the name of referring hospital.
RESULTS: In baseline scan, no significant difference in evaluation of bone regions was observed for each patients (range 49-64, mean 58±5); in II scan the variation arises (range 31-61, mean 38±11,86, but if the score 1 intensity level (suspected lesion) isn't considered, the variation lowers (range 17-31; mean 25±4,9). The concordance was near to 100% in 5/6 NM: one NM performed a pessimistic score 1 evaluation in most second scans.
CONCLUSION: This blind study confirms the validity of the evaluation form and the general concordance between NM, if score 1 level is not considered.

Ref ID: 330.1 #40

Chromogranin A (CGA), Tyrosine Hydroxylase (TH), and Protein Gene Product 9.5 (PGP9.5) Taqman“ PCR Assays Improve Detection of Neuroblastoma Cells in Blood and Bone Marrow

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Sensitive and specific assays for neuroblastoma cells in marrow and blood may improve prediction of outcome and evaluation of cells used for autologous hematopoietic stem cell transplantation (AHSCT). We evaluated CGA, TH, and PGP9.5 with Taqman“ PCR. Threshold Cycle (Ct, mean + SD), which is inversely proportional to copy number of target template (high template concentration = low threshold cycle), is shown.

Gene	Cell Lines (n=18)	Tumors (n=19)	Normal Blood Cells (n=30)	Non-nbl PBSC (n=11)
CGA	19.4±1.6	21.1±1.2	>40	>40
TH	22.3±5.4	22.0±2.1	>40	>40
PGP9.5	18.5±0.5	19.8±0.9	36.2+2.4	33.1±3.6
GAPDH	19.6±1.2	22.0±1.5	20.6+0.5	Not done

Seeding SK-N-BE2 neuroblastoma into normal blood cells showed the sensitivity of PCR and immunocytology to be 10-6 and 10-5 respectively. Marrows from 20 patients harvested for AHSCT were all positive by PCR (13 immunocytology positive). After immunomagnetic purging, tumor cells were decreased, and none were detectable in 35% by PCR (all immunocytology negative).

Gene	Before purging	After purging	P value
CGA	23.7±1.4	34.3±1.4	<0.001
TH	25.3±0.4	36.3±1.1	<0.001
PGP9.5	22.1±0.1	28.3±0.6	<0.001
GAPDH	23.4±0.3	23.8±0.2	NS

We conclude that PCR assays for CGA, TH, and PGP 9.5 detect neuroblastoma cells with greater sensitivity than immunocytology and that purging can result in less than one tumor cell per 106 marrow cells.

Ref ID: 201.1 #42

Telomere length – as a prognostic marker in neuroblastoma

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Telomeres are tandem repeats at the ends of eukaryotic chromosomes that are shortened progressively with cell divisions leading to cellular senescence and death. Telomerase is a multi subunit ribonucleoprotein enzyme that adds telomeric DNA to the ends of chromosomes. Its catalytic subunit (hTERT) expression correlates with telomerase activity. Telomerase activity (TA) has been detected in nearly all malignant tumors. We studied telomerase activity, hTERT expression and telomere length in 40
primary neuroblastoma tumors using the TRAP analysis, quantitative RT-PCR and southern blotting, respectively. High TA was detected in 50% (20/40) primary tumors and it correlated significantly with other unfavorable prognostic factors. Ten years Progression Free Survival (PFS) of the patients with Low TA was significantly longer (85%) than that of patients harboring high TA (40%) (p=0.003). High hTERT expression was identified in 35% (11/31) tumors and it correlated with high TA (p=0.009). Telomere length was compared between tumor and peripheral blood from the same patient. Shorter telomeres (ST) were detected in 41% (12/29), longer or no change (LT) in 59% (17/29). Of note, the patients with shorter telomeres had favorable prognostic factors and outcome. Furthermore, Kaplan-Meier analysis for both, TA and telomere length, could distinguish 4 significant subgroups (p=0.04). The subgroup with ST and Low TA had the most favourable prognosis (100% PFS), while LT/ Low TA and ST/high TA subgroups had intermediate prognosis, 75% and 71% PFS, respectively. The worst subgroup, with only 33% PFS, consisted of LT/High TA. Our results suggest that telomere length could play an important role in outcome of children with neuroblastoma.

Ref ID: 079.1 #41

Prognostic value of telomerase activity in neuroblastoma like tumours

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BACKGROUND: The presence of activated telomerase in neoplasm cells prevents from telomere shortening and causes the neoplasm cell immortal.
AIMS: The aim of the study was the evaluation of the influence of telomerase activity on prognosis in neuroblastoma like tumours.
PATIENTS: 33 tissue samples obtained from 30 patients aged between 1 and 75 months (19 males and 11 females) were analysed. Histologically neuroblastoma was diagnosed in 24 patients (80%), ganglioneuroblastoma in 4 (13,3%) and ganglioneuroma in 2 (6,7%) cases.
METHODS: Tissue samples were frozen immediately after the biopsy and were stored in -80ºC until the analysis. Two independent methods were used for telomerase activity evaluation: Telomerase Repeat Amplification Protocol (TRAP) and ELISA method.
RESULTS: Telomerase activity was stated in 9 tissue samples of primary tumour, in the regional lymph nodes infiltrated by neoplasm cells and in the metastatic relapse in mediastinum in single cases. Neuroblastoma with telomerase activity presented more advanced disease and also more often the prognostically unfavourable MYCN amplification was stated. Patients with telomerase activity had significantly poorer outcome in comparison to patients without telomerase activity (5-year EFS 0,61 and 0,15 respectively; p=0,01).
CONCLUSIONS: The assessment of telomerase activity could be helpful in the stratification and the choice of the optimal therapy. The influence of telomerase on clinical outcome advocates its pathogenetic role in neuroblastoma, so the enzyme could be a possible target for new antitumour drugs inhibiting it.

Ref ID: 117.1

Gene expression profiling of neuroblastoma: analysis of nonmetastatic versus metastatic tumors

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BACKGROUND: Previously we reported a significant distinction in gene expression profile between neuroblastoma (NB) tumors and cell lines, and stroma-rich and stroma-poor NB tumors. Tumors clustered into 2 groups correlating with clinically defined prognostic groups of favorable and unfavorable tumors. In this study we analyzed tumors with and without clinically proved metastatic potential.
METHODOLOGY: we present the analysis of 25 locoregional and 27 stage 4 tumors all stroma poor and >1 year of age. Microarray analysis was carried out using Affymetrix Genechip Human Genome U95 SetTM with features for 63,175 gene/EST. To calculate the ability of individual genes to distinguish between sample groups we used 1. Calculation of maximal variability and >3 standard deviation between groups and 2. Step-down permutation and false discovery rate methods. For multivariate analysis, unsupervised methods like SOMS, PCA, K-nearest neighboring, and hierarchical clustering were applied.
RESULTS: By contrast analysis, 61 out of 12,000 known annotated genes from chip A were differentially expressed in LR cases compared to stage 4. By permutation analysis only 3 out of the 12000 annotated genes showed statistical significance. Multivariate analysis showed a poor distinction between LR and stage 4 tumors with a third of samples classifying in the wrong groups. Classification models build to distinguish groups using support vector machines, k-nearest neighboring and log regression showed a cross validation accuracy that ranged from 76 to 89%.
CONCLUSIONS: the gene expression profiles of tumors presenting as LR or disseminated disease suggest the existence of more than 2 groups of tumors. A \"metastatic\" gene expression profile could not be detected.

Ref ID: 161.1 #44

Quantitative Real-Time PCR for the simultaneous determination of prognostic markers MYCN amplification, deletion 1p and 11q

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BACKGROUND: Due to the increasing clinical relevance of molecular markers, accurate assessment of the status of MYCN, 1p and 11q is essential. As two techniques are recommended to avoid artefacts we developed a PCR assay as an alternative to LOH-analyses and in addition to FISH.
METHODS: Based on real-time Q-PCR using SYBR-Green reagent (TaqManT), we developed a new assay using genomic DNA from frozen and paraffin embedded tissue as template. Determination of deletion or amplification was achieved by comparing a target gene (TG, from the region of interest) to an unaffected reference gene (RG) within the same chromosome. PCR raw data were normalized to a serial dilution standard curve and a ratio TG/RG was created. The ratio to define a deletion was set as 0.5 (=expected ratio 1TG copy/2RG copies), amplification threshold was set as >10. Data were compared to results obtained by FISH.
RESULTS: Results according to PCR and FISH were consistent in 8 tumors with deletion 1p, 11 with deletion 11q, 10 with MYCN amplification and 85 samples without aberrations. Three tumors with deletion 1p and 3 with deletion 11q were detectable by FISH but not by PCR. A single case indicated a deletion 11q by PCR only. The consistency between both techniques was 92% for 1p and 11q, 100% for MYCN. The discrepant cases are most likely due to a heterogeneous cell population in the investigated tissue.
CONCLUSION: The use of a quantitative PCR assay enables the simultaneous detection of the three most relevant chromosomal aberrations accurately as confirmed by FISH. As the assay allows the investigation of paraffin embedded material and requires no reference tissue, it can be regarded alternative to LOH- or Southern Blot analyses. Determination of the tumor cell content is crucial to avoid false-negative results.

Ref ID: 046.1 #45

Circulating MYCN DNA as a tumor-specific marker in neuroblastoma patients: Results of a blind multicentric study

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MYCN amplification is an indicator of neuroblastoma aggressiveness. It is used internationally for stratifying patients for therapy. In a monocentric study, we have previously shown that high levels of MYCN DNA sequences could be detected by PCR in the peripheral blood of patients with MYCN-amplified neuroblastomas. Furthermore, we showed that the release of MYCN sequences in the peripheral blood is an early process of disease progression. As a consequence, circulating MYCN DNA is a valuable prognostic marker able to predict relapse in neuroblastoma patients (Cancer Research 2002). To confirm the pertinence of this assay of circulating MYCN DNA detection, the present investigation examined, in a blind analysis, the MYCN DNA sequences detected in the sera obtained at diagnosis from 48 patients newly diagnosed with neuroblastoma in different centres (29 French and 19 Spanish patients) and compared with results obtained in tumour cells. A 96% correlation was observed between sera and malignant cells. As the determination of MYCN amplification is crucial for clinical decision in many therapeutic protocols, we propose the use of this new non-invasive assay to evaluate MYCN DNA sequences, in particular when malignant cells are not available for molecular analysis.

Ref ID: 171.1 #46

MAGE1 expression in primary tumor correlates with better outcome in Neuroblastoma patients

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AIM: to assess the prognostic value of TH and MAGE expression in primary tumors, peripheral blood and bone marrow at diagnosis in neuroblastoma patients.
SAMPLES AND METHODS: we analyzed 43 tumors, 166 bone marrows and 95 peripheral blood samples at diagnosis from neuroblastoma patients. Expression of TH, MAGE1 and MAGE3 was studied by RT-PCR and subsequently hybridized witch specific probes.
RESULTS: Here are shown the frequencies observed for the three markers in tumors: MAGE 1 MAGE 3 TH + - + - + - Localized disease 11 8 11 8 17 2 Disseminated disease 8 16 11 13 23 1 Total 19 24 22 21 40 3 MAGE 1 expression in the tumor at diagnosis is the only marker that correlates with a better disease free survival (p=0.006). This influence remains significant only in localized disease (p=0.013). Overall survival is significantly better in neuroblastoma patients expressing MAGE1 in primary tumors (localized disease p=0.031, disseminated disease p=0.02). We did not find any correlation between markers expression in blood or bone marrow at diagnosis and outcome.
CONCLUSION: MAGE 1 expression in primary tumor correlates with better event free survival in localized neuroblastoma and with improved overall survival in localized and disseminated neuroblastoma

Ref ID: 269.1 #47

Proliferative activity in relationships with established prognostic factors in neuroblastoma

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Stroma poor neuroblastic (Nb) tumors of 41 patients were evaluated immunochemically of Ki-67 as proliferative index (PI). PI was correlated with clinical characteristic (previous chemotherapy, age, stage, tumor's primary site, bones involving, LDH and ferritin levels) as well as the presence of MYCN amp. RESULTS: NB tumors showed differentiated values of PI (1,5-79,6%). There was statistically significant difference between average PI in tumors before and after chemotherapy (35% vs 23%). No significant correlation between PI and age, stage, primary site, bones involvement was observed. However PI was higher in children <1 than >1 year old (34% vs. 22%). Lower PI presented tumors from patients in stage III (23,9%) compared to the other stages (I+II, IV+IVs) (32% and 40%) and those localized in adrenal gland (26%) compared to retroperitoneal region (37%). Bones involvement was related to higher PI. No correlarion between PI and biochemical markers was found. Tumors with MYCN amp showed higher PI levels in comparison to nonamplifed tumors (29,5% vs 39,1%). The same analyzes of correlations performed in 27/41 tumors from patients before chemotherapy confirmed similar relationships. Worthy of note were 3 /14 tumors from pretreted patients presented high levels of PI>40% because these patients demonstrated aggressive course and poor outcome within a year.
CONCLUSIONS: Chemotherapy effects PI values in Nb. The proliferative activity should be assessed together with clinical characteristic of the disease. The further studies could evaluate the usefulness of PI as a marker of treatment response.

Ref ID: 279.1 **#48**

c-kit in neuroblastomas is not related to MYCN amplification

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BACKGROUND: c-kit (CD117) is a transmembrane tyrosine kinase representing a target for STI571 (Glivec) therapy. Some c-kit-overexpressing solid tumours have responded favourably to STI571, potentially because of the presence of c-kit-activating mutations.
METHODS: To investigate the epidemiology of c-kit overexpression in neuroblastomas, we investigated a series of 63 neuroblastomas. All tumours were analysed by immunohistochemistry in a tissue microarray format. We compared the expression of c-kit to the expression of the MYCN-gene, analysed using fluorescent-in-situ-hybridisation.
RESULTS: Nine of the 61 neuroblastomas expressed c-kit in varying amounts and intensity. Twelve of the neuroblastomas were MYCN amplified. Only 2 of the MYCN amplified tumours expressed c-kit, and only focally (p=0,5). Using Shimada pathology, we found that 9 of 31 tumours with unfavourable histology expressed c-kit, while only 1 of 24 tumours with favourable histology expressed c-kit. None of the 8 benign tumours expressed c-kit (p<0.05).
CONCLUSION: The expression of c-kit in neuroblastomas is not correlated to MYCN amplification, but still it is related to unfavourable histology, indicating that it is a prognostic factor independent of MYCN.

Ref ID: 377.1 **#49**

Serum levels of sRANKL and OPG in patients with neuroblastoma

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In previous studies we have demonstrated that neuroblatoma cells are able to induce osteoclastogenesis: a low OPG-to-RANKL ratio, that is a high expression of RANKL associated with the lack of the decoy receptor OPG, may be involved in the pathogenesis of metastatic osteolysis. The aim of this study was to evaluate the serum levels of sRANKL and OPG in neuroblastoma patients and correlate them with clinical and laboratory characteristics. A total of 55 children (age 0.5-127 months) were considered for this study. 18/27 stage IV patients (66.7%) had skeletal metastases. 35 children (age 1.2-57.3 months), who were admitted to pediatric department for minor surgical problems, was considered as control group. Serum levels of free-sRANKL and OPG were determined by an immunoenzymatic assay. OPG was found significantly lower in all patients than in control group, but it was not related to the presence of skeletal metastasis. A significant correlation was found between OPG and neuron specific enolase (R=0.74, p<0.001). Free sRANKL was found significantly higher in stage III (52.4±26; p=0.01) and IV (37.1±10; p=0.02) than in control (10.9 ± 6). sRANKL was higher in patients with skeletal metastases than in those without bone involvement; the difference was found significant when stage IV patients were considered (47.4±14 vs 18.4±7pg/mL; p=0.05). The OPG-to-RANKL ratio was significantly decreased in patients with skeletal metastasis (1819.8 ± 1173 vs 5499.5 ± 1252, p=0.05). Our results further confirm that unbalanced OPG-to-RANKL ratio could play a crucial role of in determining metastatic osteolysis. The ratio could serve as a marker for monitoring advanced neuroblastoma.

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Ref ID: 352.1 **#52**

Our experience with diagnosis of neuroblastoma using cytogenetic, molecular cytogenetic and molecular techniques

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The prognosis of neuroblastoma (NB) varies considerably with many factors: age at diagnosis, the clinical stage, the primary site, the histopathological subtype and treatment protocol. In addition, genetic parameters such as LOH 1p and MYCN amplification are important prognostic features of NB tumors. Primary NB tumors from 47 patients were genetically analysed at Mother and Child Health Institute: Dr Vukan Cupic\", Serbia between January 1997 and June 2003. 23 of 47 (49%) tumors were analysed for presence of 1p36 using FISH (19 cases) and PCR (4 cases). 24 tumors (51%) were not assessable. Nine tumors showed no 1p36 deletion by FISH and two tumors revealed no LOH 1p by PCR and by FISH. Ten tumors showed 1p deletion by FISH and two of them had the same aberration in bone marrow detected by cytogenetic analysis. 27 NB tumors (57%) were analysed for presence of MYCN amplification. 20 (43%) were not assessable. In 7 of 27 NB tumors MYCN oncogene was amplified. Two of them had HSRs and DMs in bone marrow detected by cytogenetic analysis. The authors will discuss the correlation between genetic findings and prognosis of NB patients.

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Ref ID: 211.1 **#53**

Biological studies on neuroblastoma (NB). Experience of the Italian Biology Reference Center

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It is widely accepted that MYCN amplification (MNA) and 1p36 chromosome deletion (1p36del) are linked to aggressive tumor and patient poor prognosis. Since 1995 our laboratory is the National Biology Reference Center for the biological studies of NB and member of the European Neuroblastoma Quality Assessment Group (ENQUA).Interactions among clinicians, surgeons, pathologists, cytologists are essential for a correct flow and accurate centralization of the biological material. Biological material is collected in Pediatric Italian Centers and sent to G. Gaslini Children Hospital and then to our Laboratory. The sample shipping is performed using a Biocase equipped with two different temperature compartments (+4°C and –70°C). Biological studies are performed only on material with known tumor cell content, following European guidelines. In the last three years we have analyzed MYCN amplification and 1p36 status of 356 and 315 NBs, respectively; MNA was found in 16% of NB patients, while 1p36del in 18% of cases. Both MYCN and 1p36 analyses have been performed by double color Fluorescent in Situ Hybridization (FISH) on interphase nuclei, using fresh or frozen material, paraffin embedded tissue and bone marrow smears. The progressive introduction of FISH technique on nuclei extracted from paraffin embedded tumor and on cytological evaluated bone marrow smears allowed increasing the rate of valuable cases. Since 2001 the valuable sample rate for MYCN and 1p analysis increased of 65% (p=0.0077) and 28% (p=0.0095) respectively. Our aim is to succeed in performing genetic analyses on all centralized cases. This will allow to better characterizing this tumor, to increase the number of studied patients for successful statistical analysis and to give a consistent support to clinical decisions in NB treatment,

Ref ID: 378.1 **#50**

An implication of TRK-A expression for surgical strategy in neuroblastoma

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Neuroblastoma involving the major vessels is challenging for surgeons. We retrospectively evaluated trk-A expression which reflects neuronal maturation as a role of surgical strategy. Patients and Methods: Trk-A m-RNA expression data available in 41 patients between 1995 and 2002 was subjected to this study. All tumors were either resected primarily in INSS stage 1/2 or biopsied before chemotherapy for stage 3/4/4s.These patients were divided into 3 groups according to resectability; Group I (gross complete resection>90%), Group II (partial resection 60-90%), and Group III (unresectable, biospy alone). The degree of expression of trk-A is represented as high(HG), intermediate/high(IH), intermediate(IM), low(LW) and no expression(NO). Statistical analysis was made by Fisher 's exact probability test. Results: Overall mortality/recurrence rate is higher in the LW/NO tumors(p=0.007). Group I (n=33, stage 1 :20, 2b:3, 3:3, 4:6, 4s:1) comprises 17 HG, 6 IH, 3 IM, 4 LW, and 3 NO. Three died (one each for HG, LW and NO) and two had recurrence (I/H and NO). This mortality/recurrence rate is significantly higher in LW/NO tumors(p=0.05). Group II (n=6, stage 3 :2, 2a:3 and 4:1) includes one each for HG, IH, LW and 3IM. The patient with LW had recurrence. Group III (n=2, stage 4 and 4s) showed one IM and one NO. Both died. Conclusions: 1. The patients with low or no trk-A expression tumors did not necessarily show good prognosis after gross complete resection. 2. As the prognosis was good with partial resection of tumors of intermediate and higher trk-A expression, risky attempt of complete resection of such tumors is not justified.

Ref ID: 284.2 **#51**

Prognostic impact of the International Neuroblastoma Pathology Classification (INPC) in Neuroblastoma (NB). The experience of the Spanish Neuroblastoma Registry

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BACKGROUND: In addition to clinical parameters such as age and staging, and biological factors, especially MYCN oncogene amplification, the application of INPC criteria (Shimada's modification) has also been reported as a prognostic indicator in Neuroblastoma (NB). The objective of our paper was to study the prognostic impact of INPC classification in a series of stroma poor NB and its relation with other prognostic factors.
MATERIAL AND METHODS: A total of 209 cases of NB (stroma poor tumors) diagnosed in the period 1992-2000, were collected from the files of the Spanish Neuroblastoma Registry. H&E paraffin slides were reviewed by three of the authors following the INPC criteria. NB cases were grouped into favorable and unfavorable categories taking into account morphologic features of mitosis-Karyorrhexis index, differentiation, histological subtype and age of the patient. Statistical analyses of overall survival (OS) with Kaplan-Meier curves and multivariate study using Cox regression were performed.
RESULTS: Histoprognostic evaluation was possible in 182 cases. 40.3% were considered favorable, and 59.7% unfavorable respectively. Median OS was 73 months. Histopathologically, unfavorable NB showed an OS of 57 months compared with 89 months of median OS in favorable cases. MYCN oncogene amplification as well as 1p36 deletion were more frequently observed among unfavorable NB. Finally, in our study, the Cox regression analysis demonstrated that the clinical stage and the histopathologic subtype are the two most important factors that influence the overall survival (p<0.001).
CONCLUSION: INPC classification results are major prognostic indicators in stroma poor NB and should be considered in the therapeutic stratification of NB patients

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Ref ID: 306.1 **#54**

Outcome of Children with Neuroblastoma in the South-East Poland

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From 1993 to 2003, 27 children with histopathologically proven neuroblastomas (25/27) and ganglioneuroblastoma (2/27) were admitted. The group consisted of 14 boys and 13 girls with mean age of 4,8 yr. and range of 1 months - 13 year. The primary tumour was localised in the abdomen in 19 patients and in 13 cases the primary site was found in sympathetic ganglions, adrenal gland in 3, pelvic region in 3. In 6 cases tumour was localised in thorax. INSS stage 4 disease was presented in 12/27 patients, stage 3 in 11/27, stage 2 in 4/27. Main sites of remote metastases were: bone marrow (9 pts), bones (8 pts), mediastinum (3 pts), liver (3 pts), subcutaneous (1 pts), orbital fossa (1 pts), brain (1 pts). In 2 pts tumour penetrated spinal canal. The treatment included: complete surgical removal of the origin tumour in 2 patients, and biopsy was performed in 25 ones. Chemotherapy was given in 27 patients, radiotherapy in 14. HDT and autoPBSCT was performed in 2 patients. In total 11 deaths due to progressive or relapsed disease were noted. One patient died due to car accident, 2 due to side effects of chemotherapy, 1 due to complications of the surgery. Four patients are still on chemotherapy, 2 on palliative treatment. Only 6 patients of observed group continue complete remission. Conclusion: Despite administration of intensive and burdening therapy, the results of treatment are still insufficient.

Ref ID: 375.1 **#55**

Incidence of neuroblastoma in the southeastern part of Poland

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Neuroblastoma appears with annual incidence of 7-12 per million in the developed countries. The incidence of neuroblastoma is described in relation to age and sex as well as population-derived indicators of socioeconomic level, degree of urbanization and farming activity. Aim of the analysis was evaluation incidence of neuroblastoma in the southeastern part of Poland, which is mostly agricultural region with few industrial centers located in bigger towns. From 1988 to 2000, 49 cases of neuroblastoma in children 0-17 years old were reported (55% of them were boys) in this region. It comprised 5.2% of all the neoplastic diseases in children of this region. Majority of the patients lived in town areas (61%). Mean morbidity rate in different years, ranged from 3.2 to 12.6 per million (medium 6.0). Age-standardized rate at 0-14 age children was 8.7. The highest morbidity was noted in the year 1994. Mean morbidity was higher in male than in female children (6.5 vs. 5.5 per million). Rates were highest at age 0-4 (19.4 per million, 74% of total neuroblastoma incidence) and decreased with age to 3.2 per million (14% of the total) at age 5-9, 0.9 per million (4%) at 10-14 and 2.2 (8%) at 15-17. The incidence was higher in town patients than village ones (7.7 vs 6.0 per million). Conclusions: Incidence of neuroblastoma in the southeastern Poland is similar to average reported in the developed countries. Higher incidence in patients living in urban regions probably is due to concentration of the pollution in the environment.

Poster Display
CLINICAL

Ref ID: 404.1 **#56**

A case of neuroblastoma with congenital defect of liver development in infant

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The patient was a girl 3_ month of age. Anaemia (36 g/l) and abdomen enlargement were the main clinical manifestations that caused hospitalization. Sonographical examination revealed a tumor (6x4 cm) in the left suprarenal area. On the contrary the cystic degeneration of the most of liver parenchyma was observed. The CT examination confirmed changes. In spite of that the biochemical parameters remained stable, however platelets level decreased notwithstanding prednizolontherapy. After anaemia correction and platelets mass transfusion the tumor and liver tissues biopsy was performed. On hystological examination of tumor tissue a diagnosis of neuroblastoma was made. Microscopically normal portal tracts were absent in liver tissue. A layer of rough fibrotic connective tissue and lots of fibrous septum with lymphocyte infiltration were found instead. A congenital liver development defect was detected. First chemotherapy block was performed due to POG protocol (cysplatine 90 mg/m_ and etoposid 100 mg/ m_) and child was discharged. On admitting after 21 days since the first block was held the reduction of tumor (4x2 cm) and liver size and the substitution of numerous cystic structures with parenchyma were revealed. Haematological parameters were normal too. The second chemotherapy block was held similarly. A controversial question is to perform radical operation after the sixth chemotherapy block by protocol or to make early tumorectomy and intraoperative hepar biopsy to confirm a diagnosis of liver pathology.

Ref ID: 376.1 **#57**

Parents helping parents: do psychological mechanisms work when the child is affected by high risk neuroblastoma?

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Reciprocal help relationships are very important among families with children affected by severe pediatric diseases. Communication and discussion of common problems and experiences generally allow the development of coping strategies to face new situations with subsequent adjustment and restoration of a good quality of life after diagnosis and beginning of therapy. When a child is affected by high risk neuroblastoma, this communicative approach is hardly adopted. On the basis of our 10-year experience in the observation of the behavior and the relationships of many families, we suggest the reasons for this difficulty, namely: 1) increased life expectancy of children with NB with increased number of possible relapses; 2) different time intervals between relapses and poor quality of life; 3) child´s perception of his precarious living in the absence of adequate information from both parents and doctors; 4) promiscuous presence in departments and housing facilities of patients with different disease conditions, including other tumors with more favorable prognosis; 5) spreading frustration in a group of patients having in common failure of past treatments, an example of ordeal for newly diagnosed patients accompanied by a sense of ineluctability and disease progression; 6) increased pathologic psychological defenses due to the steady reduction of the range of options and alternatives. It is necessary to suggest possible communication and behavior strategies to be adopted by physicians and other health care professionals that could improve communication with and among families of affected children and support their hope for recovery. For instance, it would be essential not to propose cure for NB as a "war", but as a cooperation to build health and not to destroy disease.

Ref ID: 101.1 **#60**

Cerebellar neuroblastoma in an infant

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Brain tumours are the most common solid neoplasms in the pediatric population. Among them, cerebellar neuroblastoma is extremely rare. A case of cerebellar neoplasm in a 4 months-old girl is presented. She had a history of strabismus for one week. Physical and neurological examinations revealed macro crania, setting sun eyes, and bilateral papilloedema. The preoperative routine complete blood count, clotting profiles, electrolytes, neuron specific enolase titration and the urinalysis were all within the normal limits. Skull x-ray showed cranial enlargement and spreading of the sutures. The CT scan revealed a marked enlargement of the lateral and fourth ventricles and a 6x4 cm mass in the posterior fossa. The MRI confirmed a circumscribed cerebellar midline tumour. She underwent gross total resection and the diagnosis of the neuroblastoma was made, on the basis of the presence of numerous synaptic vesicles in the great majority of cell processes and occasional complete synapses within the tumour tissue. N-myc amplification was negative. She did not have any further treatment. At the postoperative sixth month, the recurrence tumour was discovered on the routine radiological examination. Our patient was treated by total removal of the tumour followed by chemotherapy consisted of cisplatin, cyclophosphamide and VP-16. At the end of nine month, she is disease free, however the optimal treatment for patients with this rare tumours remain uncertain.

Ref ID: 361.2 **#61**

Changing the Course of Central Nervous System (CNS) Neuroblastoma (NB)? Not with Myeloablative Chemotherapy and Peripheral Blood Stem Cell support (PBSCs)

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BACKGROUND: We have previously reported the incidence and characteristics of patients with CNS NB treated on protocols N4, N5, N6 and N7 from 1980 1999 at MSKCC, during which time there was an increase in overall survival. The overall incidence was 4.4% (11/251 patients), with 70% representing isolated CNS recurrences.
METHODS: Attempts to eliminate the CNS as a sanctuary site incorporated high-dose myeloablative thiotepa-based regimen with autologous PBSCs and maintenance oral etoposide therapy on N8, 2000-2003.
RESULTS: There were 2 CNS relapses (5.6%) of 36 newly-diagnosed N8 patients, and 3 of 52 (5.8%) patients on protocol 94-18 treated with immunotherapy after thiotepa-based regimens. No single biological feature predicted for the development of CNS disease. All 5 of these new CNS events represented isolated CNS relapses with no evidence of systemic disease and consisted of parenchymal CNS disease only (n=3), and both parenchymal and diffuse leptomeningeal disease (n=2). The median time to diagnosis of the CNS relapse was 12-20 months from initial NB diagnosis, similar to the previously reported median time on N4-N7 protocols (12.5 months). Despite aggressive surgery and radiation therapy, the time to death was 3-10 months (n=3). Two patients are alive with disease and continue to receive systemic therapy, now 4 and 8 months since CNS disease detection.
CONCLUSION: Thus far, it does not appear that high dose thiotepa-based myeloablative therapy with PBSCs for metastatic NB protects the CNS as a sanctuary site, or has altered the incidence and characteristics of CNS relapses. Improved site-directed therapies are needed to address this complication.

Ref ID: 361.1 **#58**

Favorable Biology Neuroblastoma (NB): Leptomeningeal metastases remaining stable in a child without chemoradiotherapy

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BACKGROUND: Intrinsic tumor biological features are critical prognosticators of survival of NB. Patients with localized neuroblastoma and favorable biological parameters may be observed without treatment. Conversely, leptomeningeal metastases in patients with primary extracranial neuroblastoma is highly unusual and despite aggressive multi-modality therapies, invariably fatal. Methods: We present the case of a newly-diagnosed 7 month-old infant who presented with acute paraplegia from neuroblastoma at T12 through L4 causing complete obliteration of the spinal canal; radiographic imaging was consistent with leptomeningeal metastases.
RESULTS: The patient underwent partial surgical resection of the primary adrenal tumor and emergent spinal cord decompression. The primary tumor revealed favorable biology (favorable Shimada classification. single copy MYCN , low serum lactate dehydrogenase, and favorable VMA/HVA ratio). Metastatic work-up including extensive bone marrow examinations and bone scan were unremarkable with the exception of magnetic resonance imaging with abnormal nodular foci of intradural enhancement coating the conus medullaris and cauda equina suspicious for subarachnoid disease. An 18-fluoro-deoxyglucose (FDG) positron emission tomography scan was compatible with NB in the abdomen; longitudinal linear tracer uptake was also noted in the lower thoracic and lumbar region. The patient was observed with no cytotoxic therapy and remained well with no evidence of disease progression nearly 3 years since diagnosis. Slow recovery of motor function in the lower extremities was noted over an 18 month period.
CONCLUSION: Some infants with favorable biology neuroblastoma may be observed without treatment despite the advanced INSS stage.

Ref ID: 050.2 **#59**

Central nervous system (CNS) involvement in children with neuroblastoma (NBL) at the diagnosis and in relapse

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Neuroblastoma presents as metastatic disease in about 60% of newly diagnosed children. The usual sites of metastases are the lymph nodes, bone marrow, liver, bones; rarely lungs or CNS. The CNS tumor as the primary site of NBL has also been described. Intensification of combined treatment in children with disseminated disease leads to increase of remission rate, but also increases incidence of CNS relapse in longer surviving patients. Between January 1991 and March 2003, 75 newly diagnosed children were treated for NBL including forty stage 4 patients over than 1 year. Every patient received intensive multimodal treatment. Initial CNS involvement was found in 3 children. Two of them received intrathecal therapy additionally to normal protocol. Among 40 children, 16 remain alive, 10 in first remission including 2 with initial CNS involvement (55 and 30 months). Relapses occurred in 11 patients; including 4 with isolated CSN relapse. All these 4 children were operated on and irradiated; in 3 cases intensive chemotherapy was employed. Three children died after 18, 36 and 9 month after the diagnosis of CSN relapse. The fourth child remains in second remission, lasting 63 months. It was observed that in children with initial CNS involvement the intracranial mass was found to have continuity with the metastases localized in the skull bones. In patient with CNS relapses, all intracranial tumors were isolated.
Poor prognosis of patients with CNS relapse, no matter the satisfactory treatment use so far, should prompt us to design new therapeutic methods to prevent them.

Ref ID: 139.1 **#62**

Effectiveness of mass-screening program on neuroblastoma mortality in 1995-2000 birth cohort of Japan: Nationwide Neuroblastoma Mortality Study

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BACKGROUND: In Japan, a nationwide mass-screening program using urinary catecholamine metabolites tests at the age of 6 months has been performed since 1985. In 2003, the Japan Ministry of Health, Labour and Welfare decided to suspend the program, because of the lack of sufficient evidence on its effectiveness. We conducted a nationwide epidemiological study to evaluate the effectiveness of the program on neuroblastoma mortality.
METHODS: The study cohort was all the children who were born in Japan through 1995 to 2000. The screened children (participants of the program) and non-screened children (non-participants) were defined by the program participant lists kept by local governments. Neuroblastoma deaths were identified by death certificates that were retrieved from the national vital statistics of 1995 to 2001.
RESULTS: The study cohort consisted of 7.16 million children, of which 6.26 million children (87.4%) were in the screened group and 0.90 million children (12.6%) in the non-screened group. We identified 107 neuroblastoma deaths in the study cohort. Of the 107 deaths, 18 cases died before the age of 6 months and 11 cases were diagnosed by 6 months old (these cases were all non-screened). Therefore, 78 neuroblastoma deaths, 50 deaths in the screened group and 28 deaths in the non-screened group, were included for statistical analysis. The mortality rates were 3.21 (95%CI: 2.32-4.10) per million person-years in the screened group and 11.92 (95%CI: 7.51-16.34) per million person-years in the non-screened group. The rate ratio was 0.269 (95%CI: 0.170-0.428) and the difference of hazard between two groups was statistically significant.
CONCLUSIONS: The finding suggests the effectiveness of the screening program in terms of mortality from neuroblastoma.

Ref ID: 397.1 **#63**

Neuroblastoma screening for older children

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Background and Aims: Nation-wide 6-months neuroblastoma screening in Japan was decided to be discontinued because of lack of merit on mortality and over diagnosis. However, the reduction of mortality was not completely excluded, and, over diagnosis would be minimized if older children were screened. We analyzed clinically diagnosed and screen-detected tumors to clarify whether screening at older age would be useful. Methodology.
Clinical and biological analysis was performed on 249 of 264 cases with neuroblastoma who visited 3 hospitals between January 1, 1968 and March 31, 2002. The tumors were classified into 3 types according to DNA content and N Myc amplification (ANM); 3N without ANM as type-1, 2N without ANM as type-2, and, 2N with ANM as type-3.
Results: Fourteen cases aged younger than 6 months were clinically diagnosed: 170 were screen-detected: 42 were clinically diagnosed after negative screening: 23 were clinically diagnosed without previous screening. Of 148 type-1 cases 129(87%) were screen-detected, but all of 25 type-3 cases were clinically diagnosed. Among type-2 cases 41/66(62%) were screen-detected ones with earlier stages and better outcome than clinical ones. Type-2 clinical cases were distributed in older ages than type-3 cases.
Conclusions: Detection of children with type-2 tumors through a screening in earlier ages and stages than clinical diagnosis will be possible and result decrease in neuroblastoma mortality. Children 18 months old could be targets, when, according to our experience in wait-and-see strategy, urinary cathecholamine metabolites in screen-detected favorable cases decreased into normal level.

Poster Display
CLINICAL

Ref ID: 015.1 **#64**

Metastatic/multifocal neuroblastoma in the first year of life

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BACKGROUND AND AIM. Widespread neuroblastoma include stage 4 and stage 4s, which differ profoundly since the former requires chemotherapy for achieving cure, the latter undergoes spontaneous regression in most cases. However the distinction between stage 4 and 4s is not always clear.
PATIENTS. Children aged 0-11 months with stage 4-4s neuroblastoma diagnosed in 25 Italian institutions between 3.1991 and 11.1999 were eligible. All were evaluated at diagnosis with mIBG scintigraphy and bone marrow aspirates. Stage 4 pts received chemotherapy according to the current protocols. In absence of lifethreatening symptoms stage 4s pts received only supportive therapy.
RESULTS. Of 100 eligible pts, 68 had stage 4s (64 were £ 6mos and 27 were £ 2mos), 32 stage 4 (3 were £ 6mos). Stage 4s . Of 68 cases 20 received chemotherapy because symptomatic and 6 of them died. Other two died before treatment could be started. Of 43 not treated at onset, 10 developed PD at 4-36mos, 6 of whom died. 3/4 pts with MYCN amplification died. 53 pts survive at 18-124mos (median 65). 5-y OS is 77%, EFS is 64%. Stage 4. Of 32 cases 8 developed PD or relapse of whom one survives. 5/7 who died had MYCN amplification. One additional patient died of toxicity (brain haemorrhage). 23 pts survive from 42-118mos (median 83) and one was LFU with OS 72% and EFS 69%.
CONCLUSION. We confirm that (a) stage 4s prevails respect to stage 4; (b) stage 4s is mostly confined to the first 6 mos of life, while stage 4 is almost exclusive of the second semester; (b) as previously reported, OS and EFS are similar in both stages, (c) in both stages MYCN amplification is associated with worse prognosis. We still lack biological determinants to clearly differentiate stage 4 and 4s in infants.

Ref ID: 033.1 **#65**

Skeletal involvement in infants with neuroblastoma. A quality control attempt

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BACKGROUND: The European Infant Neuroblastoma Study (INES) has introduced a new definition of bone metastases, as sites of abnormal mIBG uptake confirmed by Xray (CT scan for skull). This change has important therapeutic implications, since it lowers the number of infants eligible for chemotherapy. However, this type of evaluation requires high quality scans and standardised procedures. We therefore retrospectively reviewed mIBG scans and related imaging of Italian cases.
METHODS: Italian infants enrolled in INES Trials 99.2, 99.3 and 99.4 between 1.2000 - 9.2002 were the object of this analysis. Thus, a team of two paediatric oncologists, one Nuclear Medicine expert and one radiologist was established. An arbitrary score (1, very poor; 2, poor; 3, intermediate; 4, good; 5, excellent) based on evaluation of (a) technical aspects of _camera; (b) type of radiopharmaceuticals, and (c) study exhaustiveness, was attributed to mIBG scans.
RESULTS: Data regarding 28 infants from 17 Centres (14 females, 14 males; median age 140 days, range 12-361) were reviewed (18- Trial 99.2, 5- Trial 99.3, 5- Trial 99.4). 12 Centres obtained scores 3 to 5, one obtained score 2, 2 got score 1. Xrays confirmed the presence of lesions in 2/8 cases with abnormal mIBG bone uptake. Of 8 patients with mIBG spots 6 had abnormal skull CTs. In 4 cases suspicious lesions not mentioned in the original reports were found. In 2 of them further investigation could have led to upstaging and consequent chemotherapy treatment. In 8 cases minor discrepancies between local reports and reviewers' comments were detected.
CONCLUSIONS: Centralised imaging review in real time and use of standardised procedures to evaluate mIBG scintigraphies could improve staging and treatment of infants with disseminated disease. Additional imaging studies (99mTc scan, SPECT) could be useful to better define particular cases.

Ref ID: 220.1 **#68**

Neuroblastoma (nbl) in adolescents and adults: analysis of a series of 33 consecutive patients

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BACKGROUND AND AIMS: NBL in adolescents is rare, and in adults is exceedingly rare. We describe the experience of our Institution.
METHODOLOGY: In the study period 1980-2002, 33 consecutive patients older than 12 with NBL at onset were admitted. Median age was 17 yrs (range 12-69). Symptoms were disregarded before diagnosis for many months (median:15). LDH level was pathologic in 15/33. Patients with stage I (n=3) were treated only with surgery, while those with stage II (n=5) underwent surgery + radiotherapy; all patients with stage III (n=9) and stage IV (n=16) received polichemotherapy. In addition, in the period 1989-1995, 10 patients with stage IV received sequential hemi-body irradiation as consolidation treatment. The local treatment for stage III-IV, was decided on individual basis.
RESULTS: The median follow-up is 43 months (range 12-264). Stage I patients are all alive and disease-free. 5-yrs EFS and OS probabilities for the other patients are: Stage II Stage III Stage IV EFS 0.67 0.40 0 OS 0.83 0.56 0.12 20 patients experienced a progression or relapse: 2 stage II, 4 stage III, 14 stage IV; 18/20 died of disease. Time to progression/relapse ranged from 3 to 58 months. In univariate analysis, the two statistically significant prognostic factors are stage and LDH level.
CONCLUSIONS: Adolescents and adults with NBL fare less well than children. In pts with locally advanced and metastatic disease the prognosis is dismal, even if the high occurrence of late relapses suggests a lower biological aggressiveness of the disease in this subset.

Ref ID: 033.2 **#69**

Ganglioneuroma in childhood: the Italian experience with 127 cases

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BACKGROUND: Ganglioneuroma represents the benign component of the Neuroblastic Tumour Family. Few series have been reported so far making its natural history poorly known.
PATIENTS: 127 cases of ganglioneuroma were enrolled into the ICGNB Registry between 1.1979 and 12.2002 (51 males, 76 females. M/F 0.67). Median age was 83 months (range 2-176). The primary tumour (in 123 cases) was abdominal in 53 patients, thoracic in 48, pelvic in 14, cervical in 8. An obvious mass was the first sign in 13 cases. Symptoms included pain (29 patients), respiratory symptoms (13), fever (11), scoliosis (5), haematuria and dysuria (7), gastro-intestinal (7), neurological (4). 45 patients were asymptomatic. Of 121 patients evaluated for surgery, 77 (64%) underwent radical tumour excision and 29 (24%) partial resection. In 15 cases (12%) a biopsy only was performed, followed by complete or partial tumour resection in 9 patients and follow-up in 6. One patient died of subarachnoideal haemorrhage soon after surgery. Nephrectomy was performed in 2 cases. Other severe non fatal complications included cerebellar haemorrhage, internal iliac artery rupture, aortic rupture (one case each) and 10 cases of Bernard-Horner syndrome (overall complication rate 13%). Median follow-up (96 patients) was 47 months (range 1-216). 76 patients (79%) are alive without disease and 19 (20%) are alive with stable tumour residue. 7 patients who developed local disease progression or relapse are all alive after partial or radical surgery. One patient developed an ovarian dysgerminoma and another a thyroid carcinoma at 9 and 17 years after diagnosis of ganglioneuroma.
CONCLUSIONS: Prognosis of children with ganglioneuroma is excellent. Early and late complications are uncommon. The only death encountered was surgery-related. Since survival is not influenced by the degree of tumour resection, an aggressive surgical approach can be avoided.

Ref ID: 274.1 **#66**

Treatment and prognostic factors of neuroblastoma in under 1-year-old infants in Japan

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OBJECTIVE: Since 1994, the Japanese Infantile Neuroblastoma Cooperative Group Study has been attempting to determine the standard optimal therapy and to analyze the prognostic factors in under 1-year-old neuroblastoma (NB) patients. Patients and Methods: The first clinical study for under 1-year-old NB patients (Protocol 9405) was started in 1994, and a revised study (Protocol 9805) was started in 1998. Of a total of 622 registered patients, 601 patients were eligible. Treatment modality was stratified on the basis of MYCN amplification (>10 copies) and the clinical stage. In Protocol 9405, after tumor resections, patients in stage 2 without MYCN amplification were randomized into two groups, one receiving surgery alone and the other receiving surgery followed by minimal chemotherapy. In Protocol 9805, after tumor resections, patients in stage 3 without MYCN amplification were randomized into two groups, one receiving no chemotherapy and the other receiving minimal chemotherapy. Results: In Protocol 9405, the clinical outcomes of stage 2 patients with and without chemotherapy were not significantly different. In Protocol 9805, the clinical outcomes in stage 3 patients were not influenced by postoperative chemotherapy. MYCN amplification, Shimada\'s UFH, low expression of TRK-A and 1p-deletion were significantly correlated with poor clinical outcomes. Conclusion: In patients with localized NB (stages 1~3) without MYCN amplification, chemotherapy after surgical excision is not required. In under 1-year-old patients with NB, MYCN amplification, Shimada's UFH, low expression of TRK-A and 1p-deletion were significantly correlated with the clinical prognosis.

Ref ID: 277.1 **#67**

Localized neuroblastoma with MYCN amplification in infants. A report of 3 cases

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Infant patients with localized neuroblastoma have good prognosis, but that with MYCN amplification is rare. We studied 3 cases who were less than 12 months of age and had localized disease with MYCN amplification. Case 1: A 9-month-old girl. When she was hospitalized for pyelonephritis, a mass was detected in left adrenal gland by an abdominal ultrasonography and was completely resection. Diagnosis: Neuroblastoma stage 1, NSE 62.3 ng/ml, HVA 21.5 μg/mg·cre, VMA 11.3 μg/mg·cre, MYCN 15 copies, DNA index triploidy, unfavorable histology. Case 2: A 1-month-old boy. Left adrenal grand mass was detecced by abdominal ultrasonography screening in 1-month-old infants. He was hospitalized and underwent total resection. At diagnosis: Neuroblastoma stage 1, MYCN 10 copies, diploidy, favorable histology. At 3 months of age, he was rehospitalized because of multiple subcutaneous, mediastinal lymph nodes, and bone marrow relapse. On relapse: NSE 57.6 ng/ml, HVA 19.3 μg/mg·cre, VMA 8.5 μg/mg·cre, MYCN 20 copies, Unfavorable histology. Case 3: An 11-month-old boy. He was hospitalized for fever and abdominal mass, and was detected by the mass screening at the period. Diagnosis: Neuroblastoma stage 3, NSE 759 ng/ml, HVA 77.5 μg/mg·cre, VMA 20.0 μg/mg·cre, MYCN 10 copies, triploidy, Unfavorable histology. At 23 months of age, he was rehospitalized because of a renal metastatic relapse. Conclusion: Localized neuroblastoma with MYCN amplification may be found in infants. We suggest, that even in infants, intense therapy at the early stages shoud be administered in cases with MYCN amplification considering the outocome of cases 2 and 3.

Ref ID: 073.1 **#70**

Neuroblastoma in the adult. A Report on 24 Cases

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BACKGROUND: Neuroblastoma in the adults accounts for < 1% of reported cases, but its incidence is probably underestimated. Clinical characteristics, biologic pattern, chemosensitivity, clinical course and outcome of such cases differ from children, but the literature at this regard is still sporadic. No specific protocols exist for such patients. Finally, there are uncertainties on whom should treat them.
METHODS: This series regards 24 adults with neuroblastoma diagnosed between 1975-2003, of whom 10 (37%) were diagnosed in the first 23 years, and 14 (63%) in the last 6, possibly as possible effect of increased referral from medical to paediatric oncologists.
RESULTS: Patients' age ranged from 18 to 32 years (median, 23). M/F ratio was 0.5. The primary tumour was abdominal in 13 pts (4 adrenal), thoracic (6), and pelvic (3). 4/16 had abnormal VMA/HVA urinary levels. 1/12 pts had MYCN amplification and 3/9 had 1p36 deletion. Histology was neuroblastoma in 15 pts, ganglioneuroblastoma in 8, and ganglioneuroma in one. There were 10 stage 1-2 pts (4 relapsed with one death, 5 are A&W, one was LFU), 4 of stage 3 (one died, 2 relapsed and are AWD, one was LFU), and 9 of stage 4 (4 DOD, 5 AWD). 5-y OS is 87% for stage 1-2, 60% for stage 3-4. 5-year PFS is 25% for stage 1-2, 0% for stage 3-4.
CONCLUSIONS: An increasing number of adults with neuroblastoma are coming to the attention of paediatric oncologists. Our data indicate that (a) these tumours have a greater propensity to relapse, even at early stages, (b) a long interval may occur between progression/relapse and death, accounting for the discrepancy between high OS and low PFS. A better interaction between medical and paediatric oncologists should be developed to design specific therapeutic and follow-up protocols for these pts.

Ref ID: 065.1 **#71**

Opsomyoclonus-ataxia syndrome occurred in regressing neuroblastoma detected through mass screening

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Opsomyoclonus-ataxia syndrome (OMA) has been observed in 2-4% of patients with neuroblastoma and known to associate with favorable prognosis. Diffuse and extensive lymphocyte infiltration with lymphoid follicles has been reported to be a characteristic histologic feature, suggesting that immune-mediated mechanisms may have a role in pathogenesis of OMA as well as favorable prognosis. Wait-and-see policy with careful observation without any treatment has been applied to mass-screened patients with a localized small neuroblastoma in Japan and spontaneous regression was frequently observed in these cases. In this report, we describe OMA occurred in regressing neuroblastoma detected through mass screening.
Case: An 8-month-old girl with the elevation of urinary levels of VMA and HVA was introduced to our hospital. The examinations revealed a localized retroperitoneal tumor without metastasis. The patient was entered into our wait-and-see pilot study with careful observation. During the initial 7 months, her tumor decreased in size from 5 cm to 3.5 cm diameter and urinary levels of VMA and HVA normalized. However, typical symptoms of OMA such as chaotic eye movements and stumbling gaits occurred. Concerning a high risk of neurologic sequelae of the patients with OMA, a laparoscopic total tumor excision was performed without delay. Microscopically, the tumor contained lymphoid follicles and was diagnosed as neuroblastoma with Shimada favourable histology. Single copy of the MYCN gene and high expression of TrkA were also observed. The neurologic symptom disappeared soon after the operation. The patient is alive without any recurrence or neurologic symptoms three years postoperatively.
The present case showed that OMA might occur suddenly during spontaneous regression, suggesting co-relation between spontaneous regression and OMA. It may support the hypothesis that the host immune response may induce spontaneous regression, but also mediate central nervous system impairment.

Poster Display
CLINICAL

Ref ID: 239.2 **#72**

Opsoclonus-myoclonus-ataxia with neuroblastoma. Follow-up of 7 cases.

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Opsoclonus-myoclonus-ataxia (OMA), also called "dancing eye, dancing feet syndrome", is a rare neurologic condition that is often a paraneoplastic manifestation of occult neuroblastoma in early childhood (2-3% of children with neuroblastoma). Among children with neuroblastoma, the occurence of OMA relates to a relatively better prognosis, but late neuropsychological impairment is often observed. In fact, despite total resection of the tumour, with or without immunosuppressive therapy, neurological outcome usually includes varying degrees of cognitive and behavioural sequelae.
We report 7 patients with OMA and neuroblastoma with a mean age at onset of 17,3 months (range 8-29 months) that we followed-up for an average of 7 years (range 4-14).
Two patients presented thoracic localisation, two had pelvic localisation and three of them had abdominal tumors. Six out of seven patients underwent radical surgery. Among the six resected patients only one also had chemotherapy, immediately following surgery, and he alone had no neurological sequelae. The patient with unoperable tumor underwent chemotherapy alone.
During follow-up 6 patients presented severe OMA which was treated with combination steroid therapy every day and intravenous immunoglobulins (IVIG) every 3 weeks. Significant improvement or remission occurred within the first 6 months of treatment in all patients but one. Attempts to decrease or eliminate therapy led to relapse in all patients. Within 1 or 2 years four patients discontinued IVIG therapy, while to date all patients are still undergoing steroid therapy. Various anti-myoclonic drugs proved to be rather ineffective.
Despite the good results obtained with treatment on OMA, 6 patients still show sequelae, including 4 with mental retardation and 2 with speech delay. It must be pointed out that the report mainly concerns follow-up of the most severe patients who were referred from all over Italy. We must emphasize how difficult it is to establish the most effective protocol.

Ref ID: 033.3 **#74**

Spinal cord compression (SCC) in neuroblastoma: the Italian experience from 1999-2003

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BACKGROUND: Incidence, clinical course, optimal treatment and late effects of SCC in children with neuroblastoma have not yet been clarified.
PATIENTS: Children with neuroblastoma presenting with SCC diagnosed in Italy between January 1999 - December 2003.
RESULTS: The 24 children of this series represent 4.5% of the neuroblastoma newly diagnosed in the above period. M/F ratio was 1 to 1,4. Median age was 15 months. 6 patients had stage 2, 12 stage 3, and 6 stage 4 disease. MYCN amplification, 1p36 deletion, 1p36 imbalance were demonstrated in 3 patients each, MYCN gain in one. Median time from first symptoms to hospital admission was 14 days (range 2 days- 12 months). SCC involved the cervico-thoracic spine in 3 cases, thoracic in 7, thoraco-lumbar in 6, lumbar, lumbo-sacral and sacral in 5, 2 and 1 patient each. All children had motor impairment: moderate (grade 1) in 5 cases, severe (grade 2) in 14, paraplegia (grade 3) in 5. Sensory abnormalities, sphincter dysfunctions and pain affected 5, 10 and 10 patients, respectively.
THERAPY: 15 patients underwent chemotherapy and 9 laminotomy. The table summarizes the responses to treatment, according to severity of motor dysfunction. One patient with score 2 deficit who had no response after laminotomy, improved with chemotherapy. Two other cases worsened after chemotherapy, and later improved following surgical decompression.

Therapy	Score of motor impairment							
	1		2		3			
Laminotomy	2	RC - RP 2 NR -	4	RC 2 RP 1 NR 1	3	RC - RP 1 NR 2		
Chemotherapy	3	RC 3 RP - NR -	10	RC 3 RP 4 NR 3	2	RC - RP 2 NR -		

CONCLUSIONS: Chemotherapy was the primary treatment in 62% of patients and proved adequate, particularly in case of moderate motor dysfunction. None of patients with severe motor dysfunction achieved full neurological recovery.

Ref ID: 036.1 **#73**

Rituximab (anti-CD20) in the Treatment of Refractory Neuroblastoma Associated Opsoclonus-Myoclonus

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BACKGROUND AND AIMS: Opsoclonus-myoclonus syndrome(OMS) occurs in 2 to 3% of children who are diagnosed with neuroblastoma. Despite excellent cancer-free survival, many are left with devastating neurologic sequelae. Scientific evidence favors an immune basis for OMS. Such evidence includes occurrence in the post-infectious state, response to immune directed therapies such as steroid and IVIG, and the presence of aggressive lymphocytic infiltration of CD20 positive B lymphocytes within the tumors of patients who experience this paraneoplastic syndrome. The current abstract describes utilization of anti-CD20 antibody as a novel therapy in the treatment of a 3 year old female with refractory OMS.
METHODS: The patient had a detailed pre-treatment evaluation including video documentation of the opsoclonus, myoclonus and ataxia utilizing a previously validated scoring system. An evaluation of sleep and mood disturbance was also performed. The patient had extensive, age appropriate neurocognitive testing. She then received 4 weekly infusions of rituximab at a dose of 375 mg/m2. Re-evaluation occurred at end of treatment and at 6 months from study entry. Additional evaluations will occur at 6 month intervals for 2 years.
RESULTS: The patient demonstrated marked improvement of the mean OMS severity scores as determined by two independent scorers utilizing the validated scale. Mean score +/- SEM: Baseline = 25.5 +/- 0.5, Week 6 = 17.5 +/- 0.5, Month 6 = 16.5 +/- 1.5. Clinical improvements are durable to 6 months and include near resolution of opsoclonus, improved neuromotor skills, greater ambulation with assistance and improvement of sleep pattern and mood. Neurocognitive evaluation is ongoing.
CONCLUSION: Rituximab anti-CD20 antibody represents a novel treatment strategy for children with refractory neuroblastoma associated OMS. This agent requires further investigation due to the potentially devastating nature of this disease with current treatment strategies.

Ref ID: 050.1 **#75**

Spinal Cord Compression in Neuroblastoma- Experience of Polish Pediatric Solid Tumors Study Group (PPSTSG)

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Neuroblastoma (NBL) usually present with a tumor mass along the sympathetic neural pathway. Children with primary lesions in the paravertebral area may exhibit spinal cord compression. Spinal cord decompression is a neurological emergency. From 1997-2003 in 10 oncology centers associated in PPSTSG, 242 children were treated because of NBL, including 28 with dumbbell tumor. In 21 children who had symptoms of the SCC, the most common was paresis of legs together with the bladder and anal sphincter dysfunction. About 30% of children required the treatment before the histopathological confirmation of diagnosis was performed because of the serious neurological symptoms. Six children required laminectomy (three of them at diagnosis); no complications of laminectomy were observed. All the children in the analyzed group required chemotherapy with or without radiotherapy. Observation were completed on December 31, 2003. Among 28 patients with dumbbell tumor, 7 died including 6 because of NBL progression. Twenty one children have remained alive for 6-80 months. At the time of the last follow-up some symptoms were still present in almost all children who had the symptoms of SCC at onset of disease. The most important aim is to establish the optimal method of treatment for dumbbell neuroblastoma to balance between the possibly highest cure rates and possibly lowest risk of late complications.

Ref ID: 257.1 **#76**

Infantile Dumbbell-type Neuroblastoma

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BACKGROUND AND OBJECTIVES: Dumbbell-type neuroblastoma, with or without neurologic symptom, is not a rare disease of infants. In the Japanese prospective study #9405 and #9805, between 1994 and 1998, we treated this neuroblastoma without intensive local treatment except for non-progressive neurological symptom.
PATIENTS AND METHODS: The thirty-three patients, presenting dumbbell-type, of 657 in both protocols. Surgical resections were preceded by the localized tumor cases in which the total excision were possible(n=15), and the patient of stage 3 received postoperative chemotherapy (n=10), the others did not received. The patients of stage 4 or unresectable tumor received preoperative chemotherapy (n=18). After surgery, some received postoperative chemotherapy.
RESULTS: Twenty-two cases of 33 were detected by the mass screening, and 11 cases clinically. Nine have neulological symptoms in the initial diagnosis. They were assigned to; stage 1, 3 cases (9%); stage 2A, 10 (30%); stage 2B, 2 (6%); stage 3, 12 (36%); stage 4, 6 (18%). There were no cases with MYCN amplification over 10 times. No cases underwent neurosargical laminectomy. The five recurred, 32 have survived except one death with malformation. Event free survival rate of only surgical resection cases is 89%, of postoperative chemotherapy 80%, and of preoperative chemotherapy 80%. Tumor has remained in 12 cases, but only one suggested tumor activity. Although the neurological symptoms remained in seven cases, the advance could not be seen.
CONCLUSION: The prognosis of dumbbell-type neuroblastoma was good, and local control was probable under conservative therapy. The treatment plan whithout laminectomy is also considered appropriate.

Ref ID: 306.2 **#77**

Neuroblastoma of paravertebral ganglions penetrating spinal canal - cases report

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Background. Epidural or intradural extension occurs in 5-16% of cases of neuroblastoma and can cause symptoms of spinal cord compression: pain, bladder and bowel dysfunction, paraparesis or paraplegia. 2/27 pts were treated due to neuroblastoma of spinal cord in our department from 1993 to 2003. Case I: The 1-month female infant was admitted because of decreased mobility of lower limbs, which was observed for a period of one week. MRI showed a tumour of lumbar region Th8-L1 (size 62x48x35mm). The biopsy was performed and neuroblastoma was diagnosed. There were not found cells with NMYC amplification with FISH method. The treatment included: chemotherapy (VCR+CTX/ VCR+CTX+ADR) with very good response and surgery surgery. The complete remission was achieved. Case II. The 6-months male infant was admitted because of flaccid paralysis of lower limbs and Horner syndrom, which lasted for 3 months. MRI showed a tumour of cervical and thoracic region C7-Th7 (size 66x54x40mm) and a tumour of posterior mediastinum. The subtotal surgical removal of the mediastinum tumour was performed and ganglioneuroblastoma was revealed. There were not found cells with NMYC amplification with FISH method. The treatment included: chemotherapy (VCR+CCDP/VCR+VP-16+CARBO), but the therapy was interrupted due to respiratory failure. The patient remains in partial remission for 20 months, but he has bladder dysfunction and paralysis of lower limbs and presents mental retardation.

Ref ID: 385.1 **#79**

Front-line topotecan and high-dose cyclophosphamide followed by ICE in high-risk neuroblastoma

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BACKGROUND: Treatment of advanced neuroblastoma remains unsatisfactory, but new agents have recently shown promising activity. A pilot study with front-line topotecan was started in April 2001.
METHODOLOGY: Two courses of topotecan 6 mg/m2 with cyclophosphamide 4.2 g/m2 were followed by two courses of ICE (ifosfamide 9 g/m2, carboplatin 800 mg/m2 and etoposide 500 mg/m2), PBSC harvest (positive selection), surgery, one course of CAV (cyclophosphamide 3 g/m2, doxorubicin 75 mg/m2 and vincristine 1.5 mg/m2), high-dose therapy with ETC (etoposide 600 mg/m2, thiotepa 750 mg/m2 and cyclophosphamide 3.6 g/m2) and PBSC rescue, hyperfractionated local radiotherapy (21 Gy over 7 days) and CRA (160 mg/m2 for 12 months). 14 consecutive pts with high-risk neuroblastoma over one year of age have been accrued, male/female 6/8, median age 32.5 mos (range 13-66). 1 stage 2 (MYCNA, 1pdel), 1 stage 3 (MYCNA, 1pdel), 12 stage 4 (2 MYCNA, one 1 pdel, one pending).
RESULTS: Median interval between TOPO-CYCLO courses was 29 days (range 23-34), toxicity was predictable and mainly hematological, 9 sepsis and 1 SIADH were recorded, observed responses [11 pts] were 1 CR, 5 PR, 5 MR (RR 55%). Median interval between ICE courses was 31 days (range 23-39), observed responses at end of induction [10 pts] were 1 CR, 6 PR, 3 MR (RR 70%). 11 pts have performed successful PBSC harvests. After surgery [10 pts] we recorded 5 CR and 5 PR. Evaluation of the first 10 pts after induction and surgery revealed a 100% RR, no toxic deaths nor PD during induction, with the potential for adequate PBSC harvests. Median interval between PBSCT and RT was 45 days (range 31-62). After a median follow-up of 11 months (range 1-25), among the 8 pts who have completed RT, we recorded 1 DOD (stage 3 MYCNA, 1pdel) 12 months after diagnosis; 4 CR (stage 4 and 2 with MYCNA) at +25, +23, +20 and +9 months; 1 VGPR (stage 4) at +11 months; 2 RD (stage 4), 25 and 14 months after diagnosis.
CONCLUSIONS: The combination TOPO-CYCLO and ICE was active and well tolerated with promising results in this high-risk population. The simple schedule and the predictable toxicity easily allow for the incorporation of biological response modifiers and other innovative therapies.

Ref ID: 215.1 **#80**

Consolidation treatment with chimeric anti-GD2-antibody ch14.18 in children >1 year with metastatic neuroblastoma

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BACKGROUND: Antibody treatment is considered tolerable and potentially effective in the therapy of neuroblastoma. We have analysed stage 4 neuroblastoma patients >1 year who underwent consolidation treatment with the chimeric monoclonal anti-GD2-antibody ch14.18.
PATIENTS AND METHODS: Stage 4 patients >1 year who completed initial treatment without event were eligible. Ch14.18 was scheduled in a dose of 20 mg/m_xd over 5 days in 6 cycles every 2 months. Patients who did not receive ch14.18 served as controls.
RESULTS: Of 334 evaluable patients, 166 received ch14.18 (784 cycles), 99 received a 12 months low dose maintenance chemotherapy (MT) instead, and 69 had no further treatment. The main side effects were fever (50% of cycles), abnormal CRP without infection (32%), cough (21%), rash (19%), and pain (14%). Univariate analysis found similar event free survival (EFS) for patients treated with ch14.18 (3-year-EFS 46.5±4.0%), with MT (44.4±4.9%), and no further therapy (37.1±5.9%, p=0.3145). Overall survival (OS) was better after ch14.18 (3-year-OS 68.5±3.9%) compared to MT (56.6±5.0%) or no further therapy (46.8±6.2%, p=0.018). Separate univariate analysis of patients with autologous stem cell transplantation revealed no difference between patients with ch14.18 treatment and no further consolidation. Multivariate analysis failed to demonstrate an advantage of antibody treatment for EFS and OS.
CONCLUSION: Consolidation treatment of stage 4 neuroblastoma with ch14.18 was associated with considerable but manageable side effects. Compared to MT or no consolidation treatment, ch14.18 had no clear impact on the outcome of patients.

Poster Display

Ref ID: 143.1 **#81**

13 cis- retinoic acid in children with high-risk neuroblastoma: Is more better?

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13 cis-retinoic acid (RA) induces differentiation in neuroblastoma (NB) cell lines in-vitro. Administration of high-dose RA (two-week pulses for 6 months) improves disease free survival among patients with advanced stage NB who are in complete remission following standard and myeloablative chemotherapy. We report our experience with prolonged RA administration in 17 high risk patients with NB. Patients received an average of 11.3 RA courses (range 2 to 26) beginning at a median of 93 days after APBSCT.
In four patients, ganglion cells appeared in bone marrow biopsies obtained during RA treatment. Six year overall survival is 48% and event free survival is 44%. With a median follow up of 59 months, eight patients have relapsed. Five of these events occurred during RA administration. One of the patients in whom ganglion cells appeared in bone marrow biopsy while on RA treatment, relapsed within three months of stopping RA. An additional relapse occurred in a child who did not tolerate RA due to drug-induced hypercalcemia. Side effects of treatment included cheilitis and dry skin in all patients and one case of severe hypercalemia as indicated above. Our cohort of very high-risk patients is exhibiting an unexpectedly good progression free interval after receiving prolonged RA consolidation therapy. In-vivo demonstration of tumor cell differentiation during therapy suggests a role for RA as consolidation treatment for patients even in the presence of small amounts of residual NB. Administration of RA for periods of longer than 6 months should be explored as part of a multi-modal approach to the treatment of high-risk NB.

Ref ID: 228.1 **#82**

Treatment results of high-risk neuroblastoma children (1995-2002). A single centre experience

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During the period of eight years (1995-2002) thirty children (16 boys and 14 girl), med. age 2,7 yrs (0,3; 7,1) was admitted to our institution. Main symptoms were bone and joint pain, abdominal tumor mass and pathological fracture. Localization of primary tumor: adrenal gland - 18, extraadrenal retroperineum - 10, mediastinum - 2. Metastases were found in bones - 29x, bone marrow - 23x, lymphnodes - 14x, liver - 3x, testis - 1x. N-Myc oncogene was investigated in 23 pts. (76,6%) and in 13 pts. more then 10 copies were found, ploidy in 16 pts. and aneuploidy was detected in 6 pts., in 3 pts del 1p was found. Induction chemotherapy constisted of cisplatin (60 mg/m2, D1), adriamycin (30 mg/m2, D3), etoposide (100 mg/m2, D3,6), cyclophosphamide (900 mg/m2, D5,6). AHSCT was planned in 29 pts., but in 24 pts. was performed (4 children had disease progression durig induction treatment, 1 child had severe chemotherapy related toxicity). Conditioning: carboplatin + etoposid + alkeran (11x), carboplatin + etoposide + alkeran + whole body irradiation (7x), busulphan + melphalan + cyclophosphamide (6x). The med. day of enfrafment was D +14 (11;21). Twenty two (73,4%) children relapsed in med. 16,2 months (5,5 - 51,8) after the date of diagnoses, 24 pts. (66,6%) relapsed in med. 9,9 mths (0,1 - 42,9) after AHSCT. All children who relapsed died. The median follow up of surviving children is 44,6 mths, 8 children (26,6%) are alive with NED.

Ref ID: 285.1 **#87**

Comparison of two induction regimens for high-risk neuroblastoma

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INTRODUCTION: Induction chemotherapy is a pivotal part of high-risk neuroblastoma treatment. Different IC regimens have been used, but the ideal one remains to be found. Continuous infusion has been proposed as a method to circumvent multidrug resistance
METHODOLOGY: Patients over 1 year of age with high risk neuroblastoma received IC with seven courses of different pairs of drugs in continuous infusion (vincristine, cisplatin, teniposide, carboplatin and tenoposide) with cyclophosphamide in short infusion. Response to IC and grade 3-4 toxicity were compared to our previous study (same drugs and doses, standard infusion). Response was evaluated according to INRC criteria.
RESULTS:

N-II-92(92-98)	N-AR-99(99-02)		
# patients	83	44	
St 3 MNA/St 4	6/77	4/40	
Mean age(range)	3,8y(0,18-11,6y)	3,8y(1,13-14y)	
1-2 year	12	11	**N.S**
Sex	53 M, 30 F	24M, 20F	
LDH>2Sd	52/81 (64%)	21/42 (50%)	
Ferritin>43 ng/ml	57/80 (71%)	31/44(68%)	
Bone, BM met	55 (66%)	52 (67%)	
MNA	12/67(18%)	13/39(33%)	
Begin IC.	81 patients	44 patients	
Toxic deaths	3 (VM-26 anaphilaxia, 2 sepsis) (3,7%)	4 (2 sepsis, 1 MOF, 1 D.I.N) (9%) **p=0,2**	
Age at Toxic death(y)	1,52/1,83/2,50	1,56/1,68/2,28/2,82	
Progression under IC	2	4 **p=0,18**	
VCR-CFM #	160	82	
VCR-CFM-toxicity	H:130(81%),I:47(29%), G:11(7%)	H:65(79%),I:21(26%),G:8(10%)	
VM26-CDDP #	238	118	
VM26-CDDP toxicity	H:57(24%),I:8(3%),G:12(5%)	H:34(29%),I:0,G:7(8,5%)	
VP16-Carboplatin #	151	75	
VP16-carboplatin tox	H:108(71%),I:27(18%),G:3(2%)	H:57(76%),I:11(15%),G:2(3%)	
Response	CR 8, VGPR 14, PR 45, NoR 14	CR 6, VGPR 12, PR 13, NoR 4	

CONCLUSIONS:
1. Continuous infusion chemotherapy obtained more patients in CR/VGPR (p<0,01) than the standard scheme.
2. Toxicity in both studies was very similar , mainly grade 3-4 haematological and infections.
3. Young patients tolerate the continuous infusion scheme badly, with more lethal toxicity.

Ref ID: 307.1 **#89**

Combinational Treatment with Chemotherapyt and 131-I-MIBG for Advanced Neuroblastoma at Diagnosis

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In an attempt to improve long term results in advanced Neuroblastoma, it may be essential during induction treatment to prevent the emergence of resistant clones. NB is a radiosensitive tumor. We report here the feasibility of a new regimen based on specific irradiation, using a fixed dose of 131-I-MIBG and the use of the most effective drugs in this disease administered within a month from diagnosis. Seven patients (4 males and 3 females, age 1 to 4 years) with 6 stage 4 and 1 stage 3 MIBG positive Neuroblastoma were treated, 6 also had bone marrow and multiple cortical bone involvement. Treatment: Cisplatin 20 mg/sqm days 1 to 4, Etoposide 100 mg/sqm c.i. days 1 to 3, Vincristine 1,5 mg/sqm days 1 and 6, Cyclophosphamide 2 gr/sqm day 4; on day 10 iv. 131-I-MIBG 200 mCi was given. For further intensification the last 3 patients also received: Vincristine weekly at same dose, Cisplatin 40 mg/sqm in 48h c.i. days 20 and 21, and Adriamycin 30 mg/sqm plus Cyclophosphamide 1,5 gr/sqm days 28 and 29 from start of treatment, with subsequent administration of G-CSF. A complete hematologic recovery was observed after 2-3 weeks. No sepsis or hemorrages occurred. No cardiac, renal or auditory toxicity were observed. All patients, after further courses of chemotherapy, between day 77 and 113 from start of treatment, underwent stem cell collection with a CD34+ yield of 4,7 to 21,1 cells/kg. Response rate: 2 CR, 3 VGPR and 2 PR, at 6 week evaluation from beginning of treatment. In conclusion, the proposed schedule represents a very effective rapid radio-chemotherapy schedule during induction treatment, associated with acceptable hematologic toxicity.

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Ref ID: 381.1 **#88**

The use of liposomal busulphan in high-dose therapy of neuroblastoma

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BACKGROUND AND AIMS: High-dose therapy regimens with busulphan may improve prognosis in neuroblastoma and Ewing's sarcoma. Busulphan is usually administered orally every six hr over four days as a part of myeloablative regimens before bone marrow transplantation or stem cell rescue. However, busulphan may cause severe toxicity such as veno-occlusive disease (VOD) and interstitial pneumonia (IP). 20-40% of treated patients experience VOD, especially young heavily pretreated children. Another problem connected with oral administration of busulphan is the wide interpatient variability in pharmacokinetics.
METHODOLOGY & RESULTS: We used a new formula of busulphan encapsulated in liposomes (LB) suitable for i.v. administration. We thereby avoid the first passage metabolism of the liver and as our recent study has shown, achieve better systemic exposure and more predictable pharmacokinetics. In the present study we included 14 patients with neuroblastoma, and 3 patients with Ewing's sarcoma, for treatment with LB combined with Melphalan as high-dose therapy followed by stem cell rescue. None of the patients developed VOD or IP. The pharmacokinetic analysis showed no significant difference between the first and the last dose of liposomal busulphan when the drug was given as a 2 hr infusion twice a day. At follow up, median 14_ months from high dose treatment, 2 patients with neuroblastoma and one patient with Ewing sarcoma had died of which one by other reasons than malignant disease. We also treated nude rats bearing human neuroblastoma xenograft tumours with LB. There was a significant reduction of tumour growth compared to untreated tumours in control rats. No signs of toxicity, except for the expected myeloablative effect, were observed.
CONCLUSIONS: The pharmacokinetics of liposomal busulphan given i.v. are more predictable than after oral administration. Liposomal busulphan is safe to use and cause minimal toxicity.

Ref ID: 303.1 **#84**

Risk adapted treatment according to the Austrian neuroblastoma Trial A-NB94

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AIMS: Risk adapted treatment according to MycN amplification, age, stage and response for children with localized or metastatic neuroblastoma and evaluation of risk factors.
PATIENTS AND METHODS: From July 1994 to January 2002 149 patients (73 females, 76 males) were registered. Staging resulted in 52x stage 1, 15x stage 2, 26x stage 3, 46x stage 4 and 10x 4s. Median age at diagnosis was 1.2 yrs(range 4days-20yrs); 64(43%) were infants. 29 infants were detected through screening. Median observation time is 4.2 yrs. Treatment strategy for MycN non-amplified patients included surgery only for Stages 1 and 2 and 6 chemotherapy courses for stage 3 and 4 including surgery after 4 courses. Stage 4 patients >1a were consolidated with megatherapy and stemcell reinfusion (MGT/PSCR). Patients with MycN amplified tumors received increased treatment intensity including N7-like induction for stage 4 patients, all local irradiation and 1 to 3 MGT/PSCR.
RESULTS: OS and EFS rates of the study population at 4 years are 85%(±3) and 79%(±4), respectively. Infants had an EFS rate of 84% (±5) and children > 1year 76%(±6). Infants with stages 1-3 including 4s had an EFS of 84%(±5) and stage 4 an EFS of 75%(±13) while patients >1yr stage 1-3 have an EFS of 100% those with stage 4 an EFS of 48%(±9). Stage, age, MYCN amplification, 1p deletion, di/tetraploidy, NSE and LDH all are significant predictors for overall survival.
CONCLUSIONS: This study achieved excellent outcome for patients >1a with loco-regional disease and improved prognosis for stage 4 patients. It is noteworthy, that these results were achieved with a markedly reduced treatment intensity for MycN non-amplified patients. Eleven infants with MycN amplified tumours appear to have taken advantage from the intensified concept.

Ref ID: 237.1 **#86**

High-dose chemotherapy consisting of thiotepa and melphalan prior to local surgery for advanced neuroblastoma

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BACKGROUND: For advanced neuroblastoma, intensive and uninterrupted chemotherapy should be administered before tumor gains chemo-resistance. In our institute, induction and consolidation chemotherapies followed by high-dose thiotepa/melphalan (HD-TT/Mel) were administered prior to local surgery for residual tumors. Radiotherapy was not performed in any patient.
METHODS AND RESULTS: Nine children with a median age of 1.5 years (range, 7 months to 4 years), who were diagnosed with neuroblastoma of stage 4, stage 3 with amplified MYCN, or refractory disease, were treated with two to five courses of chemotherapy consisting of mainly vincristine, cisplatin, cyclophosphamide and pirarubicin. Subsequently, high-dose chemotherapy consisting of TT 800 mg/m2 and Mel 280 mg/m2 was given with autologous or allogeneic hematopoietic stem cell rescue. Delayed surgery for residual primary tumors was successfully performed at the last. Although some of their extracted tumors had viable cells enclosed with calcified or fibrous tissue, no further therapy was given. One patient died of progression disease during induction chemotherapy, thus eight patients were given consolidation HD-TT/Mel. One patient with refractory disease died of progression disease at 36 days after HD-TT/Mel. Seven patients are alive with no evidence of disease at a median follow-up time of 39 months (range, 12 to 136).
CONCLUSIONS: Intensive chemotherapy including HD-TT/Mel controlled primary lesions and metastasis and enabled to perform local surgery at the end of the treatment. A nationwide feasible study is in preparation.

Ref ID: 308.1 **#91**

A single institution study of low dose 1311mIBG therapy for stage 4 neuroblastoma – therapeutic implications

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BACKGROUND: Therapeutic dose and schedule of 131ImIBG is not established for treatment of neuroblastoma. We report here our experience with low dose 131ImIBG for treatment and palliation of patients with stage 4 neuroblastoma.
METHODOLOGY: 37 patients (14 males/23 females) with stage 4 neuroblastoma received a total of 116 1311mIBG treatments - median 3 (1 to10), median dose 2.1 mCi/kg (0.5 to 9.7) over a 19 year period (1985-2004). 1311mIBG was given about every 8 weeks for the first 3 treatments and "maintenance" every 12 weeks thereafter. Follow-up is available on 29 patients. Patients were divided into 3 groups: A) Those treated with 1311mIBG post ABMT relapse (n=11), B) Those failing to achieve a CR after receiving chemotherapy (n=16), and C) those treated for pain control while on palliative care (n=9).
RESULTS: Responses: Group A) 54.5% (2CR/4PR). Group B) 50%(1CR/7PR). Excellent pain control was achieved within 1 week after receiving 131ImIBG therapy in 8/9 patients. In group A and B stable disease was obtained in 22% of patients (2SD/4SD) respectively. The longest survivor form first 131ImIBG treatment is 10 years: median survival is 16months (2-120).
CONCLUSIONS: Low Dose 131ImIBG is not only useful for control of pain but it can induce a CR or PR in 50% of patients who have failed primary chemotherapy or relapsed post ABMT. Further studies are needed to evaluate the exact dose and schedule of low dose 1311mIBG in patients with neuroblastoma.

Ref ID: 335.1 **#92**

Dosimetry in 131I-MIBG mieloablative therapy

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Aims: New high activity protocols of mIBG alone or in combination with chemotherapy are ongoing to improve the survival of patients refractory to conventional treatments. Open questions are: is the biological response invariant between two subsequent treatments? Which is the optimal waiting time for stem cells re-infusion to assure that residual bone marrow dose and dose rate do not damage them?
Methodology: biological curves were measured using a gamma-counter (blood and urine), a scintillation probe, and a gammacamera for four days. A measured correction to gammacamera count loss was developed. In one 7 years old patient, the marrow dose calculation was performed with two extreme hypothesis: a)no bone involvement; b)pathological lumbar spine uptake hypothetically extended to the whole skeleton.
Results: Total body radioactivity was satisfactorily measured by probe which avoids underestimation of activity due to saturation of gammacamera.
Treatment 2/12/2003 28/01/2004
Administered activity (GBq) 5.4 7.4
ABSORBED DOSE PER UNIT ACTIVITY (Gy/GBq)
Liver 0.69 0.58
Bladder wall 0.94 1.16
Red marrow 0.11a-0.80b 0.11a-0.53b
Total body 0.19a-0.15b 0.12a-0.12b
Tumor 7.22 3.24
ABSORBED DOSE (Gy)
Liver 3.7 4.3
Bladder wall 5.1 8.6
Red marrow 0.6a-4.3b 0.8a-3.9b
Total body 1.0a- 0.8b 0.9a-0.9b
Tumor 39 24
Conclusions: Red marrow dose is remarkably different from total body dose. WB curve is identical in the two treatments, while primary tumour dose and metastatic spine dose per GBq are much less in the second therapy. In the worst hypothesis (b), waiting re-infusion time are 4.3 and 2.6 days for 1st and 2nd treatment.

Ref ID: 273.2 **#94**

A pilot study of sequential myeloblative autologous stem cell transplantation (MA-AUTOSCT) and immunotherapy with reduced intensity allogenic stem cell transplant (RI-ALLOSCT) for high/risk neuroblastoma

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Children with high-risk neuroblastoma continue to have an unsatisfactory long-term survival. AlloSCT may be beneficial by providing a graft vs tumor effect, but is associated with significant toxicity. In this pilot study we have investigated MA-AutoSCT followed by RI-AlloSCT in newly diagnosed patients with high-risk neuroblastoma. Patients received induction chemotherapy based on the N7 regimen: cycles 1, 2, 4, 6: vincristine, doxorubicin, cyclophosphamide, and dexrazoxane (375 mg/m2); cycles 3, 5 cisplatin, etoposide, and amifostine (740 mg/m2). Patients who had a partial response to induction chemotherapy were eligible for an MA-AutoSCT (n=5), with a conditioning regimen based on the regimen of Park (Med Pediatr Oncol, 2000): topotecan (1.5 mg/m2 x 5d), carboplatin (500 mg/m2 x 3d), thiotepa (300 mg/m2 x 3d), with amifostine (910 mg/m2 x 6d). 4 of 5 patients completing AutoSCT have received a RI-AlloSCT (1 patient declined RI AlloSCT). Median time to RI-AlloSCT after AutoSCT was 82d. Donor characteristics: UCB 5/6, 6/6; related donor 5/6, 6/6 HLA matched. RI conditioning regimen: fludarabine (30 mg/m2 x 5d), busulfan IV (3.2 mg/kg x 2d), and rabbit anti-thymocyte globulin (2.0 mg/kg x 4d, UCB recipients only). GVHD prophylaxis: Tacrolimus (d-1-+60) and mycophenolate mofetil (d+1-+28). Follow-up days post RI-AlloSCT: d+282, d+59, d+8, d+1. The patient d+282 had maximal aGVHD of grade 1 (skin), is 100% donor, and is in CR after 6 cycles of cis-retinoic acid. Additional toxicity and engraftment data will be presented. In summary, MA-AutoSCT followed by RI-AlloSCT appears feasible in patients with high-risk neuroblastoma.

Ref ID: 074.1 **#93**

Immunotherapy with rHuIL-2 after autologous BMT for neuroblastoma

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For the AIEOP BMT Registry.

BACKGROUND AND AIMS: A phase II study with low doses of rHuIL-2 was performed with the aim to amplify natural immune response against neuroblastoma after autologous BMT.
METHODOLOGY: From 1/92 to 12/98, 82 consecutive pts grafted because affected by high risk neuroblastoma were enrolled. 56 pts received rHuIL-2 respectively according to a ev (1) (25 pts) and a sc (2) (31 pts) schedule, 26 pts refused or did not received IL-2 for staff decision (3). Schedule 1 consisted of two cycles of 24-h iv infusion for 5 d (2-4-6-8-8 MU/sqm) followed by 11 monthly and 6 bimonthly cycles administered sc for 5 d (2-4-4-4 MU/sqm). Schedule 2 consisted of rHuIL-2 administered sc for 5 d (4 MU/sqm), twice a month for 6 months. Before IL-2 treatment, pts were 8 in CR, 10 in VGPR and 7 in PR (schedule 1); 10 in CR, 7 in VGPR and 14 in PR (schedule 2); 15 in CR, 2 in VGPR and 9 in PR (no treatment).
RESULTS: Pts received 317 (schedule 1) and 366 (schedule 2) cycles of IL-2. Iperpirexia and trombocytopenia were the only rHuIL-2 dependent toxicity. Immunological analysis evidenced an increment of NK activity and activated T limphocyte cells number in both protocol. The 5 years DFS (IC 95%) was 28% (12-46), 41.7% (24-58) and 23.6% (15-43) respectively for the pts enrolled in schedule 1, 2 and for patients not treated.
CONCLUSION: Immunotherapy with low doses of rHuIL-2 is feasible and seems to be effective to induce immunocompetent cells proliferation. Moreover, schedule 2 of treatment seems to be more effective to improve DFS in high risk neuroblastoma after autologous BMT.

Ref ID: 361.3 **#95**

Intrathecal 131-I-labeled 3F8 antibody in patients (PTS) with GD2-expressing central nervous system (CNS) and leptomeningeal (LM) neoplasms

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BACKGROUND: Tumors metastasizing to the CNS and LM are associated with significant morbidity and mortality. We studied the pharmacokinetics, toxicity, and radiation dose to the cerebrospinal fluid (CSF) following intraventricular injection of 131-I-labeled 3F8 in pts with GD2-positive CNS/LM disease including neuroblastoma).
METHODS: 15 pts (ages 1-61) received a tracer dose (37-74 MBq, 1-2 mCi) and a second therapeutic injection (370-740 MBq, 10-20 mCi). Dosimetry was evaluated by whole-body gamma camera scanning (4, 24, 48 hrs), serial CSF and blood sampling. Pre- and post- treatment clinical, radiographic and cytologic status were evaluated.
RESULTS: Total absorbed dose to the CSF was 1.12 - 13.0 Gy, 3.2 - 41.5 Gy to the ventricles and 1.0 - 13.7 Gy to the spinal column. Clearance half-life was 12.5 hr (3.5 - 44 hr). Average ratio of the therapy/tracer administration (Gy/MBq), was 0.88 (±0.58) and 1.08 (±0.66) by CSF counting and ROI analysis, respectively. Toxicity including headache, fever, vomiting were self-limited. At the higher dose levels, transient elevations in intracranial pressure (n=1) and asymptomatic bradycardia (n=2) were seen. Of 12 evaluable patients, clinical improvement (n=2), radiographic improvement (n=2), and cytology clearing of malignant cells (n=2) was observed. No long term toxicities have been seen 19 months post-injection.
CONCLUSIONS: Intrathecal 131-I-3F8 can be safely administered. A high CSF: blood ratio is observed. Tracer studies reliably predict the therapeutic dose to the CSF. Targeted radioconjugates have potential imaging and anti-tumor properties in the treatment of GD2-expressing malignancies metastasizing to the CNS/leptomeninges.

Ref ID: 034.1 **#96**

Laparoscopic adrenal surgery for neuroblastomas in children

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Between September 2000 and October 2002, laparoscopic adrenalectomy for neuroblastoma was performed in 9 cases at a mean age of 38 months (range 2 months to 9 years). Preoperative diagnosis were neuroblastoma stage I in 4 cases, and stage IV in 3 cases and non-determined supra renal calcified masses in 2 cases. Transperitoneal approach was used. The adrenal tumour were completely excised, placed into a plastic bag, and removed through the umbilical trocar site. All the adrenal tumours were well encapsulated and completely excised. One of the 9 procedure was converted. In 1 case, a second hepatic localization was removed simultaneously, and in 3 cases, 1 or more lymph nodes were resected. Average operative time procedure was 85 minutes (range 45 to 170). There was no death. There was no postoperative complications, except one port site infection. Blood transfusion was not required. Average hospital stay was 4.5 days (range 2 to 10). Histologic analysis of the 9 specimens (maximum length of 6 cm) confirmed the diagnosis of neuroblastoma. mycN was amplified in two cases stage IV. Averagepostoperative follow-up was 15 months (range 1 to 25). There was no local recurrence or metastasis , except the case who needed conversion. In conclusion, laparoscopic adrenalectomy for neuroblastoma is safe and feasible in children with good results. Experience with advanced laparoscopic surgery is required to achieve this result in optimal cancerological conditions. Our short term results must be re-evaluated at long term and further studies are needed to compare laparoscopy to open surgical techniques.

Ref ID: 118.1 **#98**

Prospective analysis of complications central venous catheter related in paediatric patients with neuroblastoma. An experience of a single institution

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BACKGROUND AND AIMS: The use of indwelling central venous catheters (CVCs) guarantee a reliable vascular access and are essential for the management of children undergoing anticancer treatment, but they may present disadvantages such as the risk of mechanical complications, thromboses or infections. We prospectively evaluated complications of three types of CVCs inserted in children with Neuroblastoma (NB) during a 47- month's period.
METHODOLOGY: Between January 2000 and November 2003 single lumen Hickman-Broviac (SL-HB), single lumen pressure activated safety valve (PASV) catheters and ports were inserted in paediatric patients with diagnosis of NB and considered eligible for this study. CVC insertion was always performed surgically under generally anaesthesia; for each catheter were recorded data on CVC type, date of positioning, age of the patient, date and type of complications, if any, date of last examination, date and cause of catheter removal. All the children were treated according to the Italian Protocols in use at the time of diagnosis. Three groups of possible complications were a priori defined: mechanical, infections and thrombotic.
RESULTS: 74 CVCs (22 PASV, 49 SL-HB and 3 ports) were inserted in 65 children on 112 diagnosis of NB for a total of 19626 catheter-days of observation. The median age at the moment of CVC insertion was three years. The median follow-up of this group of CVCs was 326,5 days. A total of 19 complications occurred in 18 devices (overall complication rate was 0.97 per 1000 catheter–days); in particular 9 of the 49 SL-HB (18%), 8 of the 22 SL-PASV (36%) and 1 of the 3 ports (33%) presented a complication; in one PASV occurred two complications.

	SL-HB	SL-PASV	Port
mechanical	4	5	1
Infections	4	3	0
thrombosis	1	1	0
Total	9	9	1

Elective removal was performed in 7 episodes (5 related to infections, 1 related to mechanical complication and 1 related to thrombosis).
CONCLUSIONS: CVC complications occur in almost 26% of patients confirming the importance of central venous catheter adverse event in the management of children with oncological malignancies. In the group of tunnelled CVCs the rate of complications is higher in patients with PASV; they present a significative incidence of mechanical complications even if the presence of valve simplify the device maintenance procedures, while SL-HB catheters represent overall the best choice.

Ref ID: 128.1 **#97**

Long-term complications from treatment in survivors of high risk neuroblastoma

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BACKGROUND AND AIMS: Few studies have assessed late effects from treatment in neuroblastoma (NB) survivors. Methodology: 63 high-risk NB survivors were seen at the MSKCC Long Term Follow-Up Clinic since 1991.
RESULTS: Median follow-up was 7.06 years. 11 patients had stage 3 disease and 52 stage 4. All patients had surgery and received chemotherapy, 56 (89%) radiation therapy, 38 (60%) immunotherapy and 35 (56%) autologous stem cell transplant. 60 survivors (95%) had late effects and 18 (29%) experienced more than three. The most frequent complications were: hearing loss (62%), primary hypothyroidism (24%), ovarian failure (41% of females), musculoskeletal problems (22%), pulmonary (19%), visual (16%), neurocognitive (13%), dental (13%) and secondary neoplasms (6%). Grades of severity were: 1 (24%), 2 (44%), 3 (28%) and 4 (4%) (CTCAE v 3.0). Survivors who received cisplatinum were more at risk to develop hearing loss compared to those who did not receive it (OR, 9.74; 95% CI: 0.9-101.6). A total cyclophosphamide dose greater than 7.4 g was associated with ovarian failure (p=0.02). Pulmonary dysfunction was correlated with chest irradiation (p=0.009). Visual and dental problems were both associated with cranial radiation (p=0.001 and p=0.002 respectively).
CONCLUSIONS: Despite very intensive multimodality treatment, survivors of high-risk NB face
relatively moderate long-term complications. Their early identification and treatment are important for the quality of life of the survivors.

Ref ID: 382.1 **#99**

Anemia is a risk factor for cisplatin-induced hearing impairment in children with high-risk neuroblastoma

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BACKGROUND: Cisplatin is useful in chemotherapy of neuroblastoma. A frequent and sometimes dose-limiting side effect of cisplatin is permanent, sensoneural high frequency hearing loss. We experienced an increase in hearing loss in high-risk neuroblastoma patients and wanted to explore the reasons for this.
MATERIAL: Eight-teen children with high-risk neuroblastoma receiving four cycles of cisplatin during the induction treatment (rapid COJEC or OPEC/OJEC) were studied divided in 2 groups; Group 1, treated 1996 – 2000 and Group 2 treated 2001 - 2003. Hearing was measured by repeated audiology during and after induction treatment.
RESULTS: Ten children had no or minor hearing loss (Brock grade 0-1), eight suffered significant ototoxicity (Brock 2-3, 44%). Three children had cisplatin replaced by carboplatin because of rapidly progressing hearing loss. Group 2 experienced graver hearing loss (p=0.022). No conclusions could be drawn from the data concerning prehydration. Prolonging the time of prehydration did not seem to protect against
ototoxicity. Age differed significantly between the two groups but was not correlated to ototoxicity.
Haemoglobin levels prior to each course of cisplatin differed significantly with Group 2 having lower Hb concentrations (p=0.018). This difference was also present when comparing children with and without hearing impairment (p=0.014).
CONCLUSIONS: Hearing loss in high-risk neuroblastoma had increased over time. Anaemia prior to cisplatin courses seems to be a risk factor for ototoxicity. Hypoxia in the cochlea might elevate the levels of free radicals aggravating the ototoxic effects of cisplatin. Routines concerning blood transfusions might be of importance for cisplatin-induced hearing loss and should be considered further in treatment of high-risk neuroblastoma. Our significant findings in this limited number of patients warrant extended investigations in a larger cohort of children with high-risk neuroblastoma.

Poster Display
GENETICS

Ref ID: 194.1 **#100**

Gene expression profiling of neuroblastoma

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To investigate the various genetic characteristics between the early- and advanced-stage neuroblastomas (NBs) and identify the candidate genes involved in NB progression, we performed DNA microarray analysis (Gene Chip Human Cancer G110 array; Affymetrix) in a total of 20 NBs. A two-way clustering analysis based on the expression pattern of approximately 500 of 1700 genes revealed genetic subgroups in these NBs. Although 9 of 13 early-stage tumors (69%) and 4 of 6 advanced-stage tumors (67%) were classified as the same cluster, respectively, the remaining tumors showed different expression profiles. This indicates that both the early- and advanced-stage tumors were heterogeneous. Based on the microarray data, we identified the API2, p19INK4D, BAF60c and CRABP2 genes a well as MYCN, NM23-H1 and TRKA genes that are predominantly expressed in either the early- or advanced-stage of NB. These genes have been reported to be associated with apoptosis, cell cycles, the transcriptional activator and the retioic acid transportor. For better assessment of the prognostic value of these gene expression in NB, real-time PCR was carried out in 50 NBs. The expression of both the API2 and p19INK4D genes was significantly higher in the early-stage group than the advanced-stage group, whereas the expression of the BAF60c and CRABP2 expression was significantly reduced in the early-stage group. Therefore, it is possible that the API2, p19INK4D, BAF60c and CRABP2 genes are candidates as novel prognostic markers for NB. Further analysis using more than 20,000 genes is in progress in these NB samples.

Ref ID: 371.1 **#102**

Gene Expression Associated with Prognosis of Neuroblastoma

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To investigate biological features associated with prognosis of neuroblastoma, we analyzed gene expression of 59 tumor samples obtained from the patients in Japan. These tumors included 16 tumors of stage 1, 4 of stage 2, 19 of stage 3, and 20 of stage 4, and MYCN amplification was observed in 4 of the stage 3 tumors and 6 of the stage 4 tumors. For expression data acquisition, we employed an Affymetrix HG-U133A microarray which contains 22,215 probe sets. More than 60% of genes we examined were expressed in at least one of the 59 tumors. In unsupervised principal component analysis (PCA) of the expression data, the first and second components, which mainly constructed by genes associated with immune system, were not correlated to outcome of the patients. We found that the third component in PCA was clearly correlated to prognosis. Genes associated with neuronal differentiation contributed largely to this component. In t-test comparing the expression of each gene between the tumors with poor prognosis and those with favorable prognosis, 17% of the expressed genes were significantly ($p<0.01$) associated with prognosis. Interestingly, most genes except MYCN and its co-amplified genes were expressed similarly in the tumors with poor prognosis regardless of MYCN amplification status. In addition, MYCN target genes such as ODC1 and ribosomal protein genes were highly expressed even in the MYCN amplification-negative tumors with poor prognosis. These results indicate that gene expression analysis is extremely useful for outcome prediction of neuroblastoma patients, and suggest that a molecular event functionally analogous to MYCN amplification may occur in unfavorable neuroblastoma without MYCN amplification.

Ref ID: 367.1 **#107**

Gene expression profiling of neuroblastoma cell lines treated identifies genes upregulated by retinoic acid that are overexpressed in favorable neuroblastoma primary tumors.

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Biological heterogeneity of neuroblastoma is associated with a range in clinical outcomes. To identify alternations in gene expression occurring as a result of retinoic acid treatment of neuroblastoma cell lines we employed custom cDNA microarrays (6448 genes) derived from a human fetal brain library + broad human 10K microarrays (CodeLink®) to examine retinoic-acid-sensitive (SMS-KCNR, SMS-SAN) and retinoic-acid-resistant (SK-N-BE(2) and LA-N-6) cell lines treated with 10μM all-trans retinoic acid (ATRA) for 10 days. In RA-sensitive cells, hierarchical clustering showed that 232 genes were > 3-fold up-regulated and 389 genes were > 3-fold down-regulated by ATRA treatment. The expression of MYCN, TERT, CCNG1, SRC, ERBB1, and NPY were down-regulated, whereas those of transcription factors such as CBFB, GATA6, and growth arrest signals (GADD45A, NIP) were up-regulated. We confirmed these gene expression changes by real-time TaqMan quantitative RT-PCR. Interestingly, the neuronal differentiating factors including SDR1, NR0B1, CYP26A1 were remarkably up-regulated (> 10-fold) at 6 hours regulation in the RA-sensitive cell lines, but not in the RA-resistant lines. In the cells treated with 13 cis-RA, several genes including these three genes were also remarkably up-regulated. These three genes were overexpressed in 86 low-risk tumors (stage I, II, and IVS, age at diagnosis < 12 months, no MYCN amplification, favorable histology) including 45 mass-screening detected tumors, relative to the expression in 41 high-risk and intermidiate-risk tumors ($P < 0.01$). These data suggest retinoic acid causes alterations in gene expression in neuroblastoma cell lines from high-risk patients that parallel expression patterns observed in low-risk tumors.

Ref ID: 329.1 **#108**

Identification of a gene signature discriminating stage 4S and stage 4 neuroblastoma using Serial Analysis of Gene Expression

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Differentiation of various subtypes of neuroblastoma (NB) using gene expression profiling have recently been demonstrated by several publications. However, none of these studies reported on a gene signature separating neuroblastoma of stage 4S from stage 4 disease, both of which represent metastazising tumors but usually follow opposite clinical courses. To bring out the molecular differences of stage 4S NB showing spontaneous regression and unfavourable stage 4 NB without MYCN-amplification, gene expression profiles of five NB of the former and three NB of the latter subtype were generated using the technique of Serial Analysis of Gene Expression (SAGE). In total, more than 200.000 SAGE tags were sequenced with each profile comprising between 20.000 and 30.000 tags. A total of about 53.000 unique tags were cataloged, 18.000 of which were detected in at least two SAGE libraries. Transcripts corresponding to these tags are likely to represent a comprehensive portrayal of the transcriptome of the NB subtypes analysed. SAGE profiles of the two subgroups were compared using t-test statistics, which uncovered 429 tags exhibiting significantly different frequencies, 277 and 152 of which were over-represented in stage 4S and in stage 4 NB, respectively. Comparison to the UniGene reference database allowed to uniquely match 233 tags, whereas 164 tags were ambiguously assigned and 32 tags had no match in the database. Among the genes that were over-expressed in profiles of stage 4S tumors, several transcripts were detected that have been reported to be implicated in differentiation processes of neuronal tissues or to be associated with particular neuronal phenotypes, which may indicate that stage 4S and stage 4 NB develop from cells of distinct stages of neuronal differentiation.

Ref ID: 185.1 **#104**

Functional genomics in neuroblastoma

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Neuroblastoma represents the most frequent solid extracranial neoplasia in children. Cytogenetic and molecular analysis of neuroblastoma demonstrated that several chromosome abnormalities are present in these tumors. Unfortunately, the functional role of these genetic events in the development of neuroblastoma is not well defined and the involved genes are not known yet. In order to identify genes, proteins and pathways responsible for malignant transformation and progression in neuroblastoma, we performed a gene expression profiling approach of undifferentiated and retinoic acid differentiated neuroblastoma cell lines as model system. This approach is indicating new genes up and down regulated in neuroblastoma cellular models. We used the GeneChip Human Genome U133 Set (Affymetrix) for testing mRNA from LAN-5 cell line. 0.3% of the total genes were differentially expressed in LAN-5 cell line: 81 were up-regulated and 17 down-regulated in differentiated cells. The genes up-regulated at least 2.0-fold belonged in the functional categories of signal transduction, regulation of transcription, endocytosis, protein biosynthesis, apoptosis, neurogenesis, development. The genes down-regulated belonged in the functional categories of signal transduction, apoptosis and cell proliferation. 0.3% of the total genes were differentially expressed in Neuro-2a cell line: 25 were up-regulated and 18 down-regulated in differentiated cells. The genes up-regulated belonged in the functional categories of signal transduction/apoptosis, regulation of cell cycle. The genes down-regulated belonged in the functional categories of protein folding, steroid biosynthesis, proteolysis and peptidolysis, regulation of transcription
DNA-dependent, DNA packaging, oxidoreductase activity.

Ref ID: 207.1 **#105**

Use of CGH array to identify MYCN amplification and chromosome 1P36 deletion in neuroblastoma

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BACKGROUND: In the last decade, microarray technology has been extensively applied to evaluate both gene expression profile and genome imbalances. We have developed a Comparative Genome Hybridization (CGH) array to identify 1p chromosome deletion and MYCN gene amplification, two major markers of tumor aggressiveness in neuroblastoma (NB). Methods. Total DNA was purified from sixteen tumor samples containing at least 90% malignant neuroblasts and collected at the onset of disease. Pooled fluorescent-labeled reference and NB tumor genomic DNAs were hybridized to epoxy coated glass slides on in-house made cDNA microarray. The array contains PCR-probes from cDNAs of 1p36.33-36.1 chromosomal region and MYCN gene. cDNAs from 2q3-q34 and 12p13 chromosome regions were used as control and A. Thaliana genome was spotted to control for unspecific hybridization. Results and discussion. Both 1p36 chromosome deletion and MYCN amplification were detected by CGH array. A double color FISH analysis was also performed to validate results of the CGH array. For MYCN amplification, the comparison between CGH array and FISH resulted in a sensitivity of 66.7%, with a specificity of 90.1%. The sensitivity evaluated for the chromosome 1p36 deletion was 66.7%, with a specificity of 90.0%. Our study demonstrates that the CGH array can be efficiently adapted to study DNA gain and loss of specific chromosome regions. For this purpose, we have created a NB array that allows the simultaneous detection of MYCN gene amplification and chromosome 1p36 deletion.

Ref ID: 395.1 **#109**

Gene Expression Differences Among Stages in Stroma-Poor Neuroblastoma

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BACKGROUND: Neuroblastoma, a developmental tumor that originates from the neural crest, exhibits a broad spectrum of clinical behavior and histologic differentiation. Accurate stage assignment is essential for appropriate management. However, the biological basis of heterogeneity and metastasis in this tumor remains unclear.
METHODS: We performed a genome-wide expression analysis to identify genes and biological pathways associated with disease stage. 931 genes were found to be differentially expressed at a 5% false discovery threshold between local regional (stages 1, 2, 3) and metastatic (stage 4) neuroblastoma. Separately, models predictive of stage were developed using a variety of approaches, and tested in leave-one-out method. These methods included support vector machine, K-nearest neighbor, and logistic regression.
RESULTS: Genes thought to be associated with arrested differentiation during neural crest development were found to be differentially expressed. The accuracy in cross validation was 89% for the support vector machine model, 85% for K-nearest neighbor model, and 76% for the logistic regression model. DLK1 and TWIST were two of the genes in neural crest development found to be highly expressed in a subset of neuroblastomas. Their expression levels were confirmed by RTPCR and northern blot analyses. DLK1 and TWIST protein levels correlated well with transcript abundance and were detected in the neuroblastic component of tumors.
CONCLUSION: Using differentially expressed genes, a predictive model for local-regional versus metastatic stage assignment performed well, and may provide further refinement for risk-based strategies in neuroblastoma. Since some of these proteins are ligands or surface receptors on neuroblastoma cells, they may provide therapeutic targets for patients with high-risk disease.

Ref ID: 223.2 **#111**

Genome-wide transcriptome analysis for neuroblastoma gene discovery and refinement of clinical risk prediction

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Neuroblastoma risk-class prediction is integral to clinical trial design, but may be imprecise. In addition, the genes that determine aggressive clinical behavior remain largely unknown. We therefore have quantified mRNA copy numbers in a highly annotated series of 101 prospectively collected diagnostic primary neuroblastoma samples using the Affymetrix U95Av2 array. Allelic status at 1p36, 2p24 (MYCN), 11q23, and 17q23 was determined by PCR and all samples/experiments passed strict QC metrics. We first measured differential gene expression in each binary comparison of 1p36, 11q23 and 17q23-25 aberrant versus normal in order to discover the relevant region-specific expression alterations associated with genomic copy number aberrations. The majority of genes showing the most statistically significant differential expression mapped to the region being compared and these data are being used to prioritize regional candidate genes for further analyses. We next sought to determine if the expression profiles could help validate and/or refine our current risk classification system. The supervised learning algorithm Random Forests (Breiman, Machine Learning, 2001) was used to build a human neuroblastoma risk classifier, and 30,000 trees with 1178 randomly selected features at each node was stable and minimized estimated error. Overall agreement in assigning an individual sample per the current risk stratification was 78.2%, with high-risk samples accurately classified in 92% of cases. Agreement for the majority of samples showing discordance may be achieved based on analysis of additional clinical (i.e. relapse) and/or biological features. These data demonstrate the potential of transcriptome analysis for validation and perhaps refinement of clinicobiologic risk classification schemas, and suggests that our current neuroblastoma system lacks precision for patients currently assigned to low- and (especially) intermediate-risk groups. This work also shows the usefulness of combined genomic and transcriptomic analyses for cancer gene discovery.

Poster Display
GENETICS

Ref ID: 392.1 **#112**

Methylation of Multiple CpG Islands in Neuroblastomas with Poor Prognosis

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Methylation of CpG islands is deeply involved in embryonic development and tissue differentiation. In this study, CpG islands that are differentially methylated between neuroblastomas with favorable outcomes and those with unfavorable outcomes were searched for by methylation-sensitive-representational difference analysis (MS-RDA). Five CpG islands (or groups of CpG islands) were found to be differentially methylated. Kaplan-Meier analysis of 96 primary samples showed that methylation of these CpG islands, especially that of the PCDHB gene family, was closely associated with poor survival of neuroblastoma cases (Hazard ratio obtained by = 46.2; 95 % C.I.: 6.0-353.8). The CpG islands of the PCDHB gene family were located in gene bodies, and did not affect gene expression or histone modification. However, methylation of the CpG islands of the PCDHB gene family was associated with methylation of promoter CpG islands of the RASSF1A and BLU genes. It was indicated that disruption of epigenetic integrity, represented by extensive methylation of the five CpG islands, causes methylation of CpG islands in promoter regions of key genes for tumor-suppression or differentiation, and leads to poor prognosis of neuroblastomas.

Ref ID: 321.2 **#113**

Expression profiling of aggressive and benign neuroblastic tumors: is there a common pattern?

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We tested the expression pattern of aggressive neuroblastomas and their benign variant and tumors of other entities. The direct visualization of all transcribed sequences along the chromosomes was enabled by the use of the CESH (comparative expressed sequence hybridization) technique. The differential expression pattern is obtained after transcribing RNA from the tumor cells and control cells (either white blood cells or another tumor), into cDNA, after two color fluorescence labeling and the hybridisation to normal chromosomes. Thus, the total transcriptome of a given cell type can be directly visualized and assigned to specific chromosomal loci. cDNAs from neuroblastoma cell lines STA-NB-1,10,11,12,13, and corresponding tumors were analyzed, two loco-regional and one 4s neuroblastomas, STA-ET-6 and ZR75 were labeled and differentially hybridized either against leukocyte cDNA or in pairs against each other. Surprisingly, the differential hybridization of cDNA extracted from aggressive neuroblastomas against white blood cell cDNA revealed a common expression pattern among all tested neuroblastoma cell lines independent of the MYCN status and only minor differences were noted. The expression profiles of the different neuroblastoma cell lines and corresponding tumors tested displayed only minor but distinct differences compared to the tested breast carcinoma and Ewing tumor cell lines, indicating common expression profiles of malignant cells, referred to as 'malignancy-associated regions of transcriptional activation' (MARTAs). On the contrary, the expression profile of the benign variants of neuroblastico tumors displayed quite an opposite expression pattern. These data, despite the fact that only a limited number of tumors and cell lines was looked at, permit to hypothesize that malignant tumors have common hot spots of expression which totally differ from the expression profile of benign
neuroblastoma cells.

Ref ID: 154.1 **#116**

Characterization of amplicons in neuroblastoma cell lines and tumours by microarray-based comparative genomic hybridization

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Microarray-based comparative genomic hybridization is a recently developped technology that allows determination of DNA copy number alterations. In neuroblastoma, MYCN is the most frequent amplified gene but amplification at other various loci that are nonsyntenic with the MYCN locus have been observed, mainly in cases showing MYCN amplification. We have prepared and used several genomic arrays in order to precisely characterize amplicons that have been previously observed using conventional comparative genomic hybridization (CGH) in neuroblastoma cell lines and tumours. For a subset of cell lines, expression data were obtained using the Affymetrix technology and allowed to determine which genes are overexpressed in the amplified regions. Strikingly, we have observed amplifications on chromosome 1p, for which deletion is one of the most frequent genetic alterations in neuroblastoma, using conventional CGH in two tumors at bands 1p34.2 and 1p36.3, respectively. Using a medium-resolution genomic array containing 178 PACs/BACs from chromosome 1p then two high-resolution arrays, containing contigs of overlapping PACs/BACs from the amplified regions, we could precisely map and delineate both amplicons. The 1p34.2 amplicon appeared as a homogeneous amplification unit, whereas the 1p36.3 amplicon had a more complex structure with two non-contiguous highly amplified regions and several moderate amplification units. For this case, FISH analysis confirmed the amplification of several clones and indicated that the two highest amplification units corresponded to two different populations of double-minute chromosomes, one of which also contains the MYCN locus.

Ref ID: 251.3 **#117**

Isolation of normal neuroblastoma precursor cells: tools for comparative gene expression profiling

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Neuroblastoma tumour cells originate from primitive neuroblasts giving rise to the sympathetic nervous system. In order to have an appropriate normal control for real-time quantitative reverse transcriptase PCR (Q-PCR) and Affymetrix expression profiling of neuroblastoma tumours, neuroblast clusters were laser capture microdissected from snap frozen foetal adrenal cryosections (between 15 and 22 weeks of gestation). As RNA quality is critical for further downstream experiments, optimized protocols for foetal adrenal gland collection, production of cryosections, staining and RNA isolation were developed and thoroughly tested. Using a modified haematoxylin and eosin staining protocol, RNA of good quality was obtained as demonstrated by the ratio of the ribosomal RNA bands using the Agilent 2100 Bioanalyzer and the new Pico Chip kit. In a first expression analysis of the isolated neuroblast RNA, Q-PCR analysis was performed for selected neuroblastoma and sympathetic nervous system marker genes and provided further evidence for the role of neuroblasts as neuroblastoma precursor cells. Further efforts were aimed at collecting additional foetal adrenals. Approximately 100 sections were prepared from each sample and subsequently scrutinized for the presence of clusters of neuroblasts. Following laser assisted microdissection from these sections ~10 ng RNA was collected from glands of different gestational time points. Using a recently developed and validated protocol that combines two IVT amplification steps these RNA samples will be amplified in order to provide sufficient material for transcriptome wide gene expression analysis using Affymetrix chips. To this purpose we also selected representative neuroblastoma tumour samples from different genetic subgroups. Ultimately, these data will allow us to provide insights into the point of developmental arrest in the different subtypes of adrenal neuroblastomas and the deregulated biological circuits in neuroblastoma.

Ref ID: 287.1 **#114**

A Ran-binding protein, RanBPM, stabilizes p73 and enhances its pro-apoptotic activity: Its possible role in spontaneous regression of neuroblastoma

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p73 which is mapped to chromosome 1p36.2, is a newly discovered member of the p53 family. The previous analysis using p73-null mice has suggested that p73, in a balance with p53, functions as an important regulator in inducing programmed cell death of sympathetic neurons. Like p53, p73 inhibits cell cycle progression and/or stimulates apoptosis, which is in large part due to its transactivation ability. Previously, we and others demonstrated that the extreme COOH-terminal region of p73alpha plays a critical role in the regulation of its transcriptional activity and pro-apoptotic function. To better understand how p73 is regulated at a molecular level, we performed a yeast-based two-hybrid screening using the extreme COOH-terminal region of p73alpha as a bait, and identified RanBPM as a new binding partner for p73alpha. RanBPM was initially discovered as the cellular protein which was associated with Ras-like nuclear G protein (Ran). GST pull-down assay and co-immunoprecipitation analysis demonstrated that RanBPM directly interacted with p73alpha at its COOH-terminal region. RanBPM co-localized with p73alpha in the cell nucleus, and increased the intracellular levels of p73alpha. Consistent with these observations, ectopic expression of RanBPM prolonged the half-life of p73alpha, and decreased the ubiquitination level of p73alpha. In contrast, RanBPM did not bind to p53, and had no significant impact on its stability. Furthermore, RanBPM was able to enhance the p73-alpha-mediated transactivation ability as well as pro-apoptotic function. Intriguingly, RanBPM was expressed at high levels in favourable neuroblastomas as compared with unfavourable tumors. Thus, our present findings suggest that RanBPM acts as a cofactor of p73alpha to enhance its pro-apoptotic function by increasing its stability. The preferential expression of RanBPM in favorable neuroblastomas might contribute to enhance their spontaneous regression.

Ref ID: 207.2 **#115**

Identification of genes differently expressed in favorable and unfavorable histology neuroblastoma tumors

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Background. Neuroblastomas (NBs) show remarkable biological, clinical and histological heterogeneity. To survey the differences in gene expression profiles between favorable and unfavorable histology NBs, we analyzed three stroma poor NBs (SP-NBs) and four stroma rich intermixed ganglioneuroblastomas (SR-GNBs), by using Affymetrix GeneChip® technology.
Methods. Total RNA samples were amplified and labeled performing two cycles of standard cDNA synthesis combined with in vitro transcription. Human Genome 133A GeneChip® intensity data have been transformed for normalization and variance stabilization by the VSN method implemented in Bioconductor software (www.bioconductor.org). We also performed a cluster analysis by R package and the NetAffx Gene Ontology Mining Tool. Results and discussion. Hierarchical clustering clearly differentiated SP-NBs from SR-GNBs and identified three groups of 34, 23, and 32 genes highly differently expressed in the two histotypes. The clusters enclose genes involved in metabolism (protein and nucleic acid biosynthesis, modification and catabolism), cell proliferation, cell-cell signaling, signal transduction and neurogenesis. In particular, neuronal differentiating genes were detected highly expressed in SR-GNBs. Interesting, we found metalloprotease (MMP) expression in SP-NBs. They are peptidases involved in extracellular matrix degradation and they are regulated by Tissue Inhibitors of MetalloProteases (TIMPs). MMPs and their specific TIMPs have been associated with tumor cell invasion and metastasis in a number of adult tumors. Increased expression of MMP2 is also significantly associated with advanced NB stages, progressive disease and poor prognosis. Expression profiling data revealed the existence of differently up-regulated and down-regulated gene clusters in favorable and unfavorable tumors, suggesting that Schwannian stromal cells express different gene patterns respect on neuroblasts. We propose to perform gene expression profile analysis on laser capture microdissected Schwannian stromal cells and neuroblasts, in order to clarify the key genes for cell growth, regression and differentiation of NB cells.

Ref ID: 046.3 **#118**

Genome-wide analysis of gene expression in neuroblastomas detected by mass screening

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Neuroblastoma is the most common malignant disease in infancy and the third most common pediatric cancer. Although numerous factors including patient age, stage of the disease and genetic abnormalities have been shown to be predictive of the outcome in children with neuroblastoma, the mechanisms responsible for the highly variable clinical behaviour of this tumor remain largely unknown. In order to gain new insights in the biology of this tumor, we performed Affymetrix micrarray analysis and compared the gene expression pattern of neuroblastomas detected by mass screening, characterized by a high probability of spontaneous regression, to the one of metastatic neuroblastomas with a pejorative prognosis. The bioinformatical analysis revealed a set of 19 discriminatory genes, that may play a significant role in the natural progression of neuroblastoma. These genes are involved in various biological functions such as cell proliferation, cell adhesion, angiogenesis, transcriptional regulation DNA repair, splicing events or carbohydrate metabolism. The clinical pertinence of this classifier was subsequently confirmed in an independent set of samples including low and high risk neuroblastoma.

Ref ID: 346.1 **#119**

Prediction of clinical outcome using gene expression profiling and artificial neural networks for patients with neuroblastoma

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Patients with neuroblastoma (NB) are carefully risk-stratified in order to determine appropriate therapy. Despite this, patients with high-risk NB have a <30% probability of survival and it is not possible to predict which patients will respond to therapy and which will die of disease. We have utilized gene expression profiling and artificial neural networks (ANNs) to develop an accurate predictor of survival. Gene.expression profiling using 42578-clone cDNA microarrays was performed on NB samples for which outcome data was available. The profiles were used to train ANNs to predict outcome. We found that NB tumors exhibited an inherent prognostic specific gene expression profile. ANN-based prognosis prediction algorithms using expression levels of all 37920 good-quality clones achieved a performance level comparable to the current COG risk stratification. In addition, using a gene optimization strategy, we identified 34 prognostic genes that correctly predicted outcome for 98% of these patients. Moreover, these predictor genes were able to further partition COG stratified high-risk patients into two subgroups according to their survival status. Our findings provide evidence of a gene expression signature that can predict prognosis independent of currently known risk factors. We believe that our ANN-based approach using the identified predictor genes would greatly assist physicians in the individual management of patients with high-risk neuroblastoma.

Ref ID: 090.1 #120

CGH in chemoresistant neuroblastoma cell lines

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The development of resistance to cytostatic agents is a major problem of cancer therapy. One DNA-based technique that may be applicable to problem of chemoresistance is the molecular-cytogenetic technique Comparative genomic hybridization (CGH). CGH can rapidly detect and map genetic imbalances in tumor genomes and when modified to comparatively hybridize DNA from drug-resistant line to DNA from the parent non resistant line it provides very unique results already at the first examination. We studied 2 parental high risk neuroblastoma cell lines and 6 derived daughter lines that were resistant to Vincristin, Doxorubicin and Cisplatin respectively. Resistant cell lines were established by exposing cells to increasing concentrations of the respective drug. CGH profiles of both parental cell lines were obtained using a DNA from healthy volunteer as a reference DNA. Labeled DNA from each of the drug resistant daughter cell lines were hybridized together with labeled DNA from its parental cell line to obtain a comparison of gains and losses that accompanied the development of resistance for the particular drug, excluding the changes that were present in the parental cell line. Drug resistance to Doxorubicin and Vincristine is mediated not only by over expression of MDR1 gene, but also by amplification of the gene. In the cell line where amplification on the chromosome 7 occurred in the parental cell line the daughter, drug resistant, cell line had even greater amplification in the region where MDR1 gene is located. Cell lines resistant to Cisplatin did not show any amplification of the MDR1 gene which corresponds with previous findings.

Ref ID: 348.1 #121

Phylogeny of Neuroblastoma Tumor Progression from Comparative Genomic Hybridization Data

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The chromosomal locations of genomic copy number changes are non-random for specific cancers and correlates with the diagnosis and progression. This suggests that the progression of the disease leaves a characteristic signature in the pattern of affected regions. We used this idea to identify the model of tumor progression, using cDNA microarray-based Comparative Genomic Hybridization (A-CGH) data for Neuroblastoma (NB). A-CGH was performed for NB specimens, with stage 1, stage 4 both MYCN-amplified (4+) and non-amplified (4-). P-values for the presence of gains or losses were estimated by a sliding window method, where the distribution of genome-ordered observations in that window was compared by a t-test to the distribution observed for the full genome. Using this we defined recurrent genomic alterations as common, shared, or specific to the stages, and mapped these to Venn-Diagrams. We show that these diagrams have a one-to-one correspondence to tumor progression models. We used this to fully enumerate all possible progression models and identified the one model, which is compatible with our observed data. Our model indicates that stage 4+ tumors do not progress through stages 1 or 4-, while stage 1 and 4- have a common ancestor. This supports the hypothesis that the final stage of NB is pre-determined at the time they acquire specific genomic changes.

Ref ID: 360.1 #122

Genome-wide micro-array analysis of neuroblastoma links genomic alterations to expression profiles

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We carried out genome-wide expression profiling of 108 NB tumors (stages 1 trough 4, MYCN-amplified (AMP) as well as non-amplified (NA) using 42K cDNA micro-arrays. Utilizing permutation t-test we found a common set of genes that discriminate stage 1 (ST1) from tumors of higher stages in NA tumors and AMP tumors of stage 4 from NA tumors of ST4. We employed principal component analysis (PCA) to reduce the complexity from 108 to two principal components that are interpretable in terms of the tumor stage and MYCN amplification. We developed a novel approach that combines probabilistic analysis of cytoband location and gene ontology with gene expression profiles arranged in the PCA-reduced space of biologically interpretable PCs in attempt to extract important biological processes associated with amplification or stage progression in NB, as well as to associate known genomic abnormalities with the transcriptional activity in these tumors. We found an association between genes differentiating stage and amplification with genomic abnormalities (such as 1p loss and 17q gain) as well as biological processes. We also show that amplification of MYCN in ST4 strongly correlates with up-regulation of the genes involved in the ribosomal machinery. In contrast, in NA tumors, ST4 is characterized by a significant increase in the expression of the genes involved in the cell cycle, which is not found in tumors ST1-ST3.

Ref ID: 179.1 #123

Expression of the PPM1D gene is associated with poor prognosis in Neuroblastoma

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The PPM1D gene, encoding a serine/threonine protein phophatase, locates at 17q23.1, which lies within a gain/amplification region in some types of cancers such as neuroblastoma (NB), and is associated with a poor prognosis. Recently, it is reported that PPM1D is a potential target of the 17q23 gain/amplification in NB. To confirm this, we quantified the relative expression levels of PPM1D in 25 NB cell lines and 43 fresh tumors using real-time quantitative-PCR (RQ-PCR). In 25 cell lines, overexpression of PPM1D was detected in 16 cell lines (64%) whereas 19 tumors (44%) showed overexpression in 43 fresh tumors. More than 2-fold of relatively DNA copy number compared to normal controls was shown in 28% (7/25) of cell lines and 14% (6/43) of fresh tumors, respectively, by RQ-PCR.??The specimens carrying relatively higher DNA copy number also showed overexpression of PPM1D except for one cell line. Furthermore, in view of clinicopathological parameters, we found that the tumors with PPM1D overexpression showed a significantly poorer outcome compared to those with normal PPM1D expression (P=0.035). A tendency of higher expression of PPM1D was detected in advanced stage tumors, in the tumors of more than 1 year, in the tumors diagnosed clinically, and in the tumors with MYCN amplification. We also examined 7 cell lines and 16 fresh tumors of rhabdomyosarcoma, 16 cell lines and 15 fresh tumors of Ewing/PNET, however, no statistical significance was detected between PPM1D expression level and clinicopathological parameters in these tumors. Thus, these results suggest that the PPM1D gene plays a role in the pathogenesis of NB.

Ref ID: 056.1 #124

Alternative pathways of MYCN gene copy number increase in primary neuroblastoma tumors

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Neuroblastomas, tumors of the symphathetic nervous system, account for 7-10% of the cancers of childhood. Genetic studies have shown, and this study has confirmed, that neuroblastomas are very heterogeneous. One genetic aberration found frequently in this pediatric tumor is MYCN gene amplification. Recently a new subset of tumors showing MYCN gain (small increases in gene number arising from unbalanced translocation) was described. To investigate whether gain preceeds amplification or is an independent event, we surveyed a large series of primary tumors for MYCN copy number: 76% were MYCN single copy tumors, whereas 24% tumors harboured MYCN abnormalities: either MYCN amplification alone or MYCN gain alone. Among the cases with MYCN amplified gene, we found four that also showed gain. In three tumors exhibiting simultaneous gain and amplification, these two events were detected in neighboring cells. In the fourth case we detected only MYCN gain in metastatic neuroblasts in the bone marrow, but both MYCN amplification and gain in the primary tumor. The detailed study of these four cases suggests that there may be several different mechanisms leading to increase in MYCN copy number 1) direct amplification of the protooncogene, 2) gain and amplification occurring in independent cell populations (clonal evolution) within the same tumor 3) gain of copies followed by amplification occurring in the same cells.

Ref ID: 363.1 #126

RNA Interference to elucidate the mechanism of cell cycle regulation by N-myc in neuroblastoma cells

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N-myc amplification is indicative of a poor prognosis. Mechanisms by which N-myc over-expression affects cell cycle have not been fully resolved. To investigate specific N-myc effects on cell cycle, we utilized siRNA technique. Here we show that overexpression of the N-myc can be suppressed in NB cells by utilizing siRNA targeted sequences to the coding regions of N-myc mRNA. We chose 4 siRNAs, 1 localized to exon 2 and 3 spanning regions in exon 3. To screen effective siRNA sequences, we transiently co-transfected an N-myc expression vector into NIH3T3 cells with either a control or 1 of 4 different siRNA. We found that N-myc mRNA decreased 36-61% at 20hr and 44hr; while N-myc protein decreased 35-82% compared to control. We chose the two best sequences, one which was targeted to exon 2 and another to exon 3. We transfected a control and either of the 2 N-myc siRNAs into the LA-1-15N cells. We detected a 70% decrease in N-myc mRNA and protein levels. This caused a reduction in cell number that was accompanied by a decrease in E2F and an increase in p27 levels. These results are consistent with our previous data from models in which the NGF/TrkA or Retinoid pathways induced a decrease in cell proliferation that was accompanied by a decrease in N-myc, E2F and increase in p27. Thus, a number of different models show that through a common mechanism N-myc acts at multiple sites of the cell cycle machinery to alter cell cycle progression. By specifically targeting N-myc expression with siRNAs, we can explore the functions of N-myc on NB cell biology that are responsible for N-myc amplified NB patients having the worst prognosis.

Ref ID: 251.1 #128

Combined subtractive cDNA cloning and array CGH: an efficient approach for identification of overexpressed genes in DNA amplicons

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Activation of proto-oncogenes by DNA amplification is an important mechanism in the development and maintenance of cancer cells. Until recently, identification of the targeted genes relied on labor intensive and time consuming positional cloning methods. In this study, we outline a straightforward and efficient strategy for fast and comprehensive cloning of amplified and overexpressed genes. As a proof of principle, we analyzed neuroblastoma cell line IMR-32, with at least two amplification sites along the short arm of chromosome 2. In a first step, overexpressed cDNA clones were isolated using a PCR based subtractive cloning method. Subsequent deposition of these clones on a custom microarray and hybridization with IMR-32 DNA, resulted in the identification of clones that were overexpressed due to gene amplification. Using this approach, amplification of all previously reported amplified genes in this cell line was detected. Furthermore, four additional clones were found to be amplified, including the TEM8 gene on 2p13.3, two anonymous transcripts, and a fusion transcript, resulting from 2p13.3 and 2p24.3 fused sequences. To conclude, the combinatorial strategy of subtractive cDNA cloning and array CGH analysis allows cost-effective and comprehensive amplicon dissection, which opens perspectives for improved identification of hitherto unknown targeted oncogenes in cancer cells.

Ref ID: 328.1 #129

Gene expression patterns in neuroblastoma cells mediated by TrkA and TrkB expression and activation

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BACKGROUND: Neuroblastoma is a clinically and biologically heterogeneous disease. The differential expression of the receptor-tyrosine kinases TrkA and TrkB by neuroblastoma (NB) may contribute to the diverse biological behavior of these tumors and clinical outcomes. We have demonstrated previously that activation of these homologous receptors in NB cells produces primarily temporal differences in signaling pathway activation. However, preliminary analysis of downstream target gene induction revealed a few consistent patterns of divergent gene expression.
AIMS: In this study, we generated oligonucleotide-based gene expression profiles from TrkA and TrkB expressing NB cells to examine the transcriptional consequences of these signaling differences. Methods: The human NB cell line, SH-SY5Y was transfected with TrkA or TrkB, and RNA prepared from cells after activation with NGF or BDNF over a time course from 0 to 12 hours. Gene expression profiles were obtained using the Affymetrix U133A microarray platform, and raw expression data filtered through Probe Profiler to generate E-scores. Expression scores were filtered through pre-designed gene sets to examine the relative expression pattern of these functionally-grouped genes induced by specific ligand activation of TrkA or TrkB. Gene lists were grouped by their role in: cell/neuronal differentiation, apoptosis, angiogenesis, transcription factors, and cell-cycle regulation.
RESULTS: Target-genes activated by TrkA in NB included genes important in neural tissue development (CXCR4 and GATA3), and cell-cycle regulation; whereas gene targets up-regulated in TrkB expressing cells included regulators of angiogenesis (VEGF), anti-apoptosis (TR3, clusterin, IER3) and cell proliferation.
CONCLUSIONS: These data suggest that the unique transcriptional profiles of specific functionally-grouped genes resulting from TrkA and TrkB signaling may underlie the different clinical behavior of NB.

Ref ID: 373.1 #130

Application of Proteomics to Identify Novel Targets of Trk-Signaling in Neuroblastoma Cells

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Expression of Trk receptors is an important prognostic factor in neuroblastoma. Activation of the TrkA receptor by NGF mediates growth inhibition and differentiation of neuroblastoma cells, whereas TrkB/BDNF signaling promotes cell proliferation and chemotherapy resistance. Nonetheless, TrkA and TrkB exhibit a high level of sequence homology and use overlapping signal transduction pathways. To define differences in these signaling pathways and to identify novel effector molecules contributing to the characteristic phenotypes of TrkA- and TrkB-expressing neuroblastoma cells, we here used a proteomic approach of 2D gel electrophoresis and mass spectrometry. We transfected the neuroblastoma cell line SY5Y with the TrkA or TrkB cDNA or with the empty vector control (SY5Yvec). Global protein expression was analyzed in SY5Yvec, SY5Y-TrkA and SY5Y-TrkB cells activated by their specific ligands in a time course from 0-24 h. The recently introduced DIGE (fluorescence 2-D difference gel electrophoresis) system allowed reproducible identification of differentially expressed proteins between two samples in the same gel. In SY5Y-TrkA cells, we detected 8 proteins consistently up- or downregulated upon NGF-induced receptor activation (ratio > 1,3 compared to unstimulated cells, $p < 0,05$). Proteomic analysis of SY5Y-TrkB cells identified 23 proteins regulated upon BDNF-induced receptor activation (ratio > 1,5, $p < 0,05$). Differentially expressed proteins were subsequently identified by MALDI-PMF/PFF mass spectrometry. Functional assignment revealed that the majority of identified proteins is involved in cytoskeleton organisation and biogenesis. The biological function of selected proteins is currently characterized. Proteomics proved to be a promising tool to identify novel target proteins of Trk signaling in neuroblastoma cells.

Ref ID: 264.1 #131

Genes regulating drug sensitivity in human neurobalstoma

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Resistance of tumor cells to chemotherapeutic agents and high toxicity of these agents to healthy developing cells are the major limitations to chemotherapy in neuroblastoma patients. Cellular response to cytotoxic drugs is governed by different pathways ranging from drugs detoxification to apoptosis. Knowledge of the precise mechanisms of these pathways as well as their cross-communications is necessary to improve efficiency of chemotherapy and minimize side effects of chemotherapy. Amplification of MYCN is an accepted molecular prognostic factors of poor outcome and often is associated with acquired resistance to different drugs. In our previous study, we have shown that enforced in vitro expression of MYCN sensitize neuroblastoma cells to drug induced cell death. Now we implement a functional approach named "Technical Knock Out" (TKO) to study the response of the human neuroblastoma cell line Tet21N to doxorubicin. We have identified several genes that modulate the sensitivity of the MYCN overexpressing cells to doxorubicin. Among them are genes for death associated protein 3 (DAP3), lysosomal aspartil protease (CTSD), ribosomal protein (RPL27) and a microtubule binding protein (PRC1). Functional analyses using enforced expression and antisense mediated silencing demonstrate that these genes are important mediators of drug sensitivity in neuroblastoma cells.

Ref ID: 162.1 #135

MYCN signalling pathways in vivo and in vitro

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Amplified MYCN, which results in overexpression of MycN protein, is a powerful prognostic marker and identifies a group of high-risk patients with poor prognosis. MYCN amplified tumours, although sensitive to initial treatment protocols often acquire multi-drug resistance during the course of current cancer therapies. Most of the currently used chemotherapeutic drugs in neuroblastoma treatment protocols mediate cell death via p53 signalling pathways. Although p53 is rarely mutated in neuroblastoma tumours compared to other cancer types, p53 signalling pathways seem to be impaired in drug-resistant neuroblastoma tumours. The molecular pathways involved in this process are poorly understood. We provide evidence from in vitro data that targeted MYCN expression significantly sensitizes neuroblastoma cells to different death stimuli, which mediate apoptosis via p53-dependent pathways. To identify MYCN signalling pathways involved in this process we have used our in vitro neuroblastoma cell culture system (Tet21N), which allows targeted expression of MycN. Microarray technology was used to identify gene expression changes after target expression of MYCN in vitro. In addition, gene expression changes were monitored in drug-sensitive and drug-resistant neuroblastoma cells dependent on the expression of MYCN. These gene expression signatures were compared to gene expression profiles of 90 neuroblastoma tumours from different clinical subgroups. Protein analysis and functional knock down experiments were subsequently used to further characterize the role of MYCN target genes in the process of p53 activation and modulation of the p53 response.

Ref ID: 355.1 #136

Loss of hCas function, a novel neuroblastoma differentiation related gene, via LOH at chromosome 1p36.22 and functional silencing by N-myc

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Chromosome 1p deletion (LOH: loss of heterozygosity) is one of the most frequent cytogenetic aberrations in NB, and this chromosomal region has been scrutinized for putative tumor suppressor genes. In a search for genes regulating neuroblastoma differentiation, we identified a zinc finger gene, hCas, whose drosophila homolog regulates neurogenesis. hCas maps to chromosome 1p36 by FISH, and there is LOH and/or complete deletion of hCas in both NB cell lines (8/8) and primary NB tumors (3/3). During normal human fetal development hCas expression peaks at a time comparable to its expression in drsophila, and a time when migrating neuroblasts form the sympathetic ganglia and adrenal glands. In the retinoic acid (RA) induced NB differentiation model, hCas mRNA transiently increases and peaks after 24 hours of RA treatment. The increase in hCas mRNA is inversely correlated with a decrease in expression of the N-myc oncogene. In 8/9 NB cell lines there is an inverse correlation between hCas and N-myc expression ($R=-0.894$). Decreases in hCas by anti-sense hCas does not change N-myc levels. However, N-myc transfection and overexpression in NB cells consistently causes decreased hCas expression, thus N-myc negatively regulates hCas. We identified the promoter of hCas, which includes two putative N-myc binding E-boxes. A model in which one allele of hCas is deleted on 1p and the other allele is transcriptionaly silenced by the N-myc oncogene points to a potential mechanism by which two important genetic events in NB, N-myc amplification and 1p LOH, may contribute to tumorigenesis. The involvement in both drosophila and human neural development and the expression in NB differentiation raises the possibility that hCas may be a putative NB tumor suppressor gene on 1p.

Ref ID: 273.1 #132

ID2 promotes angiogenesis in neuroblastoma

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BACKGROUND: Recent evidence suggests that a critical effector of MYCN activity is Id2, which functions as a dominant-negative antagonist of bHLH transcription factors and proteins of the Rb family. We have recently found that Id2 can regulate the expression of vascular endothelial growth factor (VEGF), a key factor in tumor angiogenesis. Therefore we hypothesize that Id2 promotes angiogenesis in neuroblastoma.
METHODS: SH-N cell lines were transfected either with pCMV empty vector (EV) or pCMV-Id2, and selected for stable expression. Intrarenal xenografts were induced by implantation into the kidney of athymic mice and tumors allowed to grow for 8 weeks.
RESULTS: The incidence of tumor formation was higher in SH-N-Id2 than in SH-N-EV tumors (100% versus 55%). In tumor-bearing mice, average tumor weight was significantly greater in SH-N-Id2 than in SH-N-EV xenografts (4.7+1.2 versus 1.0+0.6 gm, $p<0.01$), and the incidence of bone marrow metastasis was also higher in Id2-expressing tumors (55% versus 22%). SH-N-Id2 tumors displayed increased proliferation as indicated by phosphorylated-H3, although there was no difference in the rate of apoptosis as determined by TUNEL assay. We found marked differences in vessel architecture in SH-N-Id2 in comparison to SH-N-EV tumors. Id2-expressing tumors displayed increased vessel density as assessed by lectin perfusion, and specific immunostaining for endothelial and vascular mural cells (PECAM-1, ASMA). This increased vascularity correlated with an increased expression of VEGF in SH-N-Id2 tumors, as demonstrated by immunohistochemistry and in-situ hybridization.
CONCLUSIONS: Our results demonstrate that Id2 promotes tumor growth and angiogenesis by increasing the expression of VEGF. These data suggest that antagonizing Id2 and its target genes may represent a novel therapeutic possibility for children with high-risk neuroblastoma.

Ref ID: 288.1 #134

Down regulation of gene-expression by N-myc in Neuroblastoma

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Amplification of the N-myc gene in neuroblastoma tumors is correlated with poor prognosis. The action of the transcription factor N-myc is dual: it can either activate transcription via dimerization with Max and binding to promoter elements (E-boxes) or repress transcription via a largely unknown mechanism. We used serial analysis of gene expression to identify which genes are down regulated by N-myc. The mRNA expression profile of a neuroblastoma cell line stably transfected with N-myc (SHEP-21N) was compared with that of the untransfected cell line (SHEP-2). A number of genes, mainly involved in cell-cell adhesion, construction of the cytoskeleton and extracellular matrix, and intracellular signalling, were significantly down regulated. We confirmed down regulation of a subset of these genes (including cdc42 and caveolin-1) using the cell line SHEP-21N, in which N-myc expression can be switched of with tetracyclin. A cell line stably transfected with the N-myc-ER chimera (SKNAS-NmycER) was used for additional analysis of genes down regulated by N-myc. In both SHEP-21N and SKNAS-NmycER we could discriminate between genes which were regulated fast (within 1-2 days) and genes which were regulated slow (after 4-5 days), pointing to different mechanisms of down regulation by N-myc. In order to find genes directly regulated by N-myc, we performed chromatin immunoprecipitation with an antibody against N-myc. Precipitates were analysed by semi-quantitative PCR with primer pairs directed against promoter regions of down regulated genes. We found that the promoter of cdc42 was specifically precipitated, pointing to direct interaction of N-myc with this promoter. Further promoter studies showed that the regulatory elements are located in the area 400 bp upstream of the transcriptional start of cdc42.

Ref ID: 399.1 #137

A Constitutional Translocation t(1;17)(p36.2;q11.2) in a Neuroblastoma Patient Disrupts NBG1, a Putative Tumor Suppressor Gene

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A constitutional translocation t(1;17)(p36.2;q11.2) in a neuroblastoma patient results in the interruption of a novel gene, NBG1 (Neuroblastoma Breakpoint Gene 1). This gene consists of 14 distinct types of coding exons, each occurring one to three times in the mRNA-transcript of 6.2 kbp. The latter encodes a protein of 139 kDa, showing no discernable functional domains or homology to any other known protein. Thorough analysis of genomic sequences revealed that NBG1 is a member of a recently duplicated, primate-specific gene family with gene copies located on human 1p36, 1p12-p13 and 1q21. All NBG gene family members are exceptionally similar to each other and show similar repetitive exon structures. Transfection experiments aiming at the isolation of stably transfected MCF7 cells overexpressing distinct NBG cDNA's were largely unsuccessful. From this we conclude that constitutive, high-level expression of NBG cDNA's is detrimental to these cells. The use of conditional expression systems may circumvent this problem. With an aminoterminal NBG domain as bait in a yeast two-hybrid screening, we identified an NBG-interacting protein that has been implicated in a pathway involved in neural crest development and oncogenesis. This interaction was confirmed in pull down experiments. We are presently investigating the functional implications of this interaction. Using additional domains of the NBG proteins as bait, we will look for more candidate interacting proteins. These results will hopefully help us to elucidate the intricate role these proteins may play in normal vs. tumoral cells.

ID: 340.1 #140

Detailed Characterization of a 1p36.3 Region Consistently Deleted in Neuroblastoma

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Substantial genomic and functional evidence from primary tumors and cell lines indicate that a consistent region of distal chromosome 1p is deleted in a large subset of human neuroblastomas, suggesting the presence of one or more tumor suppressor genes within this region. Allelic loss studies of 738 primary neuroblastomas and genotype analysis of 46 neuroblastoma cell lines defined a single region within 1p36.3 that was consistently deleted in 25% of tumors and 87% of cell lines. Two neuroblastoma patients had constitutional deletions of distal 1p36 overlapping the tumor-defined region. The tumor- and constitutionally-derived deletions together defined a smallest region of consistent deletion (SRD) between D1S2795 and D1S253. The 1p36.3 SRD was deleted in all but one of the 184 tumors demonstrating 1p deletion. Physical mapping and DNA sequencing determined that the SRD minimally spans an estimated 798 kb. Genomic content and sequence analysis of the SRD indicated the presence of 14 characterized, 10 uncharacterized, and 6 predicted genes in the region, whereas a number of previously hypothesized neuroblastoma suppressor genes mapped outside the SRD. The RNA expression profiles of eighteen of the genes were investigated in a variety of normal tissues as well as in neuroblastoma cell lines and/or primary tumor samples. The SHREW1 and KCNAB2 genes both had tissue-restricted expression patterns, including expression in the nervous system, and also low or absent expression in neuroblastoma-derived samples. In addition, a novel gene (CHD5) with strong homology to proteins involved in chromatin remodeling and histone deacetylation was expressed mainly in neural tissues, and at low levels in most neuroblastoma cell lines. Together, these results suggest that one or more genes involved in neuroblastoma tumorigenesis or progression are likely contained within this region.

Poster Display
GENETICS

Ref ID: 337.1 **#141**

Functional and expression analysis of CHD5, a candidate neuroblastoma suppressor on 1p36

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BACKGROUND: Deletion of 1p36 is a common genetic change in a substantial subset of neuroblastomas. We have defined the smallest region of consistent deletion (SRD) to ~800 kb using a large set of neuroblastoma tumors and cell lines. This region contains 14 characterized, 10 uncharacterized, and 6 predicted genes. Aims: Although we have excluded a number of these genes as candidates, CHD5 remains a viable candidate.
RESULTS: CHD5 is a novel member of the CHD gene family, which encodes proteins with chromatin remodeling, helicase and DNA binding motifs. This gene is preferentially expressed in neural tissues and is undetectable in almost all other tissues examined. CHD5 expression was consistently low or undetectable in a panel of neuroblastoma cell lines. Oligonucleotide-based mRNA expression profiling in 101 representative and highly annotated neuroblastoma samples showed significant differential expression among clinical risk groups, with low or absent CHD5 expression in the majority of high-risk tumors. Tumors with known 1p deletions (N=28) had significantly lower CHD5 expression when compared to tumors with no detectable 1p deletion (N=73; t-statistic 5.38). We transfected 18 neuroblastoma cell lines with a His-tagged CHD5 expression vector, and although all cell lines initially expressed CHD5 mRNA, only three expressed CHD5 protein, and subsequently both mRNA and protein expression was lost, suggesting that constitutive expression of CHD5 was not consistent with continued growth in vitro.
CONCLUSIONS: These findings suggest that CHD5 may be a tumor suppressor in neuroblastomas. Transfection studies using an inducible CHD5 expression vector are underway.

Ref ID: 282.1 **#142**

HsCAMTA1 and FLJ10737 within a commonly deleted region at 1p36 in neuroblastoma

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The distal portion of 1p is frequently deleted in human neuroblastomas, and it is widely assumed that this region harbors at least one neuroblastoma-related gene. A 1p36.3 common region of heterozygous deletion, bordered by D1S2731 and D1S1646, spans 441 kb and encompasses two genes, FLJ10737 and HsCAMTA1. We surveyed these for sequence variants by fluorescence based single strand conformation polymorphism (SSCP) using a panel of DNAs from 89 neuroblastomas, matching blood samples and 97 unaffected individuals. For FLJ10737, seven nucleotide variants were detected in neuroblastomas of which two dictate amino acid substitutions. For HsCAMTA1, 15 sequence variants were detected in neuroblastomas of which three dictate amino acid substitutions. Of these three, one (4007C>T, T1336I) was not detected in 97 unaffected individuals, another (3531C>G, N1177K) resides in a conserved domain of the HsCAMTA1 protein and was found hemizygous in six of 89 neuroblastomas. All variants predicted to cause amino acid substitutions were also detected in the matching blood cell DNAs of the respective patients. Real-time RT-PCR expression analysis in 86 neuroblastoma tumors and 16 neuroblastoma cell lines revealed significantly lower HsCAMTA1 expression in MYCN-amplified neuroblastomas. Our data do not provide evidence for a contribution of mutations in FLJ10737 or HsCAMTA1 to neuroblastoma formation. The functional significance of the identified variants remains to be determined. We further investigate the role of altered HsCAMTA1 expression in neuroblastoma using a vector based RNA interference (RNAi) system which allows efficient downregulation of HsCAMTA1 in neuroblastoma cell lines.

Ref ID: 213.1 **#145**

LOH analysis on microdissected neuroblastic cells reveals undetected interstitial deletions at chromosome 1p36 in neuroblastoma

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Neuroblastoma (NB) is a tumor that shows great morphological and genetic heterogeneity. NB is characterized by different cell sub-populations as well as by leukocytes infiltration, which may alter the results from tumor molecular analyses.
Laser capture microdissection (LCM) has been used to isolate malignant and normal cells from restricted areas of a tumor tissue. Molecular analyses carried out on microdissected cells can result in a more accurately detection of the genetic alterations. Chromosome 1p deletion is one of the most recurrent aberrations observed in NB and it is associated with tumor progression.
We used LCM to separate neuroblastic cells (N) from 7 stroma poor NBs (SP-NBs) and both N and schwannian stromal cells (SS) from 4 stroma rich intermixed ganglioneuroblastomas (SR-GNBs). PCR-LOH analysis of 1p36 region was performed by using 4 polymorphic markers (D1S468, D1S214, D1S244, D1S228). LOH was detected for at least one marker in N cells of 6 out of 7 SP-NBs. Interestingly, 3/6 cases did not show LOH in DNA purified from bulk tumor whereas they showed 1p36 imbalance or a low percentage of deletion by fluorescence in situ hybridization (FISH) analysis. Moreover, one case, whose deletion was not detected by FISH analysis showed 1p LOH for marker D1S244 on microdissected N cells. No LOH has been found in both N and SS cells from SR-GNBs. Our preliminary results indicate that LCM is useful to reveal LOH for those cases that would be undetectable by analysis of bulk tumor and to elucidate whether chromosome 1p deletion is a peculiar alteration of specific cell sub-populations.

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Ref ID: 075.3 **#146**

Analysis of MCL1 as a candidate oncogene within the 1Q region of gain in high-risk neuroblastomas

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We used a functional selection strategy with human neural cells to identify the BCL2 homologue, MCL1, as an oncogene that cooperates with deregulated MYCN. The selected apoptosis-resistant cell line had a clonal alteration [unbalanced t(1;7)] with trisomy 1q21-tel, and 3-fold MCL1 (1q21) overexpression sufficient to impede apoptosis and induce transformation [AACR 2003]. As 1q gain has been described in NBs we wished to investigate MCL1 as a candidate 1q oncogene. Human BAC aCGH was used to assess genomic alterations in 45 NB cell lines. 1q gain occurred in 12/36 MYCN amplified and 0/9 non-amplified lines (p=0.043) and defined a large SRO including MCL1. NBs arising in the transgenic TH-MYCN mouse were studied with murine aCGH [Hackett, Cancer Research 2003] and demonstrated gain of orthologous chromosome 3 in 16/39 (41%), with subchromosomal gain around MCL1 in additional tumors. Two of 6 TH-MYCN NB cell lines also showed chromosome 3 gain including MCL1. Human NB cell lines expressed more MCL1 mRNA than control cells, and more MCL1 than BCL2. Mcl1 protein was high in 4/4 MYCN-amplified and 2/4 nonamplified lines. Expression by RT-PCR in primary NBs was greater than fetal brain or adrenal, and the trend was higher for Stage 4 and high-risk tumors versus others, with no correlation with MYCN status. Thus, gain of chromosome 1q is common in human NBs and correlated with MYCN amplification. Gains are large and include MCL1. In support, gain of orthologous chromosome 3 occurs with the highest frequency of any alteration in TH-MYCN tumors. We will extend our MCL1 studies, including tumor tissue microarrays, to assess MCL1 expression with respect to MYCN status, tumor phenotype and 1q status or gene copy number.

Ref ID: 240.1 **#143**

Methylation studies of genes located in the chromosome region 1p36.2 in neuroblastoma tumors

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The distal part of chromosome 1p shows LOH in 20-40% of neuroblastoma tumors and has therefore been alleged to contain one or more tumor suppressor genes. We and others have previously narrowed down this region to 1p36.2-3 and more specifically to the gene region involving the genes: UBE4B-KIF1B-PGD-CORT-DFFA-PEX14. The known genes in the region have been analyzed for mutations and a few have been found in rare tumors. The genes are all, except for CORT, associated with a CpG island in their respective promoter regions. Methylation of CpG islands is a common mechanism for the inactivation of tumor suppressor genes and has been found in a wide range of tumor types. The most common way to analyze methylation status is based on bisulfite modification of DNA. In the current study, expression studies for the genes on 1p36.2 have been performed and the promoter regions of the genes that are associated with CpG islands have been analyzed for hypermethylation. Results from TaqMan expression analyses have revealed that some of the genes are less expressed in high stage tumors, as compared to lower stages. The studies show that the genes KIF1B, PGD, DFFA and PEX14 are not methylated in the neuroblastoma cell lines studied. Further studies on additional genes and also in primary neuroblastoma tumors are ongoing.

Ref ID: 162.2 **#144**

Combination of loss of heterozygosity and ploidy analysis clearly distinguishes between regions of allelic imbalance and allelic loss at 1p36 in neuroblastoma

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Surveys of tumours for loss of heterozygosity (LOH) and allelic imbalance (AI) are widely used as a tool for identifying tumour suppressor genes (TSG). LOH and AI are usually detected by either radioactive labelling of PCR products with subsequent scoring of autoradiographs or by a semi-quantitative fluorescence-based protocol. Polymorphic microsatellite loci are the most common marker type used in these studies. Even though no consensus exists as to how to evaluate such data, results from different studies are often compared and minimal common deleted regions (i.e., the smallest region of overlap, SRO) are defined, which then are used as the basis for tedious structural gene characterization analyses. In the present study, DNA from162 neuroblastoma tumour specimens (>50% tumour cells), as well as DNA from corresponding peripherial blood samples from each patient were analyzed by a semi-quantitative fluorescence-based protocol. Seven to 17 polymorphic microsatellite loci on distal 1p were used to detect LOH and AI. Ploidy status was analyzed by flow cytometry (FACS). Results were compared to interphase FISH analysis using the subcentromeric DNA probe D1Z1 at1q12 as well as D1Z2 at1p36.33. Twenty-three (14%) tumours showed LOH at distal 1p36. Forty-three (21%) tumours demonstrated allelic imbalance for more than one marker. Forty-two (98%) of these tumours revealed a DNA index ranging from 1.35-1.65 consistent with approximately three copies of each chromosome. Based on the estimated chromosome 1 copy number from the FACS analysis and the ratio comparison between neighbouring loci, we were able to calculate cut-off values for AI and LOH. From this analysis, we propose an evaluation of LOH/AI scoring which takes into account the ploidy or somy status of the investigated chromosome in each tumour. In addition, evaluation of each locus should be based on comparative analysis of neighbouring loci.

Ref ID: 071.2 **#147**

cDNA macroarrays analyses of p73-target genes reveal that Wnt8A is up-regulated by DeltaN-p73 in SH-SY5Y cells

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Ectopic overexpression of p73 promotes transcription of p53-target genes and induces tumor cell apoptosis, growth arrest and/or differentiation. In neuroblastoma (NB) cells, the p73 gene encodes 2 major isoforms: full length TA-p73alpha and N-truncated isoforms (DeltaN-p73alpha) deprived of the transactivating domain which can act as dominant negative inhibitors. We postulated that p53 mutation and an altered expression of p73 isoforms mark impaired neuronal differentiation in NB. To address this issue, we infected either full-length p73alpha or DeltaN-p73alpha recombinant adenoviruses into human NB cell lines, SH-SY5Y cells showing wild-type p53 and IGR-N-91 cells showing mutated p53. We had demonstrated previously that exogenous TA-p73alpha induces apoptosis in SH SY5Y cells and G1 arrest in IGR-N-91 cells (Goldschneider et al, 2004). Here we analysed the expression profile using the Atlas Human Cancer 1.2 Array Clontech, which is capable of detecting ~ 1,200m RNA species. Our results indicate that in both cell lines TA-p73 transactivates the expression of a group of genes associated with development and neuronal functions, such as Notch 1, MIC1/GDF-15, p75 NGFR, chromogranin B and, only in p53-proficient SH-SY5Y cells, other genes associated with growth factor, cellular adhesion, a p53-associated transcription factor and lysosomal protease function. As expected, DeltaN-p73alpha does not induce such genes leading to the likely hypothesis of an anti-differentiation role. Interestingly, Wnt8A is activated by either TA- or DeltaN-p73 only in SH-SY5Y cells, suggesting that transactivation is firstly p53 dependent and secondly independent of the NH2- terminal transactivation domain.

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Ref ID: 224.2 **#148**

p53 and JNK Apoptotic Pathways in Neuroblastoma

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Neuroblastoma tumors are generally sensitive to initial therapies but often acquire drug resistance following exposure to chemotherapeutic agents. An understanding of apoptotic pathways and how they become disrupted may lead to identification of novel targets for future neuroblastoma therapy. Two major apoptotic pathways are dependent upon the activity of either the p53 tumor suppressor protein or the c-jun NH2-terminal kinase, JNK. p53 gene mutations are rare in neuroblastoma and we have previously shown that the p53 apoptotic pathway is functional. The stress-activated protein kinase, JNK can phosphorylate and stabilize p53 following exposure to chemotherapeutic agents but the role of JNK in neuroblastoma cell death is unknown. We show here that JNK activity in unstressed NB-1643 cells expressing wild-type p53 (wtp53) is low, but that JNK activity can be induced by genotoxic stress. In contrast, isogenic cells without functional p53 (p53TDN-1 cells) exhibit high constitutive levels of JNK activity that cannot be further induced by stress. p21Waf1/Cip1 is a p53 target gene that has been shown to bind JNK and inhibit its kinase activity. These data suggest that p21 might be responsible for suppressing JNK activity in wtp53 expressing neuroblastoma cells. We are currently evaluating whether JNK mediates apoptosis in neuroblastoma independent of wtp53, and determining the interaction between p21 and the JNK and p53 apoptotic pathways.

Poster Display
GENETICS

Ref ID: 087.3 **#149**

High expression of novel HECT-type E3 ubiquitin ligases, NEDL1 and NEDL2, which target Dishevelled-1 and p73, respectively, is associated with favorable neuroblastomas with spontaneous regression

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To better understand molecular mechanism of neuroblastoma biology, we constructed the neuroblastoma cDNA libraries, from which we identified a novel gene, Nbla0078, which showed a similar structure to NEDD4, then termed it as NEDL1. We also cloned the gene with a similarity to NEDL1 and termed it as NEDL2. They encoded the HECT-type E3 ubiquitin ligases with two WW domains. NEDL1 was specifically expressed in brain and kidney, while NEDL2 ubiquitously. In vitro ubiquitination assay showed their catalytic functions similar to that of E2 and NEDD4 family. The quantitative real-time RT-PCR analysis for 99 primary neuroblastomas showed that high levels of NEDL1 and NEDL2 expression were significantly correlated with younger age less than one year (p<.00005 & p<.0002, t-tests), stages 1+2+4s (p<.00005 & p<.00005), high TrkA expression (p<.00005 & p<.00005), and single copy of MYCN (p<.00005 & p<.0013), respectively. The logrank test showed that low level of NEDL1 and NEDL2 expression was associated with an unfavorable outcome (p=.0001 and p=.028), respectively. Thus, expressions of both NEDL1 and NEDL2 are highly significant prognostic indicators of neuroblastoma. To further understand their functional roles in cancers, we then searched for the interacting molecules. Interestingly, NEDL1 was found by using yeast two-hybrid screening to physically interact with Dishevelld-1, an important Wnt signaling molecule, and to ubiquitinate it for degradation. Surprisingly, NEDL1 also bound to mutant SOD1, but not wild type SOD1, to form aggregates in the spinal motor neurons in FALS. On the other hand, NEDL2 was found to target p73 for ubiquitination. Surprisingly, however, it stabilized p73 to enhance the transactivation activity to induce apoptosis. Thus, both NEDL1 and NEDL2 E3 ubiquitin ligases may play important roles in inducing differentiation and/or programmed cell death in favorable neuroblastomas.

Ref ID: 238.1 **#150**

Shc Family Expression in Neuroblastoma

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The biological feature and prognosis of neuroblastoma (NB)   is known to be strongly associated with expression of trk receptors. However, the regulation of NB's growth, differentiation and regression has not yet been revealed. Shc family (ShcA, ShcB and ShcC), adaptor molecules of various receptors including trk receptors were found to be regulators of neuronal development. ShcA highly expressed in developing brain whereas ShcB and ShcC found to be expressed in postmitotic maturing neuronal cells. In this study, we aimed to see how these 3 genes are expressed NB cell lines, all-trans-retinoic (RA) treated line and 52 samples semiquantitative RT-PCR. In NB cell tumor samples, shcA was ubiquitously highly expressed. Little characteristics of ShcA was possibly due its expression and multivariate function. Whereas shcB was hardly expressed in NB cell lines but its after RA induced differentiation and in low-stage tumors (p=0.0095). ShcB might be affecting differentiation hence acting as a favorable factor. Expression of shcC was observed in most of NB cell lines. In stage 4 patients expression of shcC was remarkably high. Prognosis of these patients were very poor, with MYCN amplified and all died within 31 months after diagnosis. Expression of shcC was associated with patient survival (p<0.0001). ShcC seems to be associated with tumor progression by adding a malignant feature in aggressive tumors.

Ref ID: 276.1 **#153**

Somatic genetic events occurring in infant with neuroblastoma during the first year of life

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Tumor aggressiveness of neuroblastoma has mainly been associated with MYCN amplification (NMA) and chromosome 1p36 deletion (1p36del). However, tumorigenesis of neuroblastoma remains elusive. Tumors of infants represent a suitable natural model to identify the early events occuring in NB in a well-defined period of life. We observed 113 children with NB in the first year of life. We evaluated NMA and 1p36del by FISH, PCR-LOH analysis at 1p36 and DNA index in 97 available tumors. Moreover, CGH was employed to identify further DNA imbalances in tumors with 1pdel. Chromosome 1pdel was found in 12/97 (12%) tumors and NMA in 9/97 (9%). Seven out of nine NMA tumors also had 1pdel. Imbalance of chromosome 1p36 as tri-, tetra-, pentasomy but not deletion was found in 43/97 (44%) cases and a normal pair of chromosome 1p36 was observed in 42/97 (43%). Four patients, whose tumors had 1pdel died for disease progression; three of these had also NMA. The distribution of patients according to the age at diagnosis showed that during the first three months of life, only 5% (2/40) tumors had 1pdel and/or NMA; whereas, after that time the frequency of 1p36del raised up to 18% (10/57) and NMA raised up to 12% (7/57). Numerical and structural chromosome abnormalities were observed by CGH in tumors with 1p36del. Finally, four embryonal masses observed by ultrasonography had neither MNA nor 1p36del. Our results show that in neuroblastoma of infants: a) were present both numerical and structural chromosome abnormalities, and b) 1p36del and NMA are more frequently observed after the third month of life. Our observation indicates that more than one mutation is occuring in the first year of life and suggests a complex mechanism of tumorigenesis departing from both two-hit hypothesis and multistep model.

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Ref ID: 223.1 **#154**

Additional evidence for linkage of hereditary neuroblastoma to 16p13 and discovery of cooperating mutations

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Inherited predisposition to neuroblastoma shows incomplete penetrance and high lethality, resulting in relatively small pedigrees and contributing to the rarity of the condition. We previously reported linkage of familial neuroblastoma to 16p12-13 (HNB1; LOD = 3.3). We have now identified additional neuroblastoma families, including one with 7 affected individuals. This family shows evidence for linkage to 16p (cumulative LOD now 3.7), and an informative recombination event refined the HNB1 locus to D16S3075 - D16S403 (13.5 Mb). In addition, we recently identified a large pedigree with 6 siblings affected with pheochromocytoma and showing no mutations at RET, VHL, SDHD and SDHB. This family also shows evidence for linkage to 16p, and if we assume that HNB1 is a neural crest tumor predisposition locus, the combined LOD = 5.6 and the region is narrowed to D16S748 - D16S499 (4 Mb at 16p13). We have discovered several occult deletions within the putative HNB1 locus in familial, multifocal and congenital neuroblastoma tumors by array CGH. These deletions are currently being mapped with higher resolution using a 16p-specific BAC array with complete coverage across the HNB1 locus. In addition, we have recently identified a novel germline nonsense mutation in a gene critical to autonomic nervous system development in affected members of a family linked to 16p. The gene is not located on 16p, and the proband also has a previously described nonsense mutation in NF1. Additional kindreds are currently being screened for mutations. Taken together, these data strongly support the existence of a neural crest tumor predisposition locus at 16p13, but also further demonstrates that the genetic basis of neuroblastoma is complex. Our data suggest a model of oligogenic predisposition to neuroblastoma involving a gene at 16p13 cooperating with gene(s) involved in the development of the autonomic nervous system.

Ref ID: 260.1 **#151**

Hunting for a 3p tumor suppressor gene in neuroblastoma: a combined genomic and functional approach

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Recent genetic analyses have defined a new subgroup of aggressive neuroblastomas (NB) with 11q deletion and 17q gain. These tumors also exhibit a high incidence of 3p deletions. Thus far, only few studies were performed in order to map and refine the critical region for 3p loss in NB. In this study we performed high-density LOH analysis with 30 markers covering the distal half of the chromosome 3 short arm and control markers on 3q on a series of 30 NB cell lines and 100 tumors. To accomplish this study, we specifically developed a cost-effective and innovative multiplex PCR strategy for high throughput marker analysis. Because LOH patterns provide only circumstantial evidence and no ultimate proof for the localization of a suppressor gene on 3p, we also transferred an intact chromosome 3 in two neuroblastoma cell lines harboring a 3p deletion by microcell mediated chromosome transfer. Currently, the hybrid clones are characterized by marker analysis and FISH. In addition to refinement of the critical region of loss, we decided to embark upon candidate 3p gene analysis by mutation screening (VHL) and promoter hypermethylation analysis (VHL, RASSF1A, BLU, ROBO1). Although no mutations were found in the coding region and no evidence for methylation of the VHL promoter region was obtained, a sensitive real time RT-PCR assay showed significant reduced VHL mRNA expression in 3p deleted cell lines, and a significantly reduced survival probability for patients with low VHL expressing tumors. Our findings suggest that VHL haploinsufficiency contributes to the pathogenesis of 3p deleted aggressive NB.

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MSX1: a target from the MEIS1 oncogene located on the 4p16 tumour suppressor region

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BACKGROUND AND AIMS: The main goal of this project is an extensive study of the MEIS1 pathway. SAGE (Serial Analysis of Gene Expression) and DNA-microarray analysis of cell lines transfected with the MEIS1E dominant negative splice variant resulted in the identification of several hundred putative MEIS1 target genes. One important approach to study MEIS1 target genes is to focus on genes that are present in chromosomal regions which are frequently deleted or amplified in neuroblastoma. Loss of heterozygosity (LOH) is found in 20-25 % of neuroblastoma patients, and the 4p16 region was recently considerably narrowed by Perri et al. This suggests that there is a tumour suppressor gene located in this region.
RESULTS AND CONCLUSIONS: Immediately adjacent to this region the homeobox transcription factor MSX1 is found. During development, MSX1 is involved in the formation of the neural tube. MSX1 has been shown to be involved in cell cycle arrest and differentiation. SAGE analysis demonstrated that MSX1 is negatively regulated by the MEIS1 gene in neuroblastoma cell lines, and northern blot analysis of a panel of MEIS1 transfectant clones confirmed this. Mutation analysis was performed on 50 neuroblastoma samples (8 with LOH on chromosome 4p) and 22 cell lines. No mutations were discovered in the coding region of MSX1. This proposed downregulation of MSX1 by the MEIS1 gene is a first insight into the MEIS1 pathway.

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High-resolution Array-based Genomic Profiling of the Critical Region of Chromosome 17 Gain

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Unbalanced translocations resulting in gain of chromosome 17q and partial loss of the partner chromosome are the hallmark of advanced stage neuroblastomas. While partial gain of the distal part of the long arm of 17q has been proven to be the most important independent marker for poor patient survival, the responsible molecular defect remains elusive. Thusfar, candidate gene identification has been hampered by the large size (~25 Mb) of the chromosomal 17q23-qter segment for which consistent gain has been observed. In order to refine the critical region of gain, we applied high-resolution arrayCGH screening, using the Sanger set BAC clone array with a 1 Mb spacing. This array was supplemented with a quasi tile path of more than 500 BAC clones from chromosome 17, resulting in an average resolution of 150 kb along this chromosome. Cell lines with known chromosome 17q breakpoint positions were hybridized as controls, followed by ongoing arrayCGH screening of advanced stage primary tumors, including a large series which showed no chromosome 17 gain by conventional chromosome CGH. To deal with the large amounts of generated data, we developed a comprehensive and MIAME compliant MySQL-based database and data mining web tool in order to store, compare, analyze, interpret and graphically display arrayCGH results in a uniform and user friendly format. Our arrayCGHbase web tool is available online (http://medgen.ugent/be/arraycghbase/), or can be installed on a local server running the free MySQL database and PHP scripting language. Detailed results on the high resolution mapping of the 17q low copy gain amplicons will be presented.

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Regions syntenic to human 17q are gained in mouse and rat neuroblastoma cell lines

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Gain of chromosome arm 17q is the most frequent chromosomal change in human neuroblastoma suggesting that this region includes a gene, or genes, critical for tumour pathogenesis. Because the shortest region of 17q gain (SRG) encompasses >300 genes, it precludes the identification of candidate genes from human breakpoint data alone. However, mouse chromosome 11, which is syntenic to human chromosome 17, is gained in up to 30% of neuroblastoma tumours developed in a murine MYCN transgenic model of this disease. To confirm that this key genetic change indicates the involvement of a conserved molecular pathway we have used FISH to analyse sporadic cases of both mouse and rat neuroblastoma. Our results confirm the presence of chromosome 11 gain in all 3 mouse cell lines we analysed, with the SRG extending from Stat5b to tel. In addition, the rat neuroblastoma cell line harbours an extra copy of distal chromosome 10 which is also syntenic to human 17q. Comparison of the regions gained in all three species excludes ~4 Mb from the previously defined region of 17q gain in human as a likely location of candidate gene/genes, and strongly suggests that the molecular aetiology of neuroblastoma is similar in all three species. Comparative gene expression analyses from all three species are currently being performed in an effort to identify candidate genes.

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Interphase FISH reveals intratumoral heterogeneity of chromosome 17(q) status in neuroblastoma: implications for risk assessment

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Chromosome 17(q) overrepresentation is the most common genomic defect in neuroblastoma (NB). Low stage NBs are usually characterized by the presence of extra copies of chromosome 17, whereas high stage NBs typically present with overrepresentation of chromosome 17q material. Multivariate analyses have shown that chromosome 17q overrepresentation is the most powerful independent predictor for adverse prognosis. Therefore 17(q) status might become an important genetic factor in therapy stratification. Consequently, as previous studies have indicated that use of a single technique for determining a genetic marker can lead to erroneous interpretations [1], at least two reliable methods for 17(q) copy number assessment should be available. Currently, interphase FISH or quantitative PCR appear to be the methods of choice. In the present study, we evaluated the reliability of interphase FISH for determining 17(q) status on fresh NB tumour samples. Our results show that in a significant proportion of tumours, intratumour heterogeneity for chromosome 17 copy number can be detected. Apart from variation in absolute copy number of chromosome 17q or 17, we also observed, within the same tumour, FISH patterns indicating cells with either partial or whole chromosome 17 gain. These results were matched with standard CGH, arrayCGH and quantitative real time PCR data. Further studies are warranted in order to assess the effect of intratumour heterogeneity on clinical outcome and to determine the usefulness of FISH in determining 17(q) status.

1. Ambros et al. J Clin Oncol 21:2077-2084 (2003)

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Molecular cloning of unbalanced chromosome 17 breakpoints in neuroblastoma cell lines reveals distinct underlying mechanisms

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In neuroblastoma, the most frequent genetic alterations are unbalanced chromosome 17 translocations leading to gain of distal 17q, which is thought to play an important role in the oncogenesis of this tumor through a dosage effect of genes located on this chromosome. However, little is known about the mechanism leading to these unbalanced translocations, and to date, no such translocation has been characterized at a molecular level. In order to analyze their molecular structure, we have now cloned the breakpoints of the following unbalanced chromosome 17q translocations: a der(1)t(1;17)(p34;q12) and a der(1)t(1;17)(p13;q12) in cell lines CLB-Bar and CLB-Ma, a der(11)t(11;17)(q13;q11) in SK-N-AS, and a der(3)t(3;17)(p21;q11) in SK-N-BE. Our approach consisted of a delineation of the chromosome 17q breakpoints by FISH using BAC/PAC clones. Subsequently, fine mapping by FISH using PCR generated probes was performed. Southern analysis identified rearranged fragments in all cell lines, and phage libraries were constructed, which were then screened for recombinant clones containing the rearranged fragments. Positive recombinant clones were sequenced, and the exact position of breakpoints on 17q and the recipient chromosomes could be identified in all four cases. Distinct molecular features could be identified at the translocation breakpoints, suggesting that different molecular mechanisms may lead to unbalanced chromosome 17 translocations in neuroblastoma. The disrupted genes and the molecular structure at the fusion sites will be further described.

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LIM-only protein, LMO3, is up-regulated and acts as an oncogene in aggressive neuroblastomas by physically interacting with a neuronal bHLH transcription factor, HEN2

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LIM-only transcription regulators, which consist of four members including LMO1, LMO2, LMO3 and LMO4, are involved in cell fate determination and differentiation during embryonic development. Accumulating evidence suggests that both LMO1 and LMO2 act as oncogenic proteins in T-cell acute lymphoblastic leukemia, while LMO4 has recently been implicated in the breast carcinogenesis. However, little is known about the functional role of LMO3 in either tumorigenesis or development. Here we have identified both LMO3 and HEN2, which encodes a neuronal basic helix-loop-helix protein with unknown function, as the genes with oncogenic ability in neuroblastoma. RESULTS AND DISCUSSION: Our previous neuroblastoma cDNA project, which has cloned more than 5,000 independent genes, has identified LMO3 as one of the genes expressed significantly at high levels in unfavorable neuroblastomas. LMO3 mRNA was specifically expressed in brain and adrenal gland. On the analogy of LMO1 in T-cell leukemia, we hypothesized that there must be a neuronal complex of transcription factors including LMO3 and its unknown bHLH binding partner(s) like HEN1 and HEN2. Immunoprecipitation analysis demonstrated that LMO3 physically interacted with HEN2 and both proteins were co-localized in mammalian cell nucleus. The human SH-SY5Y neuroblastoma cells stably overexpressing LMO3 showed a marked increase in cell growth, a promotion of colony formation in soft agar and a rapid tumor growth in nude mice. More importantly, the increased expression of LMO3 and HEN2 was significantly associated with a poor prognosis in 87 primary neuroblastomas, albeit there was no amplification of the LMO3 gene as examined by Southern blot. Thus, our results suggest that the deregulated expression of neuronal-specific LMO3 and HEN2 contributes to the genesis and progression
of human neuroblastoma in a lineage-specific manner.

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Imbalance of transcripts encoding mitochondrial pro- and anti-apoptotic members in unfavorable neuroblastoma

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BACKGROUND AND AIMS: In an attempt to investigate the role of aberrant apoptosis in neuroblastoma (NB) progression, we sought transcripts differentially expressed unfavorable versus favorable NB tumors.
METHODOLOGY: cDNA filters consisting of 198 apoptotic genes from R&D-systems were screened in four stage 4 versus four stage 1 NB tumors. Eleven genes showing a relevant fold-change (>2.0) between groups were selected and further analyzed by real-time PCR (TaqMan) for verification in a new set of NB tumors. Another ten genes encoding factors involved in the mitochondrial apoptotic pathway were also analyzed by TaqMan for differential expression.
RESULTS: Of the eleven genes selected from the cDNA array, three mRNAs preferentially expressed in unfavorable tumors; TRAF2, CDKN1A, IL2RA, and four mRNAs preferentially expressed in favorable tumors; APAF1, NTRK1, LTA, and CASP7 with statistical significance (nominal $p<0.05$; two-tailed). By TaqMan analysis, DNCL1 and NTRK1 could be verified to be poorly expressed in unfavorable NB tumors (corrected $p<0.05$; one-tailed). Of the ten genes selected due to their mitochondrial involvement, BID (corrected $p=0.055$; two-tailed), and BCL2 (corrected $p=0.055$; two-tailed) showed nearly significantly lower mRNA levels in tumors of advanced NB stages. Several other transcripts encoding mitochondrial apoptotic mediators were also found to be lower in tumors of unfavorable biology. Conclusion. In conclusion, mRNA levels of DNCL1 and NTRK1 was significantly lower in tumors of patients with an adverse outcome. Our data also suggests that the mitochondrial pathway is suppressed at multiple steps in advanced stages of NBs, due to imbalance between anti-apoptotic and pro-apoptotic members.

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Quantitative analysis and detailed mapping of 17q gains in neuroblastomas using array CGH

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Gain of chromosome arm 17q is the most frequent cytogenetic abnormality in neuroblastomas, and is strongly associated with an adverse outcome of the disease. In search for candidate oncogenes present in 17q region, and potentially involved in tumor progression, we have built a BAC-array dedicated to the
CGH study of neuroblatomas. About 300 BAC or PAC clones were selected in regions showing frequent abnormalities in this cancer, with a high density on 17q. Clones showing a single well-mapped signal by FISH were used for arraying in 6 replicates. Control hybridization experiments, with normal human genomic DNAs, showed ratios centered on 1, with high signal to background ratio (average 7-8) and low standard deviations (0,038). Moreover, array-CGH of tumoral DNAs from cell lines precisely characterized by CGH, FISH and/or spectral caryotyping indicated a reliable evaluation of genome copy number, and an accurate delimitation of chromosomal abnormalities, with high sensitivity and reproducibility. This BAC-array is actually used to analyze a series of neuroblastomas. The tested genomic 17q region encodes for several transcription factors, genes involved in cell cycle regulation, and antiapoptotic genes. The results indicative of the smallest over-represented region will be presented, and the gene content discussed. We also developed a stoechiometric whole genome amplification method, in order to have a sufficient amount of DNA for the study of needle aspirates of tumors or laser microdissected tumoral foci.

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EMP3, A Putative Tumor Suppressor Gene Mapping At 19q13.3, Is Epigenetically Silenced In Neuroblastoma By Promoter Hypermethylation and May Have Prognostic Value

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BACKGROUND: Inactivation of tumor suppressor genes may occur by epigenetic silencing associated with the hypermethylation of the CpG islands at the promoter regions of these genes. A putative neuroblastoma suppressor gene located at 19q13.3 has not been identified to date by using standard genetic and molecular biology approaches.
METHODS: We analyzed 89 neuroblastoma tumors, 10 ganglioneuromas and 9 normal bone-marrow samples by Affymetrix Human U95v2 oligonucleotide arrays (62839 probe-sets). Candidate genes were analyzed by methylation-specific-PCR (MSP) in 116 human neuroblastic tumors, 10 neuroblastoma cell lines, 41 gliomas and 47 non-neuroblastoma cell lines.
RESULTS: 100 genes were significantly downregulated by expression array in neuroblastoma tumors when compared with ganglioneuromas and normal bone marrows, including a gene located at 19q13.3 (EMP3, epithelial membrane protein 3, involved in cell proliferation and cell-cell interactions). By MSP, EMP3 promoter was hypermethylated (24.1% neuroblastic tumors, 55.5% neuroblastoma cell lines, 39% gliomas and 40.4% non-neuroblastoma lines). Demethylating agent 5-aza-2-deoxycytidine restored EMP3 gene expression. EMP3 promoter hypermethylation was associated with poor survival (Kaplan-Meier $p=0.03$), with strong correlation with death ($p=0.03$; $r=0.19$). For both local-regional and metastatic neuroblastoma, death rate was higher with methylated EMP3 (33.3% for LR and 78.6% for stage 4) than with unmethylated EMP3 (13.3% for LR and 46.1% for stage 4).
CONCLUSIONS: Epigenetic silencing of the gene EMP3 by promoter hypermethylation is associated with a poor survival for both local-regional and stage 4 neuroblastomas. This gene could be a tumor suppressor gene located at 19q13.3.

Ref ID: 312.1 **#163**

Tumor suppressor genes methylation in neuroblastoma: RASSF1 gene is almost always methyled in primary tumors

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BACKGROUND: mechanisms of neuroblastic tumors initiation and development remain unclear. Aberrant hypermethylation has recently been showed as an important pathway for the repression of gene transcription in cancer. This study aims to define a methylation profile in childhood neuroblastoma (NB). Methodology: We investigated the methylation of 12 genes: p14ARF, p15INK4a, p16INK4a (9p21), DAPK (9q39.1), MGMT (10q26), FHIT (3p14.2), RAR b (3p24) EP300 (22q13.3), NF2 (22q12), INI1(22q11.2), TIMP3 (22q12.1) et RASSF1 (3p21.3). by methylation-specific polymerase chain reaction after bissulfite treatment of DNA in a series of 46 NB of different stages (18% stage 1, 33 % stage 2, 9% stage 3, 30 % stage 4, 9.1% stage 4S). These tumors had been analyzed by comparative genomic hybridization and molecular biology for MYCN (MYCN amplification in 24% of cases, 1p deletion in 10% and 3p deletion in 8%). Results: The frequency of methylation was 93% for RASSF1, 48% for TIMP3, 4% for RAR b and p15. Only 3 NB presented no RASSF1 methylation: 2 NB stage 1 and 1 NB stage 4S. The media of methylated genes was 2 (minimum 0, maximum 3). There was no clear association between the cytogenetic and methylation abnormalities tested untill this moment.
CONCLUSION: Methylation seems to be an important mechanism of tumor-supressor gene inactivation in neuroblastoma. It is already known that RASSF1 is methylated in the majority of lung tumors and in a proportion of breast tumors. Previous studies have observed 55% of RASSF1 methylation in neuroblastoma and in almost 100% of neuroblastoma cell lines. We were able to identify a higher percentage of RASSF1 methylation than in these previous studies. This is may be due to the systematic histological confirmation that was done before DNA extraction. Nowadays, we are studying a larger number of genes and neuroblastomas.

Ref ID: 251.2 **#164**

A putative haplo-insufficient contribution of SDHD to high stage neuroblastoma tumorigenesis

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Deletions in 11q are observed in a subgroup of neuroblastomas with poor outcome. The deleted region harbors the tumor suppressor gene SDHD that is frequently mutated in paraganglioma and pheochromocytoma, which are, like neuroblastoma, tumors originating from the neural crest. In this study, we sought for evidence for involvement of SDHD in neuroblastoma. Analysis at the genomic level of 67 tumor samples and 37 cell lines revealed at least 2 bona-fide mutations in cell lines without 11q-deletion: a 4bp-deletion causing skip of exon 3 resulting in a premature stop codon in cell line N206, and a Y93C mutation in cell line NMB located in a region affected by germline SDHD mutations causing hereditary paraganglioma. No evidence for hypermethylation of the SDHD promotor region was observed, nor could we detect homozygous deletions. Interestingly, SDHD mRNA expression was significantly lower in SDHD mutated and 11q-deleted cell lines as compared to both cell lines without 11q-deletion and normal fetal neuroblast cells. Protein analyses and assessment of mitochondrial morphology presently do not provide further clues as to the possible effect of reduced SDHD expression on the neuroblastoma tumor phenotype. Therefore further studies are needed to determine the possible role of SDHD haplo-insufficiency in high-stage neuroblastoma.

Poster Display
GENETICS

Ref ID: 356.1 **#165**

Akt pathway mediates BDNF/TrkB resistance to chemotherapy in neuroblastoma cells

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Patients whose neuroblastoma (NB) tumors expressing high levels of brain-derived neurotrophic factor (BDNF) and TrkB often associated with poor progress. Our previous studies indicated that BDNF blocked the cytotoxic effects of chemotherapeutic drugs such as Etoposide, Vinblastin, Doxorubisin and Cisplatin via phosphatidylinositol 3-kinase(PI3) kinase pathway. Akt, an important downstream target of PI3K, exerts its functions such as regulation of cell cycle, protein synthesis and apoptosis through its downstream targets. In the present study, we examined whether Akt is required and sufficient to mediate BDNF protection of NB cells from chemotherapy. BDNF caused a rapid and time-dependent phosphorylation of Akt, as well as Akt downstream targets p70S6kinase and GSK-3, and these events were inhibited by LY294002, a PI3K inhibitor. Transient transfection of a constitutively-active Akt(Myr-Akt) into the TrkB expressing SY5Y cells TB8 increased total and phosphorylated Akt and attenuated the cell death induced by Etoposide in the absence of BDNF. Furthermore expression of a dominantly-negative Akt blocked the BDNF rescue of NB cells from Etoposide-induced cell death. Besides, Forkhead family members may be the downstream targets of Akt that mediates BDNF rescue of cell death. These results indicated that Akt is a key signaling component by which BDNF protects NB cells from Etoposide-induced cell death. Novel pharmacologic inhibitors targeted to Akt are being evaluated to specifically block the effects of the BDNF/TrkB path and restore the sensitivity of NB cells to chemotherapy.

Ref ID: 339.2 **#166**

Tight regulation of retroviral gene expression by combination of advanced tetracycline-transactivator and repressor proteins

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A number of systems for conditional gene expression in mammaliancells have been described, however, their use is often difficult due to rare integration into the host genome and high background transcription in the absence of the inducing drug. We constructed a tetracycline expression system that combines a set of tetracycline-regulated transactivators with tetracycline-responsive transcriptional silencers in one retroviral vector. Tet-transactivator, tet-repressor and selection marker genes are transcribed as tricistronic mRNAs allowing efficient selection of those infected cells that express high cellular levels of transactivator/repressor-mRNA. The reporter genes are inserted in a self-inactivating viral vector that
contains a tetCMV promoter and a selection marker. More than 92% of infected neuroblastoma cells, selected for stable insertion of both virus-vectors, expressed the reporter gene EGFP in a tetracycline-regulated manner. Both silencer proteins, tetRKrab and TRSID, repressed background transcription of EGFP to auto-fluorescence. Interestingly, while cells with a tetRKrab repressor had only poor induction rates when stably integrated into the host genome, a combination of the rtTA-M2 transactivator and the TRSID repressor allowed for up to 1100 fold induction and an almost linear regulation of transgene expression in neuroblastoma cells.

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Comparative analysis of transcription factor function in pre B and neuroblastoma cells

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The childhood malignancy neuroblastoma is derived from sympathetic nervous system precursor cells arrested at immature stages of the sympathetic differentiation. To gain further insight into the molecular processes involved in these differentiation events we study the O/E-protein family of transcription factors. EBF expression is essential in B-cell development and these factors are also known to be involved in neurogenesis in Xenopus and C. elegans. The O/E-proteins are expressed in neuroblastoma cells at different levels, and EBF has been linked to the activation of transcription of the neuronal marker genes Chromogranin A (CgA) and SCG10. We investigate the expression of Olf/EBF (O/E) transcription factors in human neuroblastoma cells in comparison to expression of the same factors in pre B-cells. We here report that several transcription factors, such as Oct, Pax5 and EBF, are expressed in both cell types. However, EBF regulates the expression of different target genes in different tissues. In the neuroblastoma cell lines SK-N-BE(2)c and SH-SY5Y, EBF up-regulates the expression of CgA and SCG10, while in pre B-cells, EBF target Mb-1 and cd19. Also, the ubiquitously expressed E-proteins, which are known to be involved in many important processes, are expressed in different levels in pre B-cell compared to neuroblastoma cells. In pre B-cells the E-protein E47 cooperates with EBF in gene activation, while in neuroblastoma cells, E2-2 is the most likely partner. In order to investigate the mechanisms by which the target genes are regulated we study epigenetic control mechanisms of these promoter regions.

Ref ID: 130.1 **#170**

High-hyperdiploidy, an overlooked sub-group in neuroblastoma?

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BACKGROUND: High-hyperdiploidy defined by cytogenetic analysis is a recognised non-random sub-group in a range of childhood tumours. However, to date, neuroblastoma is not included amongst them. METHODS AND RESULTS: In a series of 53 neuroblastomas with full or partial conventional cytogenetic characterisation 9 (17%) had evidence of a high-hyperdiploidy clone or sub-clone. Six from nine cases had stage 4 disease, and the other three stage 1 or 2 disease. The tumours were characterised by the biological aberrations del(1p) 3 cases, MYCN amplification 1 case and unbalanced gain of 17q 3 cases respectively. There was frequent gain of chromosomes 1,2,6,7,12,17 and 20, which in the case of chromosome 1 was often in the form of an additional aberrant chromosome. A notable feature was the presence of karyotypic evolution/instability, often resulting in the presence of clones with different ploidy levels. Comparison with the high-hyperdiploid sub-group of other childhood tumour types showed a strong similarity to the chromosomes non-randomly gained in embryonal rhabdomyosarcoma (eRMS) and Ewing tumours (ET).
CONCLUSIONS: This latter observation leads to the suggestion that neuroblastoma may share with eRMS and ET a common mechanism/aberration that leads to high-hyperdiploidy. The overall karyotypic picture of the neuroblastoma cases indicated that the hyperdiploid clone could either derive from an earlier near-diploid clone and/or act as a precursor of a hypotriploid clone. It is this latter feature and the frequent gain of chromosome 1 may explain why high-hyperdiploidy has not been previously defined as a distinct sub-group in neuroblastoma.

Ref ID: 230.1 **#167**

NTRK1 gene polymorphisms in 30 primary neuroblastoma tumours

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Neuroblastomas with high expression of NTRK1 gene are associated with favourable prognosis, therefore investigation of polymorphic patterns of NTRK1 gene was performed to identify correlation of their occurrence with clinical and cytogenetic prognostic factors.
SSCP was used to screen for sequence variants in NTRK1 gene exons 14 and 15 (coding for the functional part of the protein - cytosolic tyrosine kinase domain) isolated from 30 primary neuroblastoma tumours. In samples in which aberrant banding pattern was identified, genetic changes were confirmed by direct sequencing. Additionally differential restriction enzyme digestion specific for known polymorphisms was performed. Results were correlated with clinical and cytogenetic data.
The most frequent polymorphism occurred at codon 628 (c1886 T>C) in 27% of tumours, followed by the one at codon 557 (c1673 G>A) – 20% and at codon 603 (c1809 C>T) – 7% tumours. The variant at codon 588 (c1766 T>C) was not detected neither by SSCP nor restriction analysis. The sequence variant at codon 603 (C>T) always occurred together with codon 557 (G>A) variant and lack of 1p deletion.
We found tendency of NTRK1 gene polymorphic variants to cluster in the group of patients with good prognosis (infants < 1y.o, disease stage other than 4, no 1p deletion). However, the examined group was too small to assess correlation with MYCN oncogene amplification and chromosome 17q gain.
To investigate correlation of NTRK1 polymorphisms with clinical outcome of the disease further enlarging of the number of analysed cases is necessary coupled with the extension of the period of clinical observation of patients.

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Notch-1 signaling in neuroblastoma cells

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Expression of embryonic genes indicates that neuroblastoma (NB) tumors arise due to perturbed differentiation of the sympathetic nervous system (SNS). It is therefore of interest to study factors that take part in SNS differentiation in order to understand the genesis of NB. Notch-1 is a transmembrane protein that restricts the number of cells that assume a neural fate. This is mediated through downstream transcription factors of the Hairy enhancer of split family (e.g. Hes-1). In the SNS, Notch signaling is important for inhibition of pre-mature neurulation and is thus needed to be downregulated in order for the cells to differentiate. During hypoxia, NB cells undergo de-differentiation as shown by induced expression of markers for early neuroblasts including Notch-1, and downregulation of neuronal marker genes. We were therefore interested in analyzing the role of Notch-1 signaling in normoxic and hypoxic NB cells.
First, we induced differentiation of the cells with TPA or RA, which led to a transient upregulation of Notch-1. Inhibition of the Notch-1 signaling cascade using a gamma-secretase inhibitor resulted in a decrease of total cell number. No increased cell death or apoptosis could be detected why the effect seems to involve a proliferation stop or a prolonged cell cycle. Hypoxic treatment of the NB cells led to upregulation of the downstream effector gene Hes-1. Reporter assays using a Hes-1 promoter coupled to a luciferase reporter gene showed that hypoxia led to activation of the reporter gene. The gamma-secretase inhibitor could partially block this activation.
In summary, our results indicate that Notch-signaling might be important for maintaining NB cells in a proliferative state and that hypoxia-induced activation of the cascade might contribute to the de-differentiated phenotype.

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Modifiers Of Tumor Susceptibility In A Mouse Model For Neuroblastoma

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BACKGROUND/AIMS: Murine models of cancer offer a genetically controlled system to discover novel genetic interactions in disease progression. Interactions among polymorphisms that individually confer marginal susceptibility, likely account for much of the population risk for neuroblastoma. These genetic interactions are thought to be mimicked in mouse models in which various strains exhibit a range of tumor incidence. The aim of this study is to identify loci that influence strain-specific susceptibility to neuroblastoma in a mouse model for this disease driven by a TH-MYCN transgene.
METHODOLOGY: Tumor incidence ranged from nearly 100% (by 5 months of age) in strain 129/SvJ, to 0% (at one year) in strain FVB/N, with no variation of transgene expression between strains. The resistant phenotype was dominant, as penetrance for tumors was 5% in the genetically homogenous F1 inter-cross generation. By backcrossing F1 mice to the tumor-sensitive 129/SvJ parent, we generated N1 animals with independently assorted alleles that showed roughly 33% tumor incidence. A genetic screen of 53 backcross mice with 250 microsatellite markers at 10cM resolution revealed sixteen markers, localizing to 7 chromosomal regions, which showed statistically suggestive correlations with tumor incidence. The most significant region contained a spread of 5 markers centered at 24 cM on chromosome 19, with an adjusted peak p-value of 0.022.
RESULTS/CONCLUSIONS: We have completed a larger screen utilizing high-throughput SNP genotyping of 90 informative SNPs to analyze a population of 336 backcross mice. Analysis of these data, which was not yet complete at the time of this submission, should localize regions that influence tumor susceptibility, which may illuminate genetic interactions relevant to human neuroblastoma susceptibility.

Poster Display
MOLECULAR BIOLOGY

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Proteomic analysis of an orthotopic neuroblastoma animal model

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Neuroblastoma is the most common extracranial solid tumour of childhood and comprises up to 50% of malignancies among infants. There is a great need of designing novel therapeutic strategies and proteome analysis is one approach for defining markers useful for tumour diagnosis, as well as molecular targets for novel experimental therapies. We started by comparing healthy adrenal glands (which are the election organs developing primary neuroblastoma, NB, tumours) and adrenal glands carrying primary NB tumours, taken from nude mice. Standard maps of healthy and tumour samples were generated by analysis with the PDQuest software. The comparison between such maps showed up- and down-regulation of 84 polypeptide chains, out of a total of 700 spots detected by a fluorescent stain, Sypro Ruby. Spots that were differentially expressed between the two groups, were analysed by MALDI-TOF
mass spectrometry and 14 of these spots were identified so far. Among these proteins, of particular interest are the down-regulated proteins adrenodoxin (21 folds), carbonic anhydrase III (8 folds) and aldose reductase related protein I (8 folds), as well as the up-regulated protein peptidyl-prolyl cis-trans isomerase A (5 folds). Moreover new proteins, which were absent in control samples (or probably too faint to be detected), were expressed in tumour samples, such as nucleophosmin and stathmin (oncoprotein 18).

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Proteomic and gene expression studies in neuroblastoma and Ewing sarcoma

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The proteomic techniques were applied in the comparison between Neuroblastoma (NB) and Ewing sarcoma (ES). Both tumours represent an interesting model because have neuroectodermic origin but are characterized by different aggressiveness. Electrophoretic spots, which are identified by image analysis, are cut and digested by trypsin directly in the gel. Tryptic digests were analysed by MALDI-TOF to obtain a peptide mass fingerprint (PMF). Uninterpreted PMFs were searched against a FASTA data base. Peptide matches are utilised by the search program to identify proteins. A number of proteins are differentially expressed in the two tumours. We focused our attention on the Heat Shock Proteins (HSPs) family because some HSPs were strongly overexpressed in neuroblastoma as evidenced by 2D gels /MALDI-TOF analysis. These results were implemented by quantitative gene expression analysis. The expression of a group of genes, including different ones codifying for HSP's family and oxidative stress proteins, was evaluated in NB and ES by relative quantification utilizing three different housekeeping genes (β-2 Microglobulin, ribosomal S14 and GAPDH) on a quantitative real-time PCR. HSP 27, HSP A5, HSP 70, Mortalin and GRP58 resulted significantly more expressed in NB than in ES. Gene expression not always correlated with differential protein analysis indicating that, in some cases, the control of the amount of HSPs found in the cells is not at the gene expression level. Interestingly overexpression of anti-apoptotic proteins correlate with the malignancy of the tumour. Several other protein were found differentially expressed in NB vs ES and necessitate further studies.

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Studies Using 17-Allylamino-17-demethoxygeldanamycin (17-AAG) to Understand the Heterogeneity of Hsp90 Linked Survival and Signalling Pathways in Neuroblastoma

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Clinical heterogeneity is a salient feature of neuroblastoma that is reflected in a wide spectrum of pathophysiological and prognostic manifestations. However, the molecular mechanisms that are responsible for the variations seen in the growth of NB cells are currently unclear. Heat shock protein 90 (Hsp90) functions as a stabilizing chaperone for many kinases, cell surface receptors and transcription factors involved in signalling, survival and proliferation of malignant cells. 17-AAG has been shown interfere with this chaperone function. We hypothesize that biologically different NB cells may use distinct cell signalling pathways and Hsp90 can be used as a bait to identify and characterize them. To test this, we used seven different cell lines with different biological properties such as N-myc amplification. We show that 17-AAG was able to induce apoptosis in all of them (IC50; 0.2 - 0.5uM/L). Western blot analysis has shown a significant reduction in survivin in all cell lines. Decrease in cellular p53 level was observed in only one cell line (IMR5) but phospho Raf-1 levels were decreased in SKNBE2 and NUB7. In contrast to p53, SHP2 levels were increased in IMR5 but not in others. We also found variations in the expression of other signalling molecules, including Src, Akt, c-Cbl, MEK, ERK1/2 and phosphoERK1/2. Our results show an emerging pattern between certain biological properties and the various signalling components that change when treated with 17-AAG. In this study, we describe a novel approach to investigate the molecular diversity of growth regulatory pathways in NB and provide evidence for the use of agents such as 17-AAG in its treatment.

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Frequent transcriptional silencing of RASSF5 (NORE1A) in human pheochromocytomas and neuroblastomas

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BACKGROUND: RASSF5 is a RAS effector and putative tumor suppressor on 1q32, which has been shown to inhibit cell proliferation, reduce transformant phenotype and induce apoptosis in a RAS dependent manner in a variety of tumor cell lines. Frequent loss of RASSF5 expression has been described by others in panels of lung, breast, colorectal, kidney and ovarian tumor cell lines as well as in primary tumors. While RASSF5 mutations have not been reported, promoter methylation has been indicated as the predominant mechanism underlying RASSF5 silencing.
AIMS AND METHODS: To investigate the involvement of RASSF5 in neural crest derived tumors we analysed a panel of 32 pheochromocytomas (adrenal and extra-adrenal) and 29 neuroblastomas as well as 7 neuroblastoma cell lines for RASSF5 mRNA expression and promoter methylation using semiquantitative RT-PCR, Real-time quantitative RT-PCR and combined bisulphite treatment and restriction analysis (COBRA).
RESULTS: We have found by semiquantitative RT-PCR that loss of RASSF5 mRNA expression occurs approximately in one third of all samples used in this study. Further characterization of RASSF5 expression by Real-time quantitative RT-PCR revealed a significantly decreased or absent expression in the majority of pheochromocytomas and neuroblastomas whereas high expression was measured in a pool of normal adrenal glands derived from 70 healthy individuals. Partial methylation of the RASSF5 promoter was detected in 2 pheochromcytomas and 1 neuroblastoma cell line, which also showed more than 90% decsreased mRNA levels compared to normal adrenal glands.
CONCLUSIONS: Our results demonstrate that the putative tumor suppressor gene RASSF5 shows frequent down regulation or loss of mRNA expression in neural crest derived tumors. Furthermore promoter methylation does not appear to be a frequent mechanism underlying RASSF5 silencing in heochromocytomas and neuroblastomas.

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The role of Wnt signaling in neural crest (NC) development

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During development of NC, which is the origin of neuroblastoma, Wnt signaling is also known to regulate cell expansion and/or determination of cell fate. To elucidate the function in the NC development, apc deficient mice and transgenic mice expressing activated beta-catenin specifically in the mouse NC were generated by using the conditional gene targeting method. These mutant mice exhibited severe craniofacial defects including skull bone malformation and died around birth. Some of them had ventricular septum defects and persistent truncus arteriosus. TUNEL staining revealed a significant increase in the number of apoptotic cells in the ventral craniofacial mesenchyme (VCM), the first bronchial arch (1st BA) and cardiac outflow tract (COT) in both mice. Both mutant embryos showed significant accumulation of beta-catenin in VCM, 1st BA and COT, where TUNEL positive cells were clustered. Therefore, it is likely that deregulated expression of beta-catenin may play a crucial role in the induction of apoptosis in cephalic and cardiac NC derived cells in the mutant mice. However, it is suggested that this apoptosis is not mediated by p53 from the analyses of Apc / p53 double deficient mice and activated beta-catenin / p53 null mice. For further understanding of Wnt signaling in NC, NC specific beta-catenin knockout mice were generated. These mutant embryos were not lethal, but died immediately after birth. All mutants also suffered craniofacial defects. Detailed examination showed these phenotype might result from the disorder of cranial NC cell proliferation and/or the increase of apoptotic cells, and beta-catenin is essential in the development of cranial NC cells. No obvious malformation of heart suggested beta-catenin may be unnecessary in the development of cardiac NC cells.

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BMP signaling in human neuroblastoma cells: the role of p27KIP1 and p53/p73 in inducing neuronal differentiation

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Bone morphogenetic proteins (BMPs) are the members of TGF-b superfamily, and have been implicated in initiating neural crest cell differentiation. However, it has been unclear whether BMPs have an ability to promote neuronal differentiation or survival in human neuroblastoma cells. We here report that BMP2 has a potent neurotrophic function for some neuroblastic cell lines by inducing morphological differentiation, whereas the downstream signaling is deregulated in some others.
RESULTS AND DISCUSSION: BMP receptors (I and II) were expressed in all 7 neuroblastoma cell lines we examined by RT-PCR and Northern bot. The treatment of 8 neuroblastoma cell lines with 1 nM BMP2 induced marked phosphorylation of Smad1/5 within 30 min in all cell lines tested. However, surprisingly, only two cell lines (RTBM1 and LA-N-5) showed a remarkable neuronal differentiation with growth arrest after treatment with BMP2, while SK-N-AS cells did growth arrest alone. In RTBM1 cells, BMP2-induced differentiation was accompanied by a significant decrease in the expression level of DAN, which is mapped to chromosome 1p35 and the product acts as an antagonist of BMP. Immunoblot analysis revealed that BMP2 treatment caused a down-regulation of both p53 and p73, and hence cyclin-dependent kinase inhibitor p21WAF1. In contrast, p27KIP1 protein was markedly increased in response to BMP2, being accompanied with down-regulation of Skp2, which is required for ubiquitin-dependent p27KIP1 degradation, during this differentiation process. Our results suggest that BMP2 signal is frequently deregulated downstream of its receptor in aggressive tumors. However, in responsive cells, increase of p27KIP1 as well as decreases of p53 and p73 may contribute to the regulation of BMP-induced growth arrest, survival and differentiation of neuroblastoma. Thus, BMP signal could be a target for developing a new therapeutic strategy against aggressive neuroblastomas.

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Involvement of endogenous ceramide in the inhibition of telomerase activity, MYCN activity, and induction of morphologic differentiation in response to all-trans-retinoic acid in human neuroblastoma cells

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Recent research has shown that the sphingolipid ceramide, in addition to its role as a structural component of cell membranes, plays important roles as a regulator of signal transduction in cell differentiation, cell proliferation, and apoptosis. We examined the role of endogenous ceramide in the inhibition of telomerase and induction of morphologic differentiation in response to ATRA in the SK-N-SH and SK-N-AS human neuroblastoma cell lines. The results showed that ATRA inhibited the activity of telomerase significantly in a time- and dose-dependent manner. Treatment of cells with ATRA also resulted in the inhibition of growth by about 30-70% after 4 and 8 days of treatment. ESI/MS/MS analysis of accumulation of endogenous ceramide showed that treatment of cells with ATRA resulted in increased levels of mainly C24:0 and C24:1 ceramides. Also, treatment of cells with ATRA in the presence of myriocin significantly blocked the accumulation of ceramide, and more importantly myriocin partially prevented the inhibition of telomerase. Mechanistically, inhibition of telomerase by endogenous ceramide in response to ATRA treatment involves, at least in part, down-regulation of the expression of telomerase reverse transcriptase (hTERT) mRNA, as determined by semi-quantitative RT-PCR. In addition, the modulation of telomerase activity by ATRA correlated with the induction of morphologic differentiation, which was also blocked by myriocin. Electrophoretic mobility shift assays demonstrated significantly decreased DNA binding activity of Myc-Max complexes in response to ATRA treatment. This effect was also blocked by myriocin. These results, therefore, reveal an important effect of ATRA on telomerase inhibition, MYCN activity, and induction of morphologic differentiation in human neuroblastoma cells. These data also demonstrate that endogenous ceramide is one of the upstream regulators of telomerase activity in human neuroblastoma cells in response to ATRA.

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Small molecule inhibitors of PI3-Kinase suppress growth of neuroblastoma cell lines by destabilizing Mycn protein

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BACKGROUND AND AIMS: BDNF and IGF-1, growth factors with mitogenic activity for MYCN amplified neuroblastoma cell lines, exert their effects in-part through upregulation of the PI3-kinase pathway. We used small-molecular inhibitors of PI3-kinase to block the growth-stimulatory effects of BDNF and IGF-1 on neuroblastoma cell lines.
METHODOLOGY: Neuroblastoma lines SH-SY5Y, SK-N-MC, Kelly, and LAN-1 were treated with LY294002, wortmannin, or siRNA directed against MYCN. Treatment doses with inhibitors were optimized to greatest efficacy (WST-1 proliferation assay) with least toxicity (Histone H-2b ELISA). Cells were analyzed by Western blotting, and by flow cytometry.
RESULTS: Flow cytometry analysis revealed substantial inhibition of cell cycling in response to treatment with LY294002, with a decreased proportion of cells in S-phase (3% S, 74% G0G1 with 20uM LY294002, 31% S, 51% G0G1 in vehicle controls; chi-square for change in S-phase fraction=2778, 1 d.f., p<0.001 for treatment compared with control). Transient transfection of siRNA targeted to MYCN caused comparable growth suppression. PI3-kinase pathway inhibition was confirmed by immunoblotting for pAkt and pGSK-3_. MYCN immunoblotting revealed decreased levels of total Mycn protein, and increased levels of phosphorylated Mycn.
CONCLUSIONS: PI3-kinase inhibitors suppressed the growth of MYCN-amplified neuroblastoma cells in-part through inhibition of Mycn. The mechanism of inhibition probably involves phosphorylation of Mycn protein by GSK-3?, which is presumably a destabilizing event. PI3-kinase pathway inhibitors should be further investigated as therapeutic agents to target the growth of MYCN-amplified neuroblastoma.

Poster Display
MOLECULAR BIOLOGY

Ref ID: 075.4 **#181**

Assessment of P19/ARF-MDM-P53 and RAS pathways for alterations in neuroblastomas arising in the transgenic TH-MYCN mouse model

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NBs arise in transgenic mice with neural crest targeted MycN [Weiss, EMBO 1997]. Tumors arise focally and stochastically within the peripheral nervous system and are genomically complex. This suggests additional mutations are required for tumourigenesis. Activated ras oncogenes cooperate with deregulated Myc to transform primary cells and ras mutations occur in Myc-driven malignancies. Similarly, spontaneous or genetically engineered lesions that abrogate p53 function (loss of p19/Arf, amplification of mdm2, or p53 inactivation) accelerate Myc initiated tumorigenesis. We therefore assessed the p19/Arf-mdm-p53 and ras pathways in NBs arising in TH-MYCN mice. Elevated p53 protein was seen in 4/13 TH-MYCN tumors (31%), and one tumor had altered p53 migration on IB. Mutational analysis of the DNA binding domain (exons 4 through 8) of murine TP53 is ongoing. Immunoblots for mdm2 and mdmX/mdm4 did not demonstrate elevated protein in any tumor suggesting that enhanced mdm-mediated p53 degradation is infrequent. PCR-based amplification of exons 1a, 1b, and 2 shared by the p19/Arf and p16 genes demonstrate no homozygous deletion. Expression studies from p19/Arf and p16 cDNA are ongoing. The entire coding sequence for all ras family genes was sequenced bidirectionally for mutation analysis. Rare silent polymorphisms but no mutations were found in Hras1 or Kras2 in 21 tumor samples. A missense mutation at codon 3 [Glu to Val] of unknown functional significance was found in 1/21 tumors (5%). Notably, activating ras mutations rarely occur in human NBs, so in this respect the TH-MYCN model faithfully recapitulates the human process. Our p53 findings await confirmation, but suggest that p53 mutation may occur with greater frequency in murine NBs. Further efforts to identify preferred secondary pathways following MYCN initiation may be of great utility in understanding NB tumourigenesis and progression.

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The neurogene BTG2 is transactivated by DeltaNp73alpha via p53 in neuroblastoma cell lines

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In neuroblastoma (NB), p53 and p73 are rarely mutated. In recent studies we showed that an overexpression of DeltaNp73alpha in the NB line SH-SY5Y with wt-p53, interestingly, induces p53 protein accumulation and as can be expected, inhibits Waf1/p21 gene expression. But, surprisingly, BTG2, another p53 target gene, is up-regulated. This effect is not observed in NB cells that express a mutated p53 (Goldschneider et al., J. Cell. Sci., 2004). To better understand the DeltaNp73-mediated transactivation of BTG2 gene expression we performed luciferase assays with 2 reporter-plasmids harboring long and short BTG2 promoter sequences in three NB lines and one cancer breast line. Our results demonstrate that BTG2 transactivation by DeltaNp73alpha depends on p53 status and cellular context since it occurs only in p53+/+ NB cells but neither in p53-/- NB nor in the p53 +/+ MCF7 breast cancer cells. Moreover, the transactivation of the BTG2 promoter sequences requires the DeltaNp73alpha C-terminus sequence since it is not observed with DeltaNp73beta. Thus DeltaNp73alpha can either inhibit or stimulate wt-p53 transcriptional activity, depending on the p53 target gene. In previous studies, we have shown that DeltaNp73alpha is the only p73 isoform expressed in undifferentiated NB tumors. In conclusion, we propose that DeltaNp73alpha not only acts as a systematic inhibitor of p53/TAp73 in NB tumors, but also cooperates with wild-type p53 in playing a physiological role through the activation of BTG2 gene expression.

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Differential expression & regulation of MXI1 isoforms in neuroblastoma

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Background: Advanced stage neuroblastoma (NB) is associated with MYCN amplification and increased N-Myc protein expression. Mxi1, a c-Myc antagonist, represses transcription of c-Myc dependent genes, and suppresses c-Myc-induced proliferation. We have previously shown that Mxi1 suppresses proliferation of NB cells. We have recently identified Mxi0, an alternatively transcribed Mxi1 isoform that lacks growth suppressive activity. While Mxi1 has a punctate nuclear distribution, Mxi0 is diffusely cytoplasmic in all cell lines examined. Since MXI0 and MXI1 mRNAs are co-expressed in both normal and tumor cells, we hypothesized that varying Mxi0 and Mxi1 levels might modulate the activity of N-Myc in NB.
Methods: Semi-quantitative multiplex RT-PCR was used to estimate relative MXI0 and MXI1 expression in 8 NB cell lines, and findings were confirmed by real time PCR. Luciferase assays were performed in NB cells to determine relative MXI0 and MXI1 promoter activity.
Results: The MXI0/MXI1 ratio was elevated in MYCN amplified cell lines (IMR-32, KCNR, GOTO, SK-N-BE) and reduced in non-amplified cell lines (SHEP, GIMEN, SH-SY5Y, SK-N-SH), indicating an association between relatively higher MXI0/lower MXI1 expression and MYCN amplification. In SHEP cells stably transfected with MYCN (SHEP/MYCN), the MXI0/MXI1 ratio was markedly elevated in comparison with SHEP cells. Furthermore, we observed relatively increased MXI0 and reduced MXI1 promoter activity in SHEP/MYCN cells compared with SHEP cells. To directly measure the effect of N-Myc on MXI0 and MXI1 promoter activity, SHEP cells were co-transfected with a MYCN expression vector and either MXI0 or MXI1 promoter constructs. In this system, MXI0 promoter activity was two-fold higher in the presence of MYCN, while MXI1 promoter activity was reduced 2.8 fold.
Conclusions: N-Myc differentially regulates MXI0 and MXI1 expression at the promoter level. N-Myc might potentiate its own oncogenic effects by downregulating levels of its antagonist, Mxi1, leading to aggressive biological behavior in MYCN amplified NB.

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The estrogen-responsive B box protein: a novel co-activator of tumor suppressor gene RARbeta in neuroblastoma

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Retinoids induce growth inhibition and neuritic differentiation in human neuroblastoma cells, and, are in clinical use in the disease. Our data indicates that one mechanism by which neuroblastoma cells evade retinoid effects in vivo is through repression of retinoic acid receptor beta (RARbeta) gene transcription. The betaRARE is the essential DNA sequence required for retinoid-induced RARbeta expression. We used protein micro-sequencing of purified betaRARE-binding proteins, to identify novel transcriptional regulators of retinoid-induced RARbeta expression. We identified the recently described estrogen-responsive B box protein (EBBP), as a betaRARE-binding protein. EBBP is homologous to several known transcriptional co-regulators involved in diverse biologic processes, such as PML and TIF1, and, preliminary evidence indicates a role in proliferation and differentiation processes of epithelial cells. Using 2-dimensional gel electrophoresis, we have shown that EBBP appears to undergo post-translational modification after retinoid treatment. EBBP protein expression is increased by retinoic acid (RA) treatment of neuroblastoma cells. A co-transfected EBBP expression vector enhances the retinoid-induced transactivation of a betaRARE-luciferase reporter construct, and, endogenous RARbeta transcription in neuroblastoma RA-sensitive cells. Retinoid-treated neuroblastoma cells showed a significant increase in the amount of nuclear EBBP protein determined by confocal immunofluorescence. We created deletion-mutant EBBP expression vectors for four different functional domains, and found that the coiled-coil domain is necessary and sufficient for retinoid-induced transactivation of betaRARE. Our results suggest that EBBP may play an important role in retinoid anticancer signalling pathway by activating tumour suppressor gene RARbeta, and, may be used as a new molecular target for retinoid therapy in neuroblastoma cells.

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Deregulated tyrosination/detyrosination cycle of a-tubulin, accompanied with decreased expression of human tubulin tyrosine ligase, is associated with malignant progression of neuroblastoma

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Among the genes obtained from our neuroblastoma cDNA project, we found a novel Nbla0660 gene, which encodes a human ortholog of tubulin tyrosine ligase (hTTL). TTL catalyses tyrosination of a-tubulin and is an important regulator of the tyrosination/detyrosination cycle of a-tubulin. The a-tubulin detyrosination reaction is catalysed by yet undetermined carboxypeptidase and produces Glu-tubulin and D2-tubulin which lack one (-Tyr) and two (-Glu-Tyr) COOH-terminal amino acids, respectively. D2-tubulin escapes from the cycle, albeit the precise mechanism is unclear. Here we found that the tyrosination/detyrosination cycle of a-tubulin is deregulated in unfavorable neuroblastomas. Results and Discussion: A full-length hTTL (GenBank No. AB071393) we cloned encoded a protein of 377aa. The catalytic activity of hTTL overexpressed in cells was confirmed by using specific antibodies against Tyr-tubulin, Glu-tubulin and hTTL we generated. Interestingly, hTTL protein was induced during induction of
neuronal differentiation in RTBM1 neuroblastoma cells after treating with BMP2 or retinoic acid. Expression of hTTL mRNA was examined in 74 primary neuroblastomas using quantitative real-time RT-PCR, resulting in that high hTTL expression was significantly associated with early stages (p=0.0069), high TrkA expression (p=0.002), a single copy of MYCN (p<0.00005), mass screening tumors (p=0.0042), and non-adrenal origins (p=0.0042). The log-rank test also showed that hTTL expression was a significant prognostic indicator of neuroblastoma (p=0.026). The immunohistochemical study demonstrated that all hTTL, Tyr-tubulin, Glu-tubulin and D2-tubulin as well as TrkA are positive in favorable neuroblastomas, whereas only D2-tubulin was positive in unfavorable tumors with MYCN amplification. Thus, the decrease of hTTL level and the resultant disturbance of tyrosination/detyrosination cycle of a-tubulin may contribute to malignant progression of neuroblastoma.

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Biological roles of hyperphosphorylated ShcC docking protein in neuroblastoma cells

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ShcC is a family member of Shc docking proteins, which contain a unique PTB-CH1-SH2 modular organization and conduct as substrates of various receptor tyrosine kinases. Recently, we showed that hyperphosphorylated ShcC detected in some of neuroblastoma cell lines, such as NB-39-nu cells, is associated with constitutively activated anaplastic lymphoma kinase (ALK) caused by the gene amplification. The ALK gene amplification was also detected in about 10% of primary human neuroblastomas. Suppression of ALK expression in NB-39-nu cells by siRNA resulted in decreased phosphorylation level of ShcC, inactivation of MAPK/Akt pathway and cell apoptosis suggesting that ALK tyrosine kinase is dominating survival signal of this neuroblastoma line. To investigate the roles of hyperphosphorylated ShcC in neuroblastoma cell lines, we established NB-39-nu cell lines whichoverexpress wildtype or mutant ShcC proteins. It was demonstrated that cell-survival and differentiation, cell-motility were markedly impaired in the NB-39-nu cells expressing the 3YF mutant of ShcC which blocks ShcC-Grb2 pathway by the dominant-negative fassion. At the same time, activation level of MAPK and Akt was severely suppressed in these cells. On the other hand, cells overexpressing ShcC as well as the 3YF mutant showed decreased transforming ability, such as anchorage independency and in vivo tumorigenicity that might suggest ShcC-specific negative effects. Loss of persistent phosphorylation of p130Cas in suspension cell culture was observed in both ShcC overexpressing cells and 3YF cells. These results suggest ShcC might negatively regulate an alternative pathway such as the Src family kinase (SFK)-p130cas pathway in addition to the authentic MAPK and Akt pathways.

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Introduction of in vitro transcribed ENO1 mRNA into neuroblastoma cells induces massive cell death

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Neuroblastoma is a solid tumour of childhood, where common genetic features in aggressive tumours are amplification of MYCN and deletions of chromosome region 1p. These genetic features are also important prognostic factors for bad outcome. The a-enolase (ENO1) gene is located in chromosome region 1p36.2, within the critical region of deletion in neuroblastoma. One of the alternative translated products of the ENO1 gene, known as MBP-1, acts as a negative regulator of the c-myc oncogene, thus making the ENO1 gene a strong candidate as a neuroblastoma tumour suppressor gene. Here we demonstrate that transfection of ENO1 cDNA into 1p-deleted neuroblastoma cell lines causes reduction of cell growth by 20 % compared to a negative control and induces apoptosis. Interestingly, a similar but much stronger dose-dependent reduction of cell growth (up to 90% of control) was observed by transfection of in vitro transcribed ENO1 mRNA into neuroblastoma cells. We can also show, by realtime-PCR, that ENO1-mRNA is present at, in average, 55% lower levels in stage 4 than in stage 2 primary neuroblastomas, regardless of 1p-deletion status. However, mutation screening of all exonic sequences, the promoter region of ENO1, and introns 4-7 in 50 primary neuroblastomas of all different stages, failed to detect any mutations. Our studies indicate that ENO1 has tumour suppressor activity and the level of ENO1 expression in neuroblastomas might be crucial for keeping the cells in a transformed state.

Ref ID: 191.1 **#189**

Constitutive and IFN-inducible expression of the Caspase-8 gene in Neuroblastoma cells is regulated through an essential Interferon-Sensitive Response Element (ISRE)

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We have recently identified a 1.2Kb DNA element (P-1161/+16), 5\' to caspase-8 exon-1, that acts as promoter in caspase-8-positive, but not in caspase-8-negative neuroblastoma (NB) cells.The P-1161/+16 DNA element regulates both constitutive and interferon (IFN)gamma-inducible caspase-8 expression. Treatments of NB cells with IFNgamma induced phosphorylation of STAT-1, increased expression of the IFNgamma-sensitive IRF-1 transcription factor, and enhanced P-1161/+16 promoter activity. Two GAS (IFNgamma activated sequence, STAT-1 binding site), three ISRE (IRF-1/-2 binding site) and two E-box (USF-1 binding site) elements were present in P-1161/+16. Elements essential for promoter activity were present in a 151 bp region 5\' flanking exon-1 (-151/+16), which contains an ISRE at position -32, a partially overlapping ISRE at -38 and two E-box elements. Site specific mutagenesis indicated that the ISRE-32 is required for both constitutive and IFN-gamma-inducible caspase-8 expression. IRF-1, IRF-2 and USF (upstream factor)-1 transcription factors bind to the (-151/+16) DNA fragment in vitro. Moreover ChIP assays showed that hyperacetylated forms of H4 histone are present in the promoter region in cells showing constitutive or IFNgamma-induced expression. In addition, IRF-1, IRF-2 and USF-1 bind to the (-151/+16) DNA region in cells which show constitutive caspase-8 expression but not in caspase-8 negative cells. In these cells, up-regulation of caspase-8 by IFNgamma was associated with induction of IRF-1 and USF-1 binding to caspase-8 promoter. Our data indicate a role of IRF-1 and USF-1, and possibly IRF-2, in caspase-8 gene expression.

Poster Display
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Ref ID: 386.1 **#190**

Silencing of MYCN expression in human neuroblastoma cell lines

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BACKGROUND AND AIMS. The most important predictor of poor outcome in neuroblastoma is amplification of the MYCN oncogene. There are indications of a direct role for the MYCN protein in neuroblastoma pathogenesis. Studies using specific antisense oligonucleotides to investigate the therapeutic potential of inhibiting MYCN expression showed that it was possible to reduce the production of MYCN protein and cell proliferation by approximately 50 percent. With a strategy using RNA interference (RNAi), which probably is more effective than the use of antisense oligonucleotides, we wanted to examine if it was possible to reduce the production of MYCN RNA and protein in neuroblastoma cell lines with MYCN amplification.
METHODOLOGY AND RESULTS. Our RNAi strategy is based on SHAGging (short-hairpin-activated gene silencing. We designed vectors expressing shRNAs targeting the coding region of the MYCN oncogene. ShRNA-expressing plasmids were transiently transfected into MYCN amplified human neuroblastoma cell lines (Kelly, SKNBE). Transfection efficiencies were examined by transfecting a plasmid expressing the green fluorescent protein (GFP). Transfection efficiencies were typically 50-70%. There was a reduction of 40-70% of the MYCN protein in transfected cells compared with control cells. Real-time RT-PCR showed that MYCN mRNA levels were reduced by approximately 40% compared with the negative control cells. This experiment confirms that the reduction of MYCN protein observed on Western blots was the result of reduced MYCN mRNA levels.
CONCLUSION. We have shown that shRNA targeting the coding region of the MYCN oncogene is able to efficiently reduce its expression. Considering transfection efficiencies of 50-60% our results indicate that the MYCN-specific shRNA eliminates the vast majority of both the mRNA and protein coded for by the MYCN gene. Therefore, treatment involving MYCN-targeted therapies may be clinically important MYCN amplified neuroblastomas.

Ref ID: 357.1 **#191**

Cyclophosphamide, but not melphalan or carboplatin, synergistically enhanced topotecan activity against neuroblastoma cell lines in hypoxia

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p21Waf1/Cip1 is a cyclin dependent kinase inhibitor that is responsible for inhibiting cdk2 kinase activity resulting in hypophosphorylation of pRb and a G1 cell cycle arrest. Following exposure to DNA damaging chemotherapeutic agents neuroblastoma cells display an attenuated G1 checkpoint, and we have previously demonstrated that this is mediated at least in part by the inability of p21 to bind cdk2. MYCN is a member of the MYC family of oncogenes that is often amplified and overexpressed in neuroblastoma tumors. MYC proteins act as both transcriptional activators and repressors, and have been shown to regulate expression of many genes involved in cell differentiation and proliferation. Here we describe an inverse relationship between MYCN and p21 protein in neuroblastoma cells suggesting that MYCN can influence p21 protein expression. Even though MYC proteins have been shown to directly bind p21, the lower levels of p21 protein measured in the MYCN expressing cells was not due to protein instability. However, measurement of p21 promoter activity when linked to a luciferase reporter revealed that MYCN expression resulted in a decreased rate of p21 transcription in neuroblastoma cells. MYC has previously been shown to repress p21 transcription by binding to Miz1. We are currently evaluating whether Miz1 is involved in the suppression of p21 expression by MYCN in neuroblastoma cells, and determining the role that MYCN plays in controlling the G1 cell cycle checkpoint in this cell type.

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Ref ID: 338.2 **#194**

Chemotherapy Induced S-Type Cell Death Via A Caspase-9 Dependant, p53 Independent Mechanism

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Neuroblastoma (NB) is the most common malignant sympathetic nervous system tumor of childhood. NB consists of two main cell populations - neuroblastic (N-type) and stromal (S-type). Doxorubicin (Dox) and Cisplatinum (CDDP) are cytotoxic drugs currently implemented in all standard therapeutic protocols for the treatment of NB. Dox and CDDP induce N-Type NB cell death through NF-kappaB and caspase-9 dependent mechanism respectively [JBC 2001; 276: 48921 - 48929]. This study focused on elucidating the effects and mechanisms of Dox and CDDP induced cell death in S-type cells. SH-EP1 cells were treated with DOX or CDDP and caspase activity was measured by flow cytometry. Caspase-9 appeared to be a more proximal death signal in the response of S-type cells to chemotherapy because it was activated 4 hours prior to caspases -3 and -8 activation. To test the functional importance of caspase-9 in chemotherapy induced S-type cell death we generated S-type cell lines stably expressing DN mutant caspase-9. DN/SH-EP1 cells were resistant to Dox and CDDP treatment. Wild-type p53 is expressed in most NB tumors and responds to chemotherapy [Cancer. 1994 Jun 15;73(12):3087-93]. We tested whether p53 mediates the caspase-9 response to Dox and CDDP in S-type cells. p53 was inactivated by
expressing human papillomavirus E6 protein in SH-EP1 cells. Compared to vector control cells, E6/SH-EP1 cells remained sensitive to Dox and CDDP. These results suggest that Dox and CDDP induce S-type cell death in a caspase-9 dependent, but p53 independent manner. Agents directly targeting caspase-9 may have potential therapeutic implications in NB treatment.

Ref ID: 224.1 **#195**

The defective G1 cell cycle checkpoint in neuroblastoma is mediated by the inability of p21Waf1/Cip1 to bind to or inhibit cyclin E-dependent Cdk2 kinase activity

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Mutations of the p53 gene in neuroblastoma are rare. However, whether the p53 pathway is functional in this tumor type is controversial. In 'normal' cells, p53 induces the expression of p21Waf-1/Cip-1 following DNA damage. p21, in turn, binds to and inhibits the function of cyclin dependent kinase-2 (cdk2) resulting in G1 arrest. We demonstrated previously that even though p53 is transcriptionally active and induces p21 in NB cells, the G1 checkpoint is attenuated. We now show that this defect is mediated by the inability of p21 to co-localize with, bind to, or inhibit cdk2 kinase activity. p21, in contrast, can bind to cdk4 in NB cells. Overexpression of the specific cdk4 inhibitor, p16Ink4a, was able only to promote a slight increase in p21-cdk2 binding and pRb phosphorylation was not altered. However, a recombinant p21 protein could inhibit cdk2 kinase activity when added to NB cell lysates in vitro. The dysfunction of p21 in NB cells represents a novel mechanism by which the G0/G1 cell cycle checkpoint can be inactivated. Cdk inhibitors currently being developed fro clinical use may be useful therapy for tumors such as NB in which endogenous cdk inhibitors are defective.

Ref ID: 351.1 **#192**

The neuronal-specific RNA-binding protein HuD regulates MYCN expression and gene copy number in neuroblastoma cells

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HuD, a member of the Hu-family of RNA-binding proteins, is expressed by cells of neuronal and neuroendocrine lineages and appears to regulate processing of specific mRNAs including the proto-oncogene N-myc. Since N-myc amplification and overexpression have been shown to play an important role in the aggressiveness of human neuroblastoma tumors, we are examining the relationship between HuD and N-myc. To test our hypothesis that HuD enhances splicing and stability of nascent transcripts, we stably transfected non-neuronal, N-myc amplified, LA1-5s cells with a sense HuD construct. HuD mRNA levels increased 8.2-fold in sense transfectants and the steady-statelevels of mature N-myc mRNA increased 2.5-fold. Cells also exhibited increased levels of N-myc processing intermediates. Increase in amount of mature N-myc mRNA is not due to an increase in half-life or transcription rate. These findings suggest that HuD regulates splicing of N-myc hnRNA leading to higher levels of mRNA. A second line of research had implicated reduced levels of HuD as a selective factor in amplification of the N-myc gene. We stably transfected N-myc nonamplified SH-SY5Y cells, which have two normal HuD alleles, with a HuD antisense construct. Both HuD protein levels and N-myc mRNA are significantly reduced although the antisense transfectants remain neuroblastic. More interestingly, the antisense transfectants appear to have increased copies of the N-myc gene. Our studies support a vital role for HuD in N-myc expression and provide evidence that the loss of HuD may lead to amplification of the N-myc gene in human neuroblastoma cells.

Ref ID: 265.1 **#193**

The role of MYCN amplification in the p53 response to DNA damage

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BACKGROUND: Our previous work has shown that some MYCN amplified neuroblastoma cell lines fail to undergo a p53 mediated G1 arrest in response to DNA damage. We hypothesised that MYCN may inhibit p53 mediated G1 arrest in neuroblastoma.
METHODS: A panel of MYCN amplified and non-MYCN amplified wild type p53 neuroblastoma cell lines were treated with 4 Gy g-irradiation. Fluorescence activated cell sorting was used to determine a G1 arrest after 24h and p53 function was measured by Western blotting for p53, MDM2, p21, BCL2, BAX and Rb 2, 6, and 24 hours after irradiation.
RESULTS: Non-MYCN amplified SJNB-1 and NB69 cells underwent G1 arrest after irradiation (defined as an increase in G1/S phase ratio), but non-MYCN amplified GIMEN cells did not. All non-MYCN amplified cell lines showed induction of p53 2 hours after irradiation from 2.2 fold (GIMEN) to 4 fold (SJNB-1), induction of MDM2 and p21 levels 6 and 24 hours after irradiation, and accumulation of hypophosphorylated Rb. MYCN amplified NBLW and LS cells failed to G1 arrest after irradiation. In LS cells (also MDM2 amplified) p53 was induced 4.8 fold 2 hours after irradiation, there were no detectable changes in MDM2 levels, p21 was induced and hypophosphorylated Rb accumulated after irradiation.
CONCLUSIONS: The G1 check point is intact in the non-MYCN amplified neuroblastoma cell lines NB69 and SJNB-1. Non-MYCN amplified GIMEN cells and two MYCN amplified cell lines (NBLW and LS) failed to G1 arrest after irradiation despite the presence of functional p53 and the accumulation of hypophosphorylated Rb. Further cell lines are being studied to confirm these findings.

Ref ID: 339.1 **#196**

FKHRL1 function in neuroblastoma cells

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The neuroblastoma (NB) is the most frequent extracranial solid tumor in childhood. Phosphatidylinositol 3`-kinase (PI3K) and PKB/Akt and its target, the forkhead transcription factor FKHRL1 have been suggested as possible downstream regulators of neurotrophin mediated cell survival in neuroblastoma cells. To analyze, whether this pathway is critical for survival and cell cycle progression we generated a retrovirus expressing a regulated FKHR-L1-ER* fusion protein and infected SH-EP and LAN-1 neuroblastoma cells and the primary tumor cell line STA-NB15. Activation of FKHR-L1 by 4-OH-tamoxifen induced apoptotic cell death in STA-NB15 and in SH-EP cells after 48 hours and caused reduced proliferation in LAN-1 as measured by propidium iodide and MTT-assay. This was associated with the induction of the cell cycle inhibitor p27Kip1 whereas expression of the proapoptotic FKHR-L1 target bim was not detected. Ectopic expression of crmA inhibited FKHR-L1 induced apoptosis until 48 hours whereas overexpression of bcl2 prevented apoptosis up to 72 hours in SH-EP cells. 4-OH-tamoxifen treated SH-EP-FKHR-L1 showed an increased chemosensitivity to cisplatin,doxorubicin, etoposide and vinblastine as measured by FACS-analysis and MTT assay. In conclusion the transcription factor FKHR-L1 critically regulates cell death and cell cycle progression in neuroblastoma cells.

Ref ID: 341.1 **#197**

Effects of recombinant TAT fusionprotein survivin T34A on neuroblastoma cells

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The neuroblastoma (NB) is the most frequent extracranial solid tumor in childhood. In presence of unfavourable prognostic parameters the event-free survival is only 30% despite high dose chemo- and radiotherapy. NB cells show complex combinations of genetic aberrations, deletion of chromosome arms 1p, amplification of the MYCN oncogene and gain of chromosome arm 17q. The gain or distal translocation of 17q has been shown to be of prognostic importance and is a significant predictive factor for an adverse outcome. The gene of the apoptosis inhibiting protein surviving located at the mainly affected segment, shows increased expression in such tumors and has been correlated with a malignant phenotype. Transgenic expression of survivin protects SH-EP cells against apoptosis by chemotherapeutica agents. Therefore the introduction of the dominant negative survivin T34A mutant into neuroblastoma cells may act as a tumour suppressor and might be an interesting therapeutic tool. The transactivator protein of HIV-1 TAT has the unique property of mediating the delivery of large proteins into cells when present in the extracellular milieu. In this study we generated a recombinant, dominant negative TAT-survivin T34A fusionprotein, purified it by affinity chromatography and tested its activity in human neuroblastoma cells. We show that TAT-survivin T34A and the control protein TAT-EGFP are transduced into neuroblastoma cells and that TAT-survivin T34A induces apoptosis in survivin overexpressing cells.

Poster Display
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Ref ID: 071.3 **#198**

Characterization of p53 mutation in neuroblastoma cell lines

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The tumor suppressor gene p53 is rarely mutated in neuroblastoma (NB) tumors at diagnosis but its dysfunction could result from non-functional conformation or cytoplasmic sequestration of the wild-type p53 (wt-p53) protein. It is suggested that the loss of p53 function due to mutations could be induced by cytotoxic therapy leading to resistance in NB tumors. To investigate the p53 functionality, we performed the functional assay using the Ade2 yeast strain, YPH500, and the transactivation test using the p21WAF-1/CIP1 Luc-promoter (Flaman et al., 1995) in 7 NB cell lines: SH-SY5Y, LAN-5, LAN-1, SK-N-BE2, SK-N-AS, IGR-N-91, and IGR-NB-8. When p53 was functionally inactive, the white yeast indicating appropriate conformation for p53 transactivation function became red. For wt-p53 cells such as SH-SY5Y and LAN-5, the p53 functional assay in yeast (FASAY) showed white yeast and the test using the p21WAF-1/CIP-1 Luc-promoter induced transactivation. For cells such as SK-N-BE2, which harbored the missense mutation in the p53 DNA-Binding Domain (DBD), the 2 tests were concordant in that they both revealed p53 in inactive conformation. When the mutation concerned the duplication of wild-type 7-9 exons in IGR-N-91 cells (Goldschneider et al., 2004), the Luc-promoter test was reliable whereas the FASAY test apparently did not show a functional defect. In p53-OD-deleted cells, the FASAY test was concordant with the pLuc-promoter transactivation test in that they both revealed functionally defective mutants. To conclude, both tests could be useful for p53-based NB therapeutics.

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Global gene-expression analysis in hypoxic neuroblastoma cells

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In neuroblastoma there is a strong correlation between low stage of tumor cell differentiation and poor outcome. Our group has previously shown that hypoxia promotes a dedifferentiated phenotype in neuroblastoma cells (Jögi, et al., PNAS, 2002, 99:7021-7026). Hypoxia correlates positively to tumor aggressiveness and promotes high metastatic potential and mutational frequency. Hypoxic tumors are also less sensitive to treatment. By use of microarray technology we have investigated hypoxic gene responses. Neuroblastoma tumor cells were treated with 1% O2 for seven time points between 0 and 72 hours. The microarray data was treated with cluster analysis methods and subsequent Gene Ontology analysis. As well as giving time-series profiles of known hypoxia regulated genes (e.g. VEGF, GLUT1, TH), several new potentially hypoxia regulated genes were identified. GeneOntology analysis showed that genes regulated by hypoxia belong to such various groups as metabolism, angiogenesis, differentiation and apoptosis.

Ref ID: 104.1 **#200**

Hypoxia transcriptionally activates the ID2 gene in human neuroblastoma

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ID (inhibitor of differentiation/ DNA binding) proteins, when deregulated, show participation in multiple fundamental traits of cancer, such as block of differentiation, increased proliferation, tissue invasiveness and angiogenesis. These proteins negatively regulate the function of basic helix-loop-helix (bHLH) transcription factors, and influence important cell cycle regulatory proteins. We previously demonstrated that hypoxia decreased expression of neuronal marker genes in neuroblastoma, but induced genes expressed in neural crest sympathetic precursors, such as ID2. Due to its involvement in proper neural crest development and ability to inhibit proneuronal bHLH proteins, the hypoxic induction of the ID2 gene was of particular interest. Here we report fast induction kinetics of the ID1 and ID2 genes in neuroblastoma cell lines under hypoxic treatment, suggesting a transcriptional mechanism. We next investigated the ID2 promoter for presence of potential binding sites for hypoxia-inducible factors (HIFs), the master regulators of gene expression and homeostasis under hypoxic conditions. Subsequent electrophoretic mobility shift assays revealed two functional HIF-1 binding sites (HBSs) within ID2 gene regulatory sequences located -725 and -1893 relative transcriptional initiation. These sites were shown to bind both endogenous and in vitro synthesized HIF-1 complex proteins (HIF-1a and ARNT). Constructs of various lengths of the ID2 gene promoter, linked to a luciferase reporter gene, were transiently cotransfected into HeLa cells together with plasmids expressing HIF-1a, in order to determine the roll of HIF-1 on hypoxic induction of ID2. The two HBSs synergistically upregulated reporter gene expression in a HIF-1 specific manner. Taken together, these observations demonstrate that the ID2 gene is actively engaged by hypoxia and a novel HIF-1 target. The hypoxia-induced ID2 expression could account in part for the previously observed dedifferentiation of hypoxic neuroblastoma cells, thereby giving rise to less mature and more aggressive tumors.

Ref ID: 100.1 **#201**

Phenotypic changes in human neuroblastoma cells induced by oxygen and energy depletion

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In a subset of neuroblastomas, a neuronal to neuroendocrine lineage shift is seen in poorly vascularized cells surrounding necrotic areas, where the tumor cells stain positively for HIF-1 alpha and HIF-2 alpha proteins. HIF-2 alpha is also expressed in the SNS during embryogenesis and HIF-2 alpha deficient mice lack tyrosine hydroxylase (TH) expression and have no catecholamine production. Previously we have investigated the effect of hypoxia on the differentiation status of human neuroblastoma cells and found that hypoxic conditions stabilized HIF-1 alpha and HIF-2 alpha proteins, correlating with activation of known hypoxia-induced genes such as VEGF, IGF-2, GAPDH, ID2 and TH. This was also observed in hypoxic regions of experimental neuroblastomas grown as xenografts in nude mice. Furthermore, expression of neuronal/neuroendocrine marker genes (ChrA/B, neurofilaments, NPY) decreased, while genes expressed in neural crest SNS progenitors (c-kit, ID2, Notch-1) were induced. Similar results were obtained when neuroblastoma cells were grown in a combination of hypoxia and hypoglycemia (low glucose levels). Thus, exposure to hypoxia and energy depletion seems to induce a de-differentiation rather than a neuroendocrine differentiation, both in vitro and in vivo. This could be a new mechanism for selection of more malignant tumor cells. To further analyze the aggressive phenotype of hypoxic neuroblastoma cells, we are currently studying the effect of hypoxia on neuroblastoma cells capacity to migrate and invade surrounding tissues.

Ref ID: 098.1 **#202**

Effect of BS RNASE on chemo resistant neuroblastoma cell lines

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There were confirmed, that bovine seminal ribonuclease (BS RNase) induces apoptosis in several malignant tumors in vivo and in vitro but not in normal cells. The aim of our study was comparing of its efficiency on chemosensitive and chemoresistant neuroblastoma cells in vitro.
METHODS: 3 high-grade neuroblastoma derived cell lines (UKF-NB-1, UKF-NB-3 and UKF-NB-4) and 3 chemoresistant (vincristine, cis-platin, and doxorubicine) derived from each of them were cultivated with different concentrations of BS RNase. Tumor cells viability was measured by MTT test. Flow cytometry was used for evaluation of apoptosis (AnexinV) and P-glycoprotein expression and function. Bcl-2 and Bax expressions were measured using real time RT PCR. We prepared BS RNase chemoresistant lines derived from UKF-NB-3 and UKF-NB-4, which grows in concentration 100 mikrog/ml (UKF-NB-3 BS Rnase res.) and 200 mikrog/ml (UKF-NB-4 BS RNase res.) while 10 mikrog/ml induce apoptosis in all BS RNase sensitive cell lines. BS RNase resistant lines did not overexpress P-glycoprotein but they have increased Bcl-2/ Bax ratio.
RESULTS: BS RNase induced apoptosis in chemosensitive and chemoresistant neuroblastoma cell lines and did not induce overexpression of P-glycoprotein. Bcl-2 overexpression play role in resistance to BS Rnase.
CONCLUSION: BS RNase significantly reduces neuroblastoma growth in vitro by induction of apoptosis, even if they are chemoresistant.

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Ref ID: 147.1 **#204**

Flavopiridol induces cell cycle arrest and apoptosis in human neuroblastoma cells. Enhancement by hypoxia and correlation with MYCN inhibition

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Neuroblastoma is the most common extracranial solid tumor of children which arises from the sympathetic nervous system. Survival rates for neurobastoma patients is low despite intensive therapeutic intervention, and the identification of new effective drugs remains a primary goal. The cyclin-dependent kinase inhibitor, flavopiridol, has demonstrated potent growth inhibitory and cytotoxic activity against various tumor cell types. Our aim was to investigate the effects of flavopiridol on advanced-stage, MYCN-amplified neuroblastoma cells. Moreover, we assessed the modulation of flavopiridol activity by hypoxia, a critical determinant of tumor progression and resistance to therapy. Exposure to flavopiridol resulted in a time- and dose-dependent decrease in the viability of a panel of MYCN-amplified neuroblastoma cell lines. Flavopiridol-treated cells displayed typical apoptotic morphology, and activation of apoptosis was confirmed by DNA fragmentation, TUNEL assay, and flow cytometric determination of DNA content. Cytotoxicity was preceded by DNA synthesis inhibition associated with cell cycle arrest at the G1 and G2 checkpoints, as determined by analysis of BrdU incorporation versus DNA content. Moreover, apoptosis induction was reversed by incubation with the pan-caspase inhibitor, zVAD-fmk and associated with rapid activation of caspase 3 and 2, increase in cytochrome C cytoplasmic content, and downregulation of MYCN mRNA and protein expression. Interestingly, exposure to hypoxia enhanced the extent of apoptosis and flavopiridol effects on cytochrome C, caspase 3, and MYCN. These results indicate that flavopiridol is effective on advanced-stage neuroblastoma in vitro, making this compound a promising candidate for the treatment of this disease.

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Ursolic Acid decreases hypoxia-mediated resistant to apoptosis in SK-N-B2 neuroblastoma cell lines by upregulating caspase-3 activity and downregulating VEGF secretion

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BACKGROUND: Anti-angiogenic agents are considered as new therapeutic agents, Neuroblastoma growth is angiogenesis driven. Angiogenesis inhibition may generate hypoxic zone in the tumor, which may select for survival of aggressive tumor cell variants.
OBJECTIVES: to develop new agent that can induce apoptosis to hypoxic tumor cells, and hence increase the efficacy of anti-angiogeneic therapy. Ursolic acid (UA) is a dietary isoprenoid having apoptotic and anti-angiogenic activity.
METHODS: SK-N-BE-2 (MYCN amplified and p53 mutated) malignant neuroblastoma tumor cell line, was exposed to hypoxia, drug resistance to conventional chemotherapeutic agents was examined by Alamar blue toxicity and caspase-3/7 activation assay. Subsequently, cells were treated with various doses of Ursolic acid (10-40µM) , and apoptosis was measured by caspase-3/7 activity assay, and confirmed by TUNEL assay. The anti-angiogenic activity of UA against hypoxia-mediated VEGF secretion was measured by an ELISA assay.
RESULTS: Cells exposed to 24hour hypoxia showed resistance to etoposide (5µM)-induced toxicity and caspase-3/7 activation . Cells treated with UA (10-20µM) under the same hypoxic condition demonstrated enhanced SK-N-BE-2 toxicity and enhanced caspase-3/7, 20uM UA increased caspase-3/7 activity by 3 fold (p=0.009), and 40uM UA increased caspase-3/7 activity by 7 fold (p=0.019).Under hypoxic condition UA was able to reverse the etoposide related resistance to apoptosis. urther investigation suggests that UA-induced caspase-3/7 activation is associated with the inhibition of hypoxia-induced VEGF upregulation. CONCLUSION: UA reduced the hypoxia-related resistance of etoposide-induced apoptosis by activating caspase-3/7 activity and decreasing VEGF secretion suggesting possible molecular link between hypoxia, angiogenesis, apoptosis and drug resistance.

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Ursolic Acid decreases hypoxia-mediated resistant to apoptosis by upregulating caspase-3 activity and downregulating VEGF secretion in a MYCN amplified neuroblastoma cell line

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BACKGROUND: The anti-angiogenic agents are recently considered as an important therapeutic option to manage advanced neuroblastoma. However, inhibition of angiogenesis might lead to hypoxia, which may increase resistance to apoptosis and subsequently further the risk of invasion, metastasis and patient mortality. Therefore, we are interested in developing novel agent that can induce apoptosis to hypoxic tumor cells, and hence increase the efficacy of metronomic therapy. Ursolic acid (UA) is a dietary isoprenoid having apoptotic and anti-angiogenic activity. UA induces apoptosis by activating caspase-3 system. Here, we examined the ability of UA to induce apoptosis of hypoxic tumor cells. Methods: A highly malignant neuroblastoma tumor cell line, SK-N-BE-2 (MYCN amplified and p53 mutated) was exposed to hypoxia, and the resistance to conventional chemotherapeutic agents was examined by Alamar blue toxicity and caspase-3/7 activation assay. Subsequently, cells were treated with various doses of Ursolic acid, and apoptosis was measured by caspase-3/7 activity assay, and confirmed by TUNEL assay. The effect of UA against hypoxia-mediated VEGF secretion was measured by an ELISA assay.
RESULTS: Cells grown in 10% serum and exposed to 24hour hypoxia showed resistance to etoposide (5uM)-induced toxicity and caspase-3/7 activation. When treated with UA (5-40uM), the same hypoxic condition enhanced SK-N-BE-2 toxicity and caspase-3/7 activity. In addition, UA was able to reverse the hypoxia-mediated resistant to etoposide-induced apoptosis. Further investigation suggest that UA -induced caspase-3/7 activation is associated with the inhibition of hypoxia-induced VEGF upregulation.
CONCLUSION: This novel effect of UA is associated with casapse-3/7 upregulation and VEGF inhibition.

Ref ID: 020.3 **#207**

Bak: A downstream mediator of fenretinide-induced apoptosis of SH-SY5Y neuroblastoma cells

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Unlike 13-cis retinoic acid, the synthetic retinoid fenretinide [N-(4-hydroxyphenyl)retinamide] induces apoptosis of neuroblastoma cells by mechanisms involving retinoic acid receptors (RARs) and oxidative stress. After screening a cDNA array for apoptosis-related genes, the Bcl2-related protein Bak was identified as a fenretinide-inducible gene in SH-SY5Y neuroblastoma cells and this was confirmed by Western blotting and flow cytometry. Although fenretinide acts synergistically in vitro with chemotherapeutic drugs, these drugs did not induce Bak expression. RAR antagonists did not block the induction of Bak by fenretinide. Conversely, Bak induction was blocked by the antioxidant, vitamin C. Overexpression of Bak increased apoptosis in both the presence and absence of fenretinide, whereas expression of antisense Bak inhibited fenretinide-induced apoptosis. Bak expression was also induced in cells over-expressing the stress-induced transcription factor GADD153, but inhibited in cells expressing an antisense-GADD153 construct. These results suggest that Bak is a downstream mediator of an oxidative stress pathway leading to apoptosis of neuroblastoma cells in response to fenretinide.

Ref ID: 020.4 **#208**

Fenretinide increases ceramide induction through acidic sphingomyelinase dependent hydrolysis of sphingomyelin resulting in downstream apoptosis of neuroblastoma cells

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Ceramide is an important lipid-signaling molecule implicated as a common intermediate of many apoptotic pathways. Fenretinide is thought to induce apoptosis and reactive oxygen species (ROS) via increases in ceramide levels; however, the mechanism of ceramide accumulation in neuroblastoma cells is unclear. Intracellular ceramide can be derived either from de novo synthesis on the endoplasmic reticulum via ceramide synthase or by hydrolysis of membrane sphingomyelin by sphingomyelinases. To identify the contribution of these pathways to fenretinide-induced ROS and apoptosis in human neuroblastoma cells, the activity and expression of ceramide synthase and sphingomyelinases was blocked using chemical inhibitors and RNA interference. Fumonisin, an inhibitor of ceramide synthase, did not block fenretinide-induced ROS or apoptosis. In contrast, a sphingomyelinase inhibitor effectively blocked apoptosis and ROS accumulation in response to fenretinide. This effect was not seen with an inhibitor of neutral sphingomyelinase, suggesting that acidic sphingomyelinase (ASMase) activity alone mediated the effects of fenretinide. \'Knockdown\' of ASMase by RNA interference blocked the fenretinide-induced increase in ceramide levels, apoptosis and ROS, and also decreased sphingomyelin levels. Furthermore, there was evidence that fenretinide increased levels of ASMase protein, and that this increase was abolished by RNA interference. These results show that, in these neuroblastoma cells, ceramide accumulation in response to fenretinide is derived from sphingomyelin hydrolysis via ASMase, but not de novo synthesis, and suggest that sphingomyelin synthetase or ASMase may be targets for future drug development.

Ref ID: 172.1 **#211**

Studies on lymphangiogenesis in human neuroblastoma

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Angiogenesis in neuroblastoma (NB) is well studied and successful reports on anti-angiogenesis as a therapeutic option are available. However, data on lymphangiogenesis in NB is missing and so far, the existence of lymphatic vessels has not been demonstrated in NB tumors. The presence of lymph node metastasis shows that next to hematogenous spread, infiltration via the lymphatics is an important mechanism in NB growth. Differentiation between blood- and lymphangiogenesis is performed by the use of specific lymphatic endothelial cell markers like VEGFR-3, LYVE-1, Podoplanin, Prox-1 and D2-40. Growth factors for blood- and lymphangiogenesis have been identified and the most important regulators for lymphangiogenesis VEGF-C and VEGF-D have been detected in some tumors. We investigated the presence of blood and lymphatic endothelial cells in 10 NB tumors by immunohistochemistry. We used the CD31 antibody which specifically binds to the surface of blood endothelial cells and the D2-40 antibody (Signet Laboratories) which recognizes a sialoglycoprotein on lymphatic endothelial cells. We showed that both, blood and lymphatic vessels are present in different amounts in all tumor specimen examined. Next, we examined the expression of VEGF-C and VEGF-D in 8 human NB cell lines and in 9 NB tumors by RT-PCR. The NB cell lines SH-EP, SH-SY5Y, Kelly, SK-N-AS, NMB express VEGF-C. Expression of VEGF-D was detected in Kelly, SK-N-FI, SK-N-AS and NMB cells. In 4 tumors, we noted expression of VEGF-C and in 2 of them co-expression of VEGF-D. Treatment of NB cell lines with 9-cis retinoic acid did not change considerably the expression of the two growth factors. In summary, we could demonstrate the presence of lymphatic vessels in NB tumors in different amounts. Furthermore, we showed that NB cell lines express VEGF-C and VEGF-D in vitro and in vivo. Therefore, anti-lymphangiogenesis could be a new option in NB therapy.

Ref ID: 028.3 **#212**

Vessel characterisation in an orthotopic model of neruoblastoma

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Orthotopic animal models allow studying organ-specific determinants and are needed to obtain relevant data for angiogenic approaches. We used sonographic and Doppler investigations to quantify atraumatically in vivo vascularization of such models. GFP-tagged human neuroblastoma cells have been injected in intra-adrenal in nude mice. EchoDoppler investigations have been conducted on an ATL HDI5000 sonograph using a 12 MHz linear probe. Mice were sonographed once a week during 78 days after tumor transplantation. For each mouse, tumoral dimensions were quantified and a Doppler sequence recorded in the longitudinal axis of the tumor has been transferred to a computer via a network. Vessel counting was post-processed on the numerical sequence using the HDILab software. Intratumoral and peripherical vessels have been counted separately. Rapid increase in the tumor volume, between 43 and 50 days after neuroblastoma cells injection, was positively correlated with the density of intratumoral vessels. Peripherical vessels were generally detected since the first day and their number increased with tumor development. This study demonstrates the ability of high frequency ultrasound investigations to detect deep and small tumors such as orthotopic neuroblastoma. The strong correlation between tumor growth and tumoral neovessel density was confirmed by immunohistochemistry staining for CD31. Interestingly, CD61 immunostaining reveals that endothelial cells of orthotopic tumors vessels express alphaV-beta3 integrins, by contrast to heterotopic control tumors, making this model particularly suitable for the evaluation of antiangiogenic approaches.

Ref ID: 393.1 **#209**

Induction of the BH3-only HRK and BBC3 Correlates with Sensitivity to Fenretinide in Neuroblastoma Cell Lines

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Fenretinide (4-HPR) is a synthetic retinoid whose apoptosis-inducing effects have been demonstrated in vitro and is currently being tested in clinical trials for a number of malignancies including neuroblastoma (NB), breast, ovarian, colorectal and prostate cancer. In NB, 4-HPR has been shown to induce apoptosis in retinoid-sensitive and -resistant cell lines, but the mechanism responsible for its apoptotic action is not fully understood. In the present study, we performed cDNA microarray experiments to investigate the temporal changes in gene expression during 4-HPR-induced cell death in an ATRA resistant cell line. Analysis of the data through K-means clustering and gene ontology searches identified 5 major temporal expression patterns; each one was enriched for genes that reflected the biological processes that were impacted during treatment with 4-HPR. Analysis of the apoptotic-related genes demonstrated an early up-regulation of Harakiri (HRK) and Puma (BBC3), two transcriptionally regulated pro-apoptotic members of the BCL2 family. Viability assays performed on a set of MYCN amplified and non-amplified NB cell lines, showed that two 4-HPR treated cell lines out of eight were resistant to the drug. RT-PCR showed that HRK and BBC3 were only induced in the 6 sensitive lines suggesting that the induction of these genes play a role in 4-HPR-induced cell death in NB cell lines.

Ref ID: 390.1 **#210**

Cell death-associated genes in neuroblastoma: evidence for a cell type-specific effect of ataxin-2 in doxorubicin-induced apoptosis

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Understanding the molecular pathways that regulate apoptosis of neuroblastoma cells may provide important information about drug resistance mechanisms and thus, may eventually lead to the development of novel therapeutic strategies. We have been studying the transcriptional profiles of pre-apoptotic neuroblastoma cells, employing subtractive cDNA cloning and an antisense-based functional assay (´technical knock-out´). Recently, two putative apoptosis-related genes, ataxin-2 and SOXN, were identified (Wiedemeyer et al., Oncogene 2003; Wittke et al., Cancer Research 2003).
Ataxin-2 has been implicated in RNA editing and is mutated by polyglutamine expansion in the neurodegenerative disease SCA2. We found high ataxin-2 expression in pre-apoptotic Tet21N neuroblastoma cells and in non-MYCN-amplified neuroblastoma tumors while low protein expression levels were detected in MYCN-amplified neuroblastoma specimens. Furthermore, overexpression of wildtype ataxin-2, containing a polyglutamine tract of 22 amino acids, sensitized Tet21N cells for apoptosis following serum starvation and interferon g treatment. In contrast, ataxin-2 mutants with 79 glutamines (as in SCA2) or 1 glutamine residue (as in mouse ataxin-2) lost their proapoptotic activity, indicating that apoptosis sensitization is a wildtype function in this setting and probably mechanistically different from cell death induction by expanded ataxin-2 in SCA2. Extending our studies to other cell lines and apoptosis-inducing agents, we now show that ataxin-2 also sensitizes for doxorubicin-induced apoptosis and that it does so in a cell type-specific manner. The molecular characterization of ataxin-2-mediated apoptosis reveals a link to the apoptosis-inducing machinery and offers a possible explanation for the observed proapoptotic effect of ataxin-2 in neuroblastoma cells.

Ref ID: 032.1 **#213**

Bone Marrow Derived Matrix Metalloproteinase-9 Regulates Maturation of Neuroblastoma Vasculature

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We have previously shown that matrix metalloproteinase-9 (MMP-9) is markedly elevated in advanced stages of neuroblastoma and is expressed by perivascular stromal cells in the tumor. We have demonstrated that MMP-9 knockout (MMP-9-/-) mice, orthotopically implanted with human neuroblastoma tumors, have a defect in vascularization characterized by small, immature vessels with minimal pericyte coverage. Here we have determined the source of MMP-9 production and its role in neuroblastoma vascularization by performing EGFP labeled bone marrow transplant, using MMP-9-/- and MMP-9+/+ recipient and donor combinations, followed by orthotopic neuroblastoma implantation into the left adrenal gland after full bone marrow engraftment. We observed a complete restoration of vascular architecture with a 74% increase in pericyte area (p=0.0006) and more than a two-fold increase in pericyte/endothelial cell ratio (p=0.00003) in MMP-9-/- recipients transplanted with MMP-9+/+ donor bone marrow in comparison to tumors where both recipients and donors were MMP-9-/-. However, when MMP-9+/+ recipients were transplanted with bone marrow from MMP-9-/- donors, vessels were immature with few pericytes, indicating that bone marrow derived MMP-9 is required for pericyte coverage. Whereas we could not find evidence that bone marrow cells become pericytes, we documented that some endothelial cells are derived from bone marrow and that vasculogenesis requires the expression of MMP-9 by bone marrow cells. Thus MMP-9 plays multiple roles in neuroblastoma vascularization by regulating vessel maturation and contributing to vasculogenesis. The data suggest that inhibition of MMP-9 could contribute to neuroblastoma therapy by altering tumor vascularization.

Ref ID: 032.4 **#214**

Absence of plasminogen activator inhibitor-1 inhibits angiogenesis and progression in neuroblastoma

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Inhibition of plasminogen activator (PA) in cancer has been expected to inhibit progression. However in many cancers, including neuroblastoma (Cancer Research 59:1327-1336, 1999), higher rather than lower levels of the PA inhibitor-1 (PAI-1) have been reported associated with more aggressive disease. Interestingly, in neuroblastoma, this inhibitor is exclusively expressed by stromal cells. In this study, we have examined the role of PAI-1 in tumor progression in human neuroblastoma tumors orthotopically implanted in PAI-1 deficient and immunosuppressed mice. We observed an 80% reduction of tumor growth during the first 5 weeks after tumor implantation in PAI-1-/- mice compared to PAI-1+/+ mice. This growth inhibitory effect was associated with a 3 fold reduction in the vascular endothelial cell area of these tumors. Concomitantly, we observed a 2 to 4 fold decrease in the number of BrdUdr positive tumor cells and a 3 to 8 fold increase in TUNEL positive (apoptotic) endothelial cells. To understand the mechanism underlying endothelial cell apoptosis in the absence of PAI-1, we downregulated PAI-1 by siRNA in human brain microvascular endothelial cells. Suppression of PAI-1 expression in these cells inhibited cell spreading and decreased the formation of endothelial tubes in vitro by 78%. This effect was associated with a significant increase in spontaneous apoptosis and in the levels of Caspase 3. The data, which suggest that PAI-1, in addition to inhibiting plasminogen activation, may be a survival factor for endothelial cells, provide a mechanism explaining the proangiogenic effect of PAI-1 and its association with an unfavorable prognosis in neuroblastoma.

Ref ID: 013.1 #215

Inhibition of Angiogenesis by the Epidermal Growth Factor (EGF)-module of the Follistatin Domain of SPARC is Conformation-Dependent

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BACKGROUND: SPARC (Secreted Protein Acidic and Rich in Cysteine) is a multi-functional matricellular glycoprotein. Previously, we demonstrated that neuroblastoma (NB) tumor-derived Schwann cells produce SPARC and that purified SPARC protein potently inhibits angiogenesis and impairs NB growth in vivo. The SPARC protein is comprised of three highly conserved domains that are independently folded by a complex pattern of disulfide bonds. To investigate the biologic activity of these domains, peptides were synthesized and tested for their ability to inhibit angiogenesis.
METHODS: SPARC domains were synthesized as cysteine-linked peptides FS-E, FS-K, and EC-N designed to correspond to the EGF-like module, part of the Kazal module of the follistatin domain, and the conserved α-helix in the C-terminal extracellular calcium-binding domain, respectively. A non-folded peptide FS-E in which the cysteines were not linked during the synthesis, and a scrambled peptide FS-E, were used as controls. The folded peptides and controls were tested for their ability to inhibit endothelial
cell migration and angiogenesis in vivo.
RESULTS: Peptide FS-E strongly inhibited basic fibroblast growth factor-(bFGF) induced endothelial cell migration (ED50=10 pM) and potently blocked angiogenesis in vivo in the rat corneal assay and the Matrigel plug assay. The anti-angiogenic activity was completely abrogated with the non-folded and scrambled FS-E peptides. Peptides FS-K and EC-N had minimal to no inhibitory activity.
CONCLUSION: Our data demonstrate that the EGF-like module of the SPARC follistatin domain is a powerful inhibitor of angiogenesis, and that its structural conformation is essential for this biologic activity. Because of its physiological relevance to Schwann cells, SPARC peptides like FS-E may be promising candidates for the development of anti-angiogenic treatment strategies for NB.

Ref ID: 170.1 #216

Anti-angiogenic effects of neuroblastoma tumour vessels-targeted liposomal chemotherapy

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Solid tumours recruit new blood vessels to support tumour growth and unique epitopes expressed on tumour endothelial cells can function as targets for anti-angiogenic therapies. An NGR peptide targeting aminopeptidase N, a marker of angiogenic endothelial cells, was coupled to the surface of liposomal-doxorubicin (NGR-SL[DXR]) and was used to treat orthotopic neuroblastoma (NB) xenografts in mice. These long-circulating liposomes showed time-dependent uptake into tumour, being 10 times higher than that of non-targeted liposomes. No uptake was observed into tumours of mice treated with the mismatched peptide ARA-targeted SL[DXR] or those co-injected with an excess of soluble NGR. NB-bearing mice treated with NGR-SL[DXR] partly outlived the control mice, displaying tumour regression and inhibition of metastases growth. All animals showed tumour mass reduction, pronounced destruction of the tumour vasculature and blood vessel density suppression. Double staining of tumours with TUNEL and anti-factor VIII antibody or anti-human NB, demonstrated endothelial cell as well as tumour cell apoptosis. Moreover, NGR-SL[DXR], alone or in combination with GD2-targeted liposomal doxorubicin, has been used against a more aggressive experimental metastatic model of NB. Long term survival was obtained only in mice treated with the combination of the two formulations, with improved vascular damage and anti-angiogenic effects. Further investigations as well as other in vivo combined therapies are on running, whose results will be presented at the meeting.

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Epidermal Growth Factor Receptor Mediated Survival of Human Neuroblastomas

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BACKGROUND: Neuroblastoma is a common solid tumor of childhood and derived from the neural crest. Expression of epidermal growth factor receptors (EGFRs) has been associated with enhanced cell growth and aggressive behavior in other tumor systems. Aims: Here we examined the expression profile of EGFRs in 13 neuroblastoma cell lines and 16 primary tumors. Results: We found that all neuroblastoma cell lines and primary tumors examined expressed EGFR1 (HER1) at readily detectable levels. We show here that EGF has a significant proliferative effect on neuroblastoma cell lines SY5Y and NLF in vitro by MTT assay. EGF exposure phosphorylates HER1 and activates both MAPK and PI3K/Akt pathways, demonstrating the HER1 pathway in neuroblastoma is functional. Exposure to 0.5 µM ZD1839, a HER1-specific inhibitor, caused a 40%-50% reduction in survival of SY5Y and NLF cells, as measured by MTT assay ($p<0.01$), and a two to three fold increase in apoptosis, as measured by cell death ELISA. Even at a concentration of 0.1µM, ZD1839 partially inhibited autophosphorylation of HER1 by EGF in NLF cells. This concentration also blocked phosphorylation of Akt but not MAPK. Additional studies demonstrated that the PI3K/Akt-specific inhibitor LY294002, not the MAPK-specific inhibitor U0126, completely abolished the proliferation effect by EGF. This indicates that the PI3K/Akt pathway may be the main signaling pathway responsible for the survival and growth effects of EGF in neuroblastomas. Conclusions: Our results also indicate that ZD1839 has potent growth inhibitory effect in neuroblastoma cells, and so it could be a useful, biologically-based therapeutic agent for neuroblastoma.

Ref ID: 085.1 #218

Effects of Human Endostatin on Neuroblastoma Xenograft: Lessons for Clinical Use

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We examined the efficacy of recombinant human endostatin (rhEndostain) on human neuroblastoma xenograft by 3 different ways of administration, and compared its efficacy with those of cytotoxic agents. In the first experiment, when tumors (TNB9) on nude mice reached a weight of 90-95 mg, rhEndostatin 10 mg/kgMW/day was administered subcutaneously to the mice (n=5) daily for 10 consecutive days (total, 100 mg/kgMW). Secondly, the same daily dose of rhEndostatin was administered continuously to the TNB9-bearing mice (n=6) with pumps for 3 consecutive days (total, 30 mg/kgMW). Thirdly (n=6), the period of the administration was extended to 9 days with the pumps replaced every three other days (total, 90 mg/kgMW). In addition to HE, nestin and factor VIII expressions were studied immunohistochemically. The mode of pharmacological actions of 23 chemotherapeutic agents was compared with that of rhEndostatin. In the first experiment, there was a significant decrease in relative tumor weight (RTW) in the experimental group on day 2 only ($p<0.05$). In the second experiment, RTW was significantly less than that of controls on day 2 through 10 ($p<0.01$ or 0.05), with a maximum inhibition rate of 60.7%, indicating efficacy. In the third experiment, RTW was also significantly less than that of controls on days 6, 9 and 12 ($p<0.01$ or 0.05). The number of the intratumoral vessels immunostained with anti-factor VIII antibody was noticeably reduced in tumors on rhEndostatin-treated mice, and nestin staining showed a loss of fibrillar vascular structure in treated tumors. No side effects were observed throughout experiments. Measurement of RTW and immunohistochemical studies clarified that rhEndostatin is effective only when administered continuously, whereas 12 of the 23 chemotherapeutic agents showed efficacy by intermittent administration.

Ref ID: 187.1 #219

Antiangiogenic effects of chemotherapeutics, rapamycin and fenretinide in neuroblastoma

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Angiogenesis plays a critical role in sustaining growth metastatic potential of many solid tumours, including neuroblastoma (NB). Recent evidences suggested that antiangiogenesis is an attractive and effective strategy against NB. Here, we evaluated the antiangiogenic activities in NB of two cytoskeleton-toxic chemotherapeuticals (vinblastine and docetaxel), the mTOR inhibitor rapamycin and the synthetic retinoid fenretinide, tested at low doses, accordingly to the metronomic therapeutic schedule. To this purpose, we investigated in vitro and in vivo potential of these molecules on angiogenesis induced by the conditioned media (CM) derived from two human NB cell lines MYCN-amplified and nonamplified, such as Htla-230 and SH-SY5Y, respectively. We found that Htla-230-derived CM strongly increased endothelial cell (EC) proliferation, whereas only a weak induction by SH-SY5Y-derived CM was observed. A strong EC growth inhibition was observed in a time- and dose-dependent manner with IC50 values in the range of 0.1-1pM for vinblastine and docetaxel, 10-50nM for rapamycin and 0.5-1M fenretinide. Comparable antiproliferative effects were also obtained when EC were preincubated with NB cell-derived CM. Apoptosis was evident by treating EC with both chemotherapeuticals and fenretinide while a cell cycle inhibition with only marginal apoptotic effect was observed in rapamycin treated EC. Moreover, Htla-230 CM seems to delay the effects induced by the different molecules. Similar results were obtained in vivo by using the chick embryo choriallantoic membrane assay, where the angiogenic response induced by NB CM, NB tumour xenografts in mice or human NB biopsy samples was significantly inhibited by administration of the four molecules. Further studies to investigate the potential synergistic antiangiogenic effect of these molecules administred in combination are in progress in our laboratories.

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Fenretinide induces sustained-activation of JNK/p38 MAPK and apoptosis in a reactive oxygen species-dependent manner in neuroblastoma cells

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Fenretinide, which mediates apoptosis in neuroblastoma cells, is being considered as a novel therapeutic for neuroblastoma. However, the cytotoxic mechanisms of fenretinide have not been fully elucidated. Sustained-activation of JNK and p38 MAPK signaling has recently been shown to have a pivotal role in stress-induced apoptosis. Whether fenretinide activates the signaling in neuroblastoma cells is not known. In the present study, fenretinide induced sustained-activation of both JNK and p38 MAPK in neuroblastoma cells. Pretreatment with the antioxidant L-ascorbic acid almost completely inhibited the accumulation of fenretinide-induced intracellular reactive oxygen species (ROS), activation of JNK and p38 MAPK, and apoptosis. On the other hand, intracellular ROS production and activation of stress signaling was not altered by fenretinide in resistant neuroblastoma cells. Our study demonstrates that in neuroblastoma cells, fenretinide induces sustained-activation of JNK and p38 MAPK in an ROS-dependent manner, and indicates that JNK and p38 MAPK signaling might mediate fenretinide-induced apoptosis. Our results also indicate that suppression of the fenretinide-induced ROS productive system and the downstream JNK and p38 MAPK signaling pathways causes neuroblastoma cells to become resistant to fenretinide.

Ref ID: 163.1 #220

Targeting neuroblastoma with bi-specific antibodies against the neural cell adhesion molecule (NCAM)

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BACKGROUND: Cell membrane localized neural cell adhesion molecules (NCMA) are found in nearly 100 % of all neuro-blastoma cells (Roy et al, 1996). Recent research revealed that antibody binding to NCAM inhibits neuro-blastoma cells growth in a direct way due to signal transduction (Krushel et al, 1998, Dehal et al, 2002). Thus NCAM may be a promising target for antibody based immunotherapy.
AIM: A bispecific CD3xNCAM antibody was generated. The cytotoxic activity against neuroblastoma cells as well as its immunomodulatory effects was investigated.
RESULTS: Bispecific CD3xNCAM antibodies demonstrated distinct cytotoxic activity against neuroblastoma cells. Unexpectedly normal NCAM+ Natural Killer cells (NK cells) were found helping T cells to proliferate and enforcing T cells attack against neuroblastoma cells. As a consequence of bispecific T cell recruitment NK cells itself were depleted. CD3xNCAM bispecific antibodies were demonstrated pushing T cells into a CCR7-/CD45RA-/CD62L- effector memory T cell (EM cells) differentiation. EM cells are usually capable of actively migrating into malignant tissues were they can exercise strong cellular cytotoxicity.
Further investigations using very low doses of CD3xNCAM bispecific antibodies in an antigen specific T cell response model (tetanus toxoid) provided evidence for synergism indicating that bi-specific antibodies might also enhance T cell receptor (TcR) specific responses, which probably could result in a long lasting anti-tumor immunity when used in vivo. Thus far upregulation of CD25, CD86 and CD95 (weak) on CD14+ cells (monocytes, macrophages) indicated co-activation of these important T cell supporting by-stander cells.
CONCLUSIONS: The distinct cytotoxic properties against neuroblastoma cells and the strong immunomodulatory effects of CD3xNCAM bispecific antibodies make them promising new tools for immunotherapy of neuroblastoma.

Ref ID: 369.1 #223

Distinct involvement of reactive oxygen species and caspase activation in apoptosis pathways in a neuroblastoma cell line, NB-39-nu, induced by retinoic acid or fenretinide

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BACKGROUND: In a few of neuroblastoma cell lines, retinoic acid (RA) can induce apoptosis independently of differentiation, but the precise mechanism(s) remains unknown. NB-39-nu is a human neuroblastoma cell line that has the oncogene MYCN amplification and a near-triploid karyotype. In the previous ANR meeting, we presented that, in this cell line, RA not only down-regulated MYCN protein, but also induced apoptosis independently of differentiation. Aimes: In this study, we further investigated if RA or fenretinide-induced apoptosis of this cell line would be dependent on generation of reactive oxygen species (ROS) or activation of caspases.
METHODS: After cells were cultured for three days in the presence of RA or fenretinide at 5 microM in combination with or without N-acethyl-L-cysteine (NAC), apoptosis was evaluated by measuring the subG1 fraction by PI staining and FACS analysis, and the expression of poly (ADP-ribose) polymerase (PARP) and caspases (3, 8 and 9) were examined by Western blot analysis.
RESULTS: A combination of NAC reduced two-thirds of subG1 fraction in fenretinide-treated cells, while NAC had no effect on that of RA-treated cells. Cells treated with RA or fenretinide showed the cleaved form of PARP. Moreover, cells treated with fenretinide showed the cleaved form of caspase 8, but little activation of caspase 8 was detected in cells treated with RA.
CONCLUSIONS: Although the cleavage of PARP suggested that RA or fenretinide-induced apoptosis of NB-39-nu was dependent on caspase 3 activation, its upstream apoptosis pathway in RA-induced apoptosis may be distinct from that induced by fenretinide in which ROS generation and caspase 8 activation were involved. It is now under investigation to determine whether alteration in mitochondria property is involved in RA-induced apoptosis of NB-39-nu.

Ref ID: 020.2 **#224**

GADD153 Mediates Apoptosis in Response to Fenretinide but Not Synergy Between Fenretinide and Chemotherapeutic Drugs in Neuroblastoma

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Fenretinide [N-(4-hydroxyphenyl)retinamide] induces apoptosis of neuroblastoma cells in vitro and interacts synergistically with the chemotherapeutic drugs cisplatin and etoposide. The stress-inducible transcription factor GADD153 is induced in response to fenretinide, and in other cell types modulates apoptosis via pro- and anti-apoptotic members of the BCL2 family. Since BCL2-family proteins are important in apoptosis induced by chemotherapeutic drugs, GADD153 may be a key mediator of synergy between fenretinide and chemotherapeutic drugs. To investigate this, GADD153 cDNA in sense and antisense orientations was stably transfected into SH-SY5Y neuroblastoma cells using a tetracycline-inducible vector. Increased expression of GADD153 raised the background level of apoptosis, and increased apoptosis induced by fenretinide or the chemotherapeutic drugs cisplatin and etoposide. However, there was no increase in synergy between fenretinide and chemotherapeutic drugs. Conversely, expression of antisense-GADD153 virtually abolished the induction of apoptosis in response to fenretinide, but, overall, had no significant effect on apoptosis induced by chemotherapeutic drugs. The effect of antisense-GADD153 on synergy between chemotherapeutic drugs and fenretinide varied with the drug used: there was no effect on synergy between fenretinide and cisplatin, but the combination of fenretinide with etoposide became antagonistic. These results suggest that mechanisms mediating synergy between fenretinide and chemotherapeutic drugs lie upstream of GADD153.

Ref ID: 151.1 **#225**

Retinoids in Experimental Neuroblastoma Therapy

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Retinoids have documented activity against various malignant cell types. Neuroblastoma shows a complex clinical and biological heterogeneity, with poor outcome despite intensive multimodal therapy. We investigated effects of retinoid treatment in vitro on human neuroblastoma cells, and in vivo on human neuroblastoma xenografts in nude rats. The ultimate goal was to find a new retinoid treatment for children
with neuroblastoma. Oral treatment with 9-cis RA in vivo resulted in a significant inhibition of neuroblastoma tumour growth, but with major toxic side effects. Further experiments showed that 9-cis RA might not be suitable for clinical use in children with neuroblastoma, because of its short half-life, low bioavailability and toxic profile in rats. Ro 13-6307 was established to be a morphologically differentiating retinoid, able to reduce proliferation and induce G1 growth arrest in both MYCN amplified and non-amplified neuroblastoma cell lines in vitro. Further experiments showed that oral Ro 13-6307 could inhibit neuroblastoma tumour growth in vivo with limited toxicity. In vitro and in vivo results indicated that Ro 13-6307 was at least as effective as the clinically established retinoid 13-cis RA. No significant reduction in neuroblastoma tumour growth was observed after oral treatment with fenretinide in vivo, despite promising in vitro results. Five different doses were evaluated, but no significant inhibiting effect on tumour growth or morphological changes were found in treated compared to untreated tumours. In conclusion, retinoids inhibited growth of human neuroblastoma both in vitro and in vivo, however the effect depends on the retinoid in use. Dosing, scheduling, and toxicity are important factors determining the therapeutic efficacy of retinoids in vivo. Other alternatives for fenretinide administration should be investigated in future experimental and clinical studies. Ro 13-6307 may be a retinoid for future clinical therapy of children with neuroblastoma.

Ref D: 219.1 **#229**

Digoxin inhibits neuroblastoma tumor growth in mice

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Epidemiological data have shown that carcinoma of the breast in women taking cardiac glycosides due to congestive heart failure has a more favorable outcome. The aim of this study was therefore to investigate whether digoxin could inhibit growth of various cancers in mice or not, and if so, whether the mechanism is cytotoxicity or inhibition of angiogenesis Digoxin inhibited human neuroblastoma xenografts growth in mice by 44% (p= 0.008), and syngeneic neuroblastoma by 19% (p=0.007) whereas LS174T colonic cancer xenografts and syngeneic Lewis lung cancer tumors were less responsive.
This neuroblastoma specificity was confirmed in vitro, where the human neuroblastoma cell lines SH-SY5Y and SK-N-AS were the most sensitive to digoxin among the cell lines tested. Digoxin also inhibited fibroblast growth factor-2 (FGF-2) stimulated bovine endothelial cell growth in vitro with a 50% cell survival (IC 50) at 53 ng/ml. Treatment with 1 μg/filter of digoxin inhibited FGF-2-induced angiogenesis in the chick chorioallantoic membrane (CAM) assay. Our data suggest that digoxin may be a specific neuroblastoma growth inhibitor and an unspecific inhibitor of angiogenesis. This makes cardiac glycosides an interesting basis for development of new anticancer agents.

Key words: angiogenesis - digoxin- mice - neuroblastoma-tumor

Ref ID: 035.2 **#230**

Transcriptional Therapy for Neuroblastoma (NB)- The HDAC inhibitors MS-275 and Depsipeptide decrease N-myc levels and inhibit NB tumor growth in vitro and in vivo

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Deacetylated histones are associated with cell growth while hyper-acetylated histones are associated with cell growth arrest and differentiation. Histone deacetylase inhibitors (HDACI) are a class of compounds that have a broad spectrum of anti-tumor activity and are in Phase I clinical trials. We have evaluated the effects of MS-275 and depsipeptide in a number of NB cell lines in in vitro and in vivo models. The growth of 5 NB cell lines (both N-myc amplified and single-copy) is inhibited by either MS-275(IC50= 0.3-8uM) or depsipeptide(IC50= 1-6ng/ml) after 72hrs of treatment. By 4-8 hours there is marked increase in Acetylated-histone H-3 (AC-H3) that plateaus by 24hrs. The decrease in cell growth is marked by an increase in apoptotic cells and cleavage of the caspase-3 substrate PARP. Increases in p21 and decreases in N-myc mRNA occur within 4hrs of depsipeptide and 18hrs of MS-275 treatment. Both HDACI were active in single-copy and N-myc amplified cell lines. In vitro, combinations of MS-275 and retinoids are not advantageous. In vivo, MS-275 inhibited the growth of 3/3 NB cell lines evaluated from 50-80% and therapeutically relevant levels of MS-275 were achievable in vivo. Tumor xenografts from MS-275 treated mice had decreased expression of N-myc and increased expression of p21 compared to tumors in placebo treated mice. The molecular mechanisms by which these drugs modulate gene transcription in vitro and in vivo is currently underway. These studies indicate that HDACI may be class of compounds that are efficacious for Neuroblastoma patients.

ID: 325.1 **#226**

Inhibition of neuroblastoma cell proliferation by combination of STI-571 with 9-cis retinoic acid in vitro

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Neuroblastoma (NB) originates from sympathetic neurons derived from the neural crest. Stage IV disease is related to poor prognosis although multidisciplinary therapy such as surgery, chemotherapy, differentiation with retinoic acid (RA) and radiation is used. Therefore, novel anticancer drugs are urgently needed. STI-571 is a selective inhibitor of several structurally related receptor tyrosine kinases including c-Abl, Bcr-Abl, c-Kit and the platelet-derived growth factor receptors (PDGF-receptors). This new agent is used in the treatment of chronic myeloid leukemia and gastrointestinal stromal tumors. Potential therapeutic activity is also described for other c-Kit positive malignancies.
We investigated 6 NB cell lines (SH-EP, SH-SY5Y, Kelly, LAN-5, SK-N-FI, SK-N-LO) for expression of c-Kit and PDGF-receptor alpha and beta by FACS analysis. Expression of c-Kit is present in SK-N-FI and SK-N-LO cells. Expression of PDGF-receptor alpha is absent in all cell lines examined, whereas SH-EP, SH-SY5Y, LAN-5 and Kelly cells express PDGF-receptor beta. In vitro cell growth following treatment with 1, 5, 10 and 20 µmol STI-571 for 72 hours was measured by MTT assay. Proliferation of all 6 NB cell lines is inhibited significantly by 20 µM STI-571. LAN-5 and SK-N-FI cells are also inhibited by lower concentrations of STI-571. Taken together, expression of c-Kit or PDGF-receptors does not correlate with the inhibitory effect of STI-571 in NB cell lines. The proliferation of SH-EP, SH-SY5Y and Kelly cells but not of the other three NB cell lines examined is inhibited by treatment with 1 µmol 9-cis retinoic acid for 72 hours. Addition of STI-571 in all concentrations during treatment with 1 µmol 9-cis retinoic acid significantly increased this inhibitory effect. In conclusion, NB cell lines sensitive to RA are potential candidates for a combination therapy with STI-571.

Ref ID: 218.1 **#227**

Continuous low-dose treatment with imatinib mesylate as a possible therapeutic concept for neuroblastoma

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Many neuroblastomas express the tyrosine kinase receptors for PDGF and c-kit, both of which can be targeted by imatinib mesylate We evaluated dose- and time-dependent effects of treatment with imatinib on the proliferation of neuroblastoma cells in vitro and neuroblastoma xenograft growth in vivo. When given as short-term treatment (< 72 hours) imatinib was ineffective against neuroblastoma in vitro, in concentrations below 15 µM. However, prolonged administration of imatinib for five days or more was significantly more effective than short-term treatment against SH-SY5Y and IMR-32 cells ($p<0.001$). Treatment with 5 µM imatinib significantly stabilized the growth of SH-SY5Y cells and completely inhibited the growth of IMR-32 cells, respectively, when given continuously for 7 days. Continuous exposure to low-dose imatinib (2.5-5 µM) for one week resulted in a near complete inhibition of neuroblastoma cell clonogenic survival ($p<0.001$) for all cell lines tested (SK-N-BE(2), SH-SY5Y, SK-N-SH, SK-N-AS, SK-N-DZ). In contrast, 48 hours incubation with 2,5 µM imatinib was ineffective in preventing clonogenic survival. Combination treatment with imatinib and cytotoxic agents or radiation (60Co) resulted in additive inhibition of nuroblastoma cell survival. Treatment of rats with imatinib (50 mg/kg i.p. twice daily) for 10 days significantly inhibited the growth of established SH-SY5Y xenografts ($p<0.01$). Our findings suggest that imatinib could be a potential candidate drug for neuroblastoma. Whereas short-term treatment is likely to require unrealistically high plasma concentrations of the drug in order to be efficacious, our findings indicate that continuous treatment with imatinib could be efficacious also in concentrations < 5 µM, which is known to be clinically achievable from previous studies in patients with CML.

Ref ID: 168.1 **#231**

Anti-gene peptide nucleic acid (PNA) specifically and persistently blocks N-myc expression in neuroblastoma cells leading to cell-cycle inhibition and apoptosis

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We developed an anti-gene sense peptide nucleic acid (PNA) targeted against a unique sequence in the antisense DNA strand of exon 2 of N-myc and linked at its N-terminus to a nuclear localization signal (NLS) peptide, designed for selective inhibition of N-myc transcription in neuroblastoma cells. Fluorescent microscopy showed specific nuclear delivery of the PNA in three human neuroblastoma cell lines: GI-LI-N and IMR-32 (N-myc-amplified) and GI-CA-N (N-myc-unamplified). A relevant anti-proliferative effect was observed already after 24h (60% and 70% in GI-LI-N and IMR-32 respectively), increased at 48h and reached a maximum at 72h (80% in GI-LI-N and 90% in IMR-32); no reduction was recorded for GI-CA-N (control). In N-myc-amplified GI-LI-N and IMR-32 cells, the PNA determined NMYC inhibition demonstrated by Western blotting. Following a single, initial dose exposure, these inhibitory effects increased over 3 days. After 24h the PNA induced accumulation of cells in G1 ( ) and apoptosis ( ) maintained for 3 days. Selective activity of the PNA was demonstrated by altering three point mutations, and by the observation that an anti-gene antisense PNA targeted against the complementary sense DNA strand did not show any effect. These findings could encourage development of an anti-gene PNA-based tumour-specific agent for neuroblastoma (or other neoplesia) with N-myc over-expression.

Ref ID: 197.1 **#233**

Sensitising effect of MYCN to different chemotherapeutic drugs in neuroblastoma cells

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Multiple drug resistance is a major complication especially in patients with advanced neuroblastoma tumours with amplified MYCN. Dysfunctional apoptotic pathways appear to be involved in this process. On the other hand in vitro data suggests that targeted MYCN expression sensitises for drug-induced apoptosis. Our aim is to study the drug response of chemotherapeutic drugs targeting different cellular pathways dependent on MYCN expression in neuroblastoma cells. To elucidate drug responding mechanisms in neuroblastoma cells, we used our human neuroblastoma cell system, which allows a directed MYCN expression. The susceptibility of neuroblastoma cells to varying concentrations of cytotoxic drugs was measured dependent on MYCN expression. Specific apoptosis was determined by fluorescence activated cell sorting (FACS). In neuroblastoma cells exposed to Doxorubicin, Etoposide, Cisplatin, Carboplatin, Vindesin, Vincristin or Paclitaxel, targeted MYCN expression led to higher specific apoptotic rates compared to cells not expressing MYCN. Among these agents Vindesin, Paclitaxel and Vincristin could most effectively induce apoptosis followed by Doxorubicin, Cisplatin, Etoposide and Carboplatin. These results expand the notion that MYCN overexpression sensitises for drug-induced apoptosis. It could be shown that drug response varies among the applied chemotherapeutic agents. Ongoing studies focus on the identification of cytostatic compounds, which induce apoptosis independently from MYCN expression. Future research aims at establishing highly drug resistant neuroblastoma cell lines, which could be used as a comparative in vitro system together with drug sensitive cell lines.

Poster Display
TRANSLATIONAL

Ref ID: 319.1 **#234**

Dramatic reduction of MYCN gene copy number and telomere lengths in F-cells induced by hydroxyurea

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INTRODUCTION: MYCN amplified neuroblastoma cell lines exhibit two morphologically distinct cell types, the neuronal cells (N-cells) and fibroblastoid like so-called flat cells (F-cells). It has been shown that F-cells are generated by active expulsion of extrachromosomally amplified MYCN copies by micronuclei formation resulting in tumor cell revertance. We tested whether it is possible to induce F-cells by the exposure of cells to low concentrations of hydroxyurea and whether these morphological changes are associated with changes in the MYCN copy number, telomere lengths, p16 and SA-ß-gal expression.
MATERIAL AND METHODS: The two cell lines STA-NB-9 and STA-NB-10 were treated with 75 - 150 mM hydroxyurea for several weeks. Automatic IQ-FISH (interphase quantitative fluorescence in situ hybridization) was performed using MYCN and telomere specific probes and appropriate references. The quantification of p16 expression was performed by static intensity measurement on immunofluorescence stained cells.
RESULTS: F-cells showed, in contrast to N-cells, a dramatically reduced MYCN copy number or single copies of MYCN while 1p deletion and 17q gain was found in both sublines. While p16 was highly expressed in N-cells and no SA-ß-gal activity had been observed, the F-cells showed a marked reduction of p16 expression but were positive for SA-ß-gal.
CONCLUSION: The induced F-cells are characterized by a dramatic reduction of the MYCN copy number and of the telomere lengths. Together with the upregulation of SA-ß-gal and the reduced p16 expression in F-cells we hypothesize that the replicative pathway is operating but that the p16 senescence pathway is not involved. The entering of the tumor cells into cellular senescence can be enhanced by the drug hydroxyurea.

Ref ID: 394.1 **#235**

Smac agonists as novel therapeutics to overcome resistance of neuroblastoma cells against radiation therapy

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Resistance of neuroblastoma to radiotherapy still remains a major concern and may be caused by defective apoptosis programs. Here, we report for the first time that Smac agonists may be a novel strategy to target radioresistance of neuroblastoma. We developed Smac agonists, e.g. cell-permeable Smac peptides using the TAT protein transduction domain, that strongly sensitized resistant neuroblastoma cells for radiation-induced apoptosis. Cell-permeable Smac peptides even bypassed the Bcl-2 block in tumor cells with high expression Bcl-2, which prevented the release of Smac from mitochondria into the cytosol. Smac peptides even sensitized resistant neuroblastoma cells lacking caspase-8 or resistant patient-derived primary neuroblastoma cells ex vivo. Most importantly, Smac peptides strongly enhanced the antitumor activity of TRAIL in vivo in a mouse xenograft model. Complete eradication of established tumors and survival of mice was only achieved upon combination treatment with Smac peptides. Importantly, Smac peptides exerted no detectable toxicity on nontransformed, primary human cells of different lineages. Also, injection of Smac peptides into normal tissue in vivo caused no cytotoxic effects. Interestingly, injection of FITC-labelled Smac peptides into normal tissue or into human tumors grown in mice revealed no difference in the distribution of Smac peptides in normal tissue compared with tumor tissue showing that Smac peptides did not preferentially localize to tumor cells. These findings indicate the specificity, and thus the potential safety, of the sensitization effect of Smac peptides for tumor cells, but not for normal cells. Thus, Smac agonists represent novel promising cancer therapeutics to overcome radioresistance of neuroblastoma without detectable toxicity to normal tissues.

Ref ID: 188.1 **#242**

The novel 7-substitued camptothecin analog ST1481 (GIMATECAN) is active in preclinical models of human neuroblastoma

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Gimatecan (ST1481, 7-tert-Butoxyiminomethylcamptothecin), is a novel lipophilic camptothecin analog showing a better pharmacological profile and a lack of cross-resistance to topotecan and irinotecan. Gimatecan is currently under evaluation in Phase I/II clinical trials administered by oral route. In the present study we compared the in vitro antitumour activity of gimatecan, SN38 (the active metabolite of irinotecan) and topotecan in a panel of neuroblastoma cell lines (SK-N-DZ; BE(2)M17; LAN-1; RNGA and BE(2)c). Gimatecan was about 1.4-4 times and up to 40-fold more cytotoxic than SN38 and topotecan respectively. All analogues induced a dose-dependent arrest in G2-M phase of the cell cycle after 1h incubation and 24/48/72 hours of recovery in drug-free medium. Gimatecan was more efficient than SN38 and topotecan in inducing apoptotic death as shown by tunel assay and sub-G0 cell accumulation. DNA strand breaks induced by the drugs (detected by the alkaline Comet assay) was dose-dependent and was up to 4-fold higher for gimatecan at equitoxic doses (10xIC50). In the in vivo study, where gimatecan was administered orally at 0.6mg/Kg and 0.9 mg/Kg doses and q4dx3 schedule, the drug showed a complete tumour regression in 100% of mice. Toxicity was negligible with no toxic deaths and less that 10% in weight loss. > Taken as a whole, our findings show that gimatecan appears very active in neuroblastoma with limited toxicity. We suggest that the drug warrants further clinical development in patients with neuroblastoma.

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Ref ID: 041.1 **#243**

Cytotoxic T lymphocytes activated on third party targets induce cell death in neuroblastomas in a MHC-independent fashion

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BACKGROUND AND AIMS: A large body of evidence indicates that the MHC class restricted processing and presentation pathway is inefficient in neuroblastoma (NB) cells, therefore the usefulness of the MHC class I restricted CD8+CTL responses in the elimination of NB cells has been debated. We have asked whether CD8+CTLs can lyse NB cells in a MHC class I-independent fashion.
RESULTS: We found that a number of NB cell lines are susceptible for lysis by terminally differentiated CD8+CTL clones in a MHC class I un-restricted manner. CTL killing was partially blocked by Fas-ligand neutralizing antibody and soluble TRAIL-receptor, indicating that signaling mediated by surface death receptors contributes to this phenomenon. Furthermore TNFalpha blocking agents in part reduced killing by CTLs. We further demonstrate that supernatant of activated CTLs ("activated supernatant") is capable of inducing death in NB in vitro, suggesting that soluble effector molecules contribute to T cell mediated killing of NB in our model system. We observed that activated supernatant induces both caspase-dependent and caspase-independent cell death of NB. Caspase-dependent cell death was monitored at 18/24 hours of treatment and was associated with caspase-3 activation, PARP cleavage and could be blocked by TNFalpha blocking agents to different extents depending on the cell line tested. Caspase-independent cell death was detected at 48 hours post exposure to activated supernatant and was blocked by neither Z-VAD-fmk nor TNFalpha-, IFNalpha- or IFNgamma-blocking agents.
CONCLUSIONS. Our data suggest that bystander lysis by CTLs may prove useful as an immunotherapeutic modality for NB, a tumor with low MHC class I expression and frequently mutated key molecules of the caspase-mediated apoptotic pathway.

Ref ID: 331.1 **#237**

Sensitivity of neuroblastoma cell lines to celecoxib and in combination with other chemotherapeutic agents

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Celecoxib, a selective cyclo-oxygenase-2 (COX-2) inhibitor, is currently being studied for both chemoprevention and treatment of adult cancers in numerous clinical trials. It has been demonstrated that celecoxib doses of at least 20 mM are required to significantly inhibit cell growth in vitro, concentrations that cannot be attained in vivo. In animal studies, substantial inhibition of tumor growth is seen at plasma concentration of 2 - 3 mM. Since it is unclear whether COX-2 inhibitors demonstrate activity in neuroblastoma, we asked whether neuroblastoma cells were sensitive to celecoxib alone, or in combination with other chemotherapeutic agents (doxorubicin, cisplatin, etoposide and camptothecin). We found that celecoxib inhibited the growth of the neuroblastoma cell lines IMR-5 (N-myc amplified) and SK-N-AS (single copy N-myc) after 72 h of treatment, with an IC50 ranging from 40 mM to 65mM. Low doses of celecoxib (9-18mM) alone did not inhibit neuroblastoma cell growth. However, low dose celecoxib in combination with doxorubicin resulted in synergistic effects when compared with treatment of doxorubicin alone. In contrast, combinations of celecoxib with cisplatin, etoposide or camptothecin exhibited antagonistic effects. In conclusion, syngergism between low dose celecoxib and doxorubicin may provide an effective regimen for treatment of neuroblastoma and further studies are warranted to investigate optimal combinations and the mechanisms by which celecoxib inhibits neuroblastoma tumor growth.

Ref ID: 368.1 **#241**

Multidrug resistance in neuroblastoma cell lines is not associated with altered expression of ceramide pathway enzymes mRNA

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BACKGROUND: Ceramide is a sphingolipid involved in death signalling that can be metabolized to less toxic forms. We have correlated drug resistance in neuroblastoma (NB) cell lines with mRNA over-expression of g-glutamylcysteine synthetase (g-GCS) and GSH-s-transferase µ (GSTµ), that code for glutathione synthesis and utilization enzymes.
METHODS: We studied 20 NB cell lines containing 10 sensitive and 10 multi-drug resistant lines. Sensitivity to melphalan, cisplatin, carboplatin, doxorubicin and etoposide was determined by digital imaging microscopy microassay. 10 of the cell lines constitute 5 pairs established from 5 patients at diagnosis and after therapy. We measured basal mRNA expression by quantitative RT-PCR for 11 enzymes and subunits involved in ceramide synthesis [LCB-1 and LCB-2 subunits of Serine Palmitoyl Transferase, Acid and Neutral Sphingomyelinase, Dihydrocramide Desaturase] and metabolism [Sphingosine Kinase, Sphingosine Kinase 2, Ceramide Kinase, Acyl Ceramide Synthase, Glucosyl Ceramide Synthase and Acid Ceramidase]. We also determined basal mRNA expression of g-GCS, GSTµ and MDR-1.
RESULTS: No correlations between basal mRNA expression of the ceramide pathway enzymes and drug resistance were found ($0.05<p<0.9$). However, significant mRNA over-expression was observed in the multi-drug-resistant lines for g-GCS and GSTµ ($P < 0.001$), and for MDR-1 ($P=0.007$).
CONCLUSIONS: Multidrug resistance in NB cell lines was associated with basal over-expression of mRNA for glutathione enzymes and MDR-1, but not for ceramide pathway enzymes. These data provide further evidence in support of non-cross-resistance between ceramide modulators and currently employed chemotherapy. However, they do not preclude an association of induced mRNA for ceramide-associated enzymes under drug treatment, or changes in enzymatic activity due to post-translational regulation.

Ref ID: 145.1 **#244**

Analysis of susceptibility of Neuroblastoma to NK-mediated killing and identification of a novel cell surface antigen expressed by Neuroblastoma

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Neuroblastoma is the most common solid tumor of childhood that can arise anywhere along the sympathetic nervous system. Although many efforts have been made, Neuroblastoma remains the tumor with the highest risk of death in children. Our aim was to value fresh neuroblastoma cells susceptibility to Natural Killer (NK) cells cytotoxicity. This analysis could provide solid basis for possible new NK-mediated immunotherapeutic approaches. A first step was represented by Neuroblastoma cells purification. Then the identification of markers useful for specific recognition of Neuroblastoma cells represented an essential step of our analysis. To this aim mice were immunized with a Neuroblastoma cell line and after cell fusion, hybridoma supernatants were screened by indirect immunofluorescence and cytofluorimetric analysis for surface reactivity with a panel of Neuroblastoma cell lines. Using this experimental approach, a mAb termed 5B14 was selected that stained all Neuroblastoma cell lines tested as well as tumors of different origin, including melanomas and carcinomas. Importantly, in bone marrow aspirates derived from stage 4 patients, infiltrating Neuroblastoma cells were specifically recognized by 5B14 mAb and in no instances 5B14-reactive molecules could be detected in normal CD45+ cells. This mAb specifically recognized a 90-100 kD surface molecule displaying high numbers of N-linked glycosylations.
Thus we have identified a novel surface marker to be used either alone or in combination with GD2 to identify Neuroblastoma infiltrates by cytofluorimetric analysis.
Purified neuroblastoma cells were used as target cells in cytotoxicity assays in which effector cells were represented by polyclonal activated NK cells. By these experiments we determined the existence of heterogeneity among different patients for susceptibility to NK-mediated lysis. We also explored the molecular mechanisms that are responsible for such heterogeneity.

Ref ID: 058.1 **#247**

Preclinical evaluation of autologous dendritic cells transfected with tumor mRNA or loaded with apoptotic cells for immunotherapy of high risk neuroblastoma

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Children with high-risk neuroblastoma (NB) have a poor clinical outcome. The purpose of the present study was to evaluate different strategies for immunotherapy of high risk NB based on vaccination with antigen-loaded dendritic cells (DCs). DCs are professional antigen-presenting cells with the ability to induce anti-tumor T-cell responses. We have compared DCs either loaded with apoptotic tumor cells or transfected with mRNA from the NB cell line HTB11 SK-N-SK, for their capacity to induce T-cell responses. Monocyte-derived DCs from healthy donors were loaded with tumor antigen, matured and co-cultured with autologous T cells. After one week, T-cell responses against antigen-loaded DCs were measured by ELISPOT assay. DCs loaded with apoptotic NB cells or transfected with NB-cell mRNA were both able to efficiently activate autologous T cells. The results indicate that loading of DCs with apoptotic NB cells or transfection with tumor mRNA represent promising strategies for development of cancer vaccines in treatment of NB.

Ref ID: 184.1 **#248**

Anti-neuroblastoma immunotherapy with GD2 Peptide Mimotope DNA Vaccines

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The tumor-associated antigen disialoganglioside-GD2 is expressed on neuroblastoma and melanoma and is an established target for immunotherapy. Carbohydrates and glycolipids are T cell-independent antigens (TI) and usually evoke a poor immune response in tumor-bearing hosts. In order to overcome T-cell independency, we identified peptides mimicking the structure of glycolipid GD2, i.e. GD2 mimotopes. This was accomplished by biopanning experiments of a phage-display library displaying circular decapeptides (kindly provided by L. Mazziacchelli, Bern, Switzerland) against the human/mouse chimeric anti-GD2 antibody (Ab) ch14.18. Thirteen independent phage clones were identified which bind to ch14.18 with high specificity and harbor mimicry potential with GD2. The subsequent immunization strategy to break peripheral tolerance is based on the construction of DNA minigenes encoding for the two best mimotopes of the selection procedure, mimotope A (MA) and and mimotope D (MD), including a T cell epitope from HIV-1 gp 120 referred to as the T1 peptide. T1 is suggested to bind both MHC class I and class II and thus might stimulate the cellular arm of anti-tumor response. The final minigens were generated by overlapping PCR, Klenow reaction and cloning into the leader sequence containing pSecTag2-A-Vector (pSA). Then, pSA-MA and pSA-MD were transferred into attenuated Salmonella typhimurium (SL 7207), which were already successfully used as oral vaccines for neuroblastoma antigens. Based on these data, we test whether oral vaccination with pSA-MA and pSA-MD induces an anti-neuroblastoma immune response capable of eradicating spontaneous neuroblastoma metastases. Results will be presented at the meeting.

Ref ID: 134.1 **#249**

Production of dimeric small immunoproteins specific for neuroblastoma-associated antigen GD2

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GD2 is a disialoganglioside expressed at high density on the surface of malignant cells of neuroectodermal origin, in particular neuroblastoma and melanoma. Since its expression in normal tissues is very restricted, GD2 represents an excellent target for neuroectodermal tumor targeting. Mini-antibody technology allows the production of dimeric single-chain antibodies, also called small immunoproteins (SIPs), which are composed of a scFv fused to a dimerizing domain of immunoglobulin heavy chains. Dimerization results in an increase of the total apparent affinity and a slower clearance in vivo than scFvs. In this study, we have isolated the variable regions from an anti-GD2 monoclonal antibody and exploited the SIP technology to generate two novel anti-GD2 SIPs suitable for diagnostic and/or therapeutic neuroectodermal tumor targeting. The first anti-GD2 SIP is a fully murine molecule containing the CH3 domain of mouse IgG1, whereas the second construct is a hybrid mouse-human molecule containing the CH4 domain of human IgE. Both mini-antibodies were successfully produced and shown to be secreted as dimers that retain binding specificity as well as an affinity comparable to that of the original antibody. Because of their high tissue penetration, superior to complete immunoglobulins, SIPs can be considered very promising tumor imaging reagents. In addition, their slower clearance, which results in a high tumor/blood ratio, makes them very attractive molecules also for therapeutic purposes, once they are conjugated to radioisotopes, toxins or other biologically active molecules. Moreover, their dimeric nature allows them to deliver two effector molecules at the tumor site, thus increasing their biological effect. Fusion proteins SIP/chemokines are presently being studied to this purpose.

Ref ID: 345.1 **#253**

IL-27 mediates complete regression of primary and/or metastatic murine neuroblastoma tumors: role for CD8+ T cells

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We have shown previously that IFN-g-inducing cytokines such as IL-12 mediate potent antitumor effects against murine neuroblastoma. IL-27 is a newly-described IL-12-related cytokine that triggers clonal expansion of naïve T cells and synergizes with IL-12+/-IL-2 to induce IFN-_ production by activated naïve T and/or NK cells. Therefore, we hypothesized that IL-27 might also mediate potent immunoregulatory and therapeutic antitumor activity in vivo. TBJ neuroblastoma cells engineered to overexpress IL-27 demonstrated markedly delayed growth and underwent complete durable regression in over 90% of mice bearing either subcutaneous or orthotopic tumors compared to 15% of control mice. IL-27 expression also mediated complete regression of induced metastatic disease in 40% of mice versus 0% in control mice. IL-27-induced tumor regression occurs in conjunction with local-regional T cell activation. Tumor-draining lymph node-derived lymphocytes from mice bearing subcutaneous TBJ-IL-27 tumors were primed to proliferate more readily when cultured ex vivo with anti-CD3/anti-CD28 compared to those from mice bearing control tumors. Further, potent upregulation of local IFN-g gene expression and marked infiltration of CD8+ T cells is observed within TBJ-IL-27 versus control TBJ tumors. Functionally, in vivo depletion of CD8+ but not CD4+ T cells or NK cells abrogates the anti-tumor effects of IL-27. Collectively, these studies demonstrate that IL-27 can enhance immune function in vivo, induce complete regression of both primary and metastatic neuroblastoma tumors via mechanisms that are critically dependent on CD8+ T cells, and suggest that IL-27 could be used to therapeutically potentiate the host antitumor immune response in patients with malignancy.

Ref ID: 347.3 **#255**

IL-18 Induces Complete Regression of Orthotopic Primary and Metastatic Murine Neuroblastoma Tumors and Potentiates Antitumor Immune Reactivity in Combination with IL-2

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Several potent immunoregulatory cytokines, including IL-12 family members (IL-12, IL-23 and IL-27) as well as IL-18, are generated during early activation of the immune response, and function in a coordinated fashion to link non-specific immune surveillance mechanisms (innate immunity) and the subsequent generation of a productive T-cell mediated immune response (adaptive immunity). We have initiated studies to investigate the antitumor efficacy and mechanisms of action by IL-18 using an orthotopic model of murine neuroblastoma. Administration of IL-18 induces marked increases in serum IFN-gamma levels, and potentiates proliferative responses and IFN-gamma production by murine splenocytes in vitro. Administration of IL-18 induces complete durable tumor regression in up to 60-70% of treated mice bearing established orthotopic or subcutaneous primary TBJ neuroblastoma tumors or induced hepatic and/or pulmonary neuroblastoma metastases. Like IL-12, another potent IFN-gamma-inducing antitumor cytokine, the immunoregulatory effects of IL-18 can be dramatically potentiated by IL-2. IL-18 and IL-2 synergistically enhance proliferative responses and/or IFN-gamma production by murine splenocytes in vitro. In turn, treatment of TBJ-bearing mice with IL-2 markedly enhances IFN-gamma production by splenocytes treated subsequently ex vivo with IL-18, and these responses are further potentiated by concurrent restimulation ex vivo with irradiated TBJ tumor cells. These observations suggest that IL-18 and IL-2 may interact favorably in vivo, and that IL-18 may be particularly effective in potentiating antigen-specific responses to neuroblastoma tumors. Collectively, these studies demonstrate potent immunoregulatory and/or therapeutic effects by IL-18+/-IL-2 in mice bearing neuroblastoma tumors, and suggest that clinical investigation of the antitumor activity of IL-18 in children with neuroblastoma may be warranted.

Ref ID: 221.1 **#250**

Isolation and characterisation of peptide mimotopes of the neuroblastoma-associated carbohydrate antigen GD2 ganglioside

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Aberrant glycosylation is a universal feature of cancer cells. There are quantitative and qualitative changes in the expression of gangliosides observed in tumors of the neuroectodermal origin e.g., neuroblastoma, melanoma, astrocytoma. The presence of large amounts of GD2 ganglioside on neuroblastoma cells, as compared to normal cells, opens the possibilities to use the tumor-associated carbohydrate antigen in diagnosis and immunotherapeutic approaches. Our goal is to develop active specific immunotherapy of neuroblastoma with peptide mimics of GD2 ganglioside. The mimotopes could be used as surrogate antigens to elicit specific anti-GD2 ganglioside humoral and cellular immune responses. In our studies, we have performed four rounds of affinity purification of peptides from the phage-displayed peptide library LX-8 (12-mer containing disulphide bridge) with biotinylated anti-GD2 ganglioside mAb 14G2.a. In the solution-phase panning experiments, we have applied different concentrations of the screening antibody and various times of incubation of the phage-antibody complexes in streptavidin-coated wells to discriminate between the low and the high affinity phage-borne peptides. Several individual clones of phages that bind mAb 14G.2a have been identified with immunoblotting. The phage-borne peptides have been tested for their anti-GD2 ganglioside antibody binding activity with ELISA (also under reduced-conditions to determine the role in the peptide binding played by disulphide bridge). Additionally, we have examined whether the peptides could compete for binding to mAb 14G2.a with GD2 ganglioside present on the neuroblastoma cell lines.

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Ref ID: 261.1 **#252**

Combined targeted GM/CSF and IL/2 therapy switches innate to adaptive anti-neuroblastoma immunity

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A major goal of active tumor immunotherapy is to induce T-cell dependent eradication of disseminated metastases. We previously demonstrated that targeted IL-2 to the tumor microenvironment is effective in inducing an NK-cell dependent immune response. Here we extend these findings by showing that also neuroblastoma targeted GM-CSF induces an innate response mediated by NO radicals produced from activated macrophages. Based on these findings we tested the hypothesis that combined targeting of GM-CSF and IL-2 to neuroblastoma may switch innate to adaptive anti-neuroblastoma immunity. For this purpose, two types of genetically engineered antibody cytokine fusion proteins were constructed specific for mouse transferrin receptor (ch17217-mGM-CSF and ch17217-mIL-2) and ganglioside GD2 (ch14.18-mGM-CSF and ch14.18-hIL-2), respectively. All fusion proteins were characterized by determination of binding and cytokine activity. Efficacy and mechanism of combined targeting of GM-CSF and IL-2 was determined in an experimental and spontaneous hepatic metastasis model of murine neuroblastoma using NXS2 cells. We demonstrate that only combinations of targeted GM-CSF and IL-2 can completely eradicate established experimental and spontaneous hepatic metastases in contrast to targeted GM-CSF and IL-2 used as monotherapy showing a reduction in tumor growth. The anti-tumor effect observed in the combined targeted GM-CSF and IL-2 treatment groups was abrogated in T-cell deficient SCID mice. Furthermore, splenocytes isolated from mice receiving the combined targeted GM-CSF and IL-2 revealed MHC class I restricted target cell killing in contrast to monotherapy controls. These findings indicate a mechanism primarily dependent on T-cell. In conclusion these data demonstrate that combining targeted GM-CSF and IL-2 improves anti-neuroblastoma efficacy and therefore may have important clinical implications for the design of future clinical trials.

Ref ID: 186.1 **#256**

Neuroblastoma immunotherapy with IL-12 and IL-15-gene modified NB cells

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We used Neuro2a cells engineered with IL-12 and/or IL-15 genes as prophylactic or therapeutic vaccine in a syngeneic metastatic NB model. A single s.c. injection of IL-12-modified Neuro2a cells (Neuro2a/IL-12) induced resistance to a subsequent i.v. wild type (wt) Neuro2a cell challenge in 45% of mice, while Neuro2a/IL-15 vaccine only protected 28% of mice. To increase vaccine efficacy we co-expressed IL-12 and IL-15 in Neuro2a cells, but this approach did not lead to any improvement. However, sequential vaccination using Neuro2a/IL-12 and, after 15 days, Neuro2a/IL-15 induced protection in 71% of animals challenged with wt Neuro2a. Protection correlated with CTL activity and IFNgamma production by MLTC re-stimulated splenocytes from immunized mice. We then used engineered Neuro2a cells as therapeutic vaccine. A single dose of Neuro2a/IL-12 injected 3 days after induction of w.t. Neuro2a micro-metastases only prolonged the mean survival time (from 23+/-4 days to 46+/-20 days). The use of Neuro2a/IL-15 or Neuro2a/IL-12/IL-15 had no significant effects. By contrast the use of a two-step vaccination protocol using Neuro-2a/IL-12 (day+3) followed by Neuro2a/IL-15 (day+13) showed that 43% of mice remained disease-free for more than 90 days, without signs of recurrence by histological analysis. CTL activity against wt Neuro2a was observed in splenocytes from all treated mice. In addition, cell depletion experiments showed that CD8+ cells were strictly necessary for the vaccine effect, while CD4+ or NK cells were not required. The superior effect of sequential vaccination with IL-12 and IL-15-engineered cells may relate to a Th1 priming effect of IL-12, followed by optimal CTL expansion, mediated by IL-15.

Ref ID: 262.1 **#257**

Low dose interferon-g producing human neuroblastoma cells show reduced proliferation and delayed tumorigenicity

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IFN-g directs T helper-1 cell differentiation and mediates anti-tumor effects in pre-clinical models. However, high dose IFN-g is toxic in vivo, and IFN-g-transfected neuroblastoma cells secreting high amounts of the cytokine may be lost due to cell apoptosis or differentiation.Two human neuroblastoma cell lines (ACN and SK-N-BE2(c)) differing as to genetic and phenotypic features were transfected with the human IFN-g gene and selected on the ground of the low concentrations of IFN-g produced. In both IFN-g-transfected cell lines, autocrine activation of IFN-g-mediated pathways occurred, leading to markedly reduced proliferation rate, to increased expression of surface HLA and CD40 molecules and of functional TNF binding sites. More importantly, all these changes occurred through paracrine mechanism in parental NB cells co-cultured with the IFN-g-transfected cells. ACN/IFN-g cells showed a significantly delayed tumorigenicity in nude mice as compared to parental cells. ACN/IFN-g tumors were smaller, with extensive necrotic area as a result of a damaged and defective microvascular network. In addition a significant reduction in the proliferation index was observed. This is the first demonstration that IFN-g inhibits in vivo proliferation of neuroblastoma cell by acting on the tumor cell itself. This effect adds to the immunoregulatory and anti-angiogenic activities operated by IFN-g in syngeneic tumor-bearing hosts.

Poster Display
TRANSLATIONAL

Ref ID: 202.5 **#258**

In vitro and in vivo antitumour activity of the synthetic polymer P10

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We synthesised an amphiphilic polymer based on a polyvinylalcohol backbone (P10). Initially intended as a particulate vehicle for anticancer drugs, P10, by itself, exerted a potent activity on different neuroblastoma (NB) and melanoma cell lines. We observed dose- and time-dependent cytotoxicity of P10, with IC50 between 2.5 and 10 _g/ml after 24 h. P10 did not affect normal cells. Treated tumour cells underwent detachment from the flasks, became smaller and showed nuclear condensation. Cytofluorimetric assay indicated that a substantial number of cells had sub-G1 DNA content, as confirmed by DNA internucleosomal cleavage. Activation of caspase cascade was assessed by fluorescence-based pan-caspase activity assay, and caspase 3 cleavage by Western blot; the upstream pathways involved are currently investigated. We tested the effects of P10 in nude mice injected with 105 NXS2 murine NB cells i.v., to mimic the metastatic spreading of NB. Treatment with P10 (300mg/Kg, i.v. at days 1 and 7 after NB cell challenge) significantly increased the lifespan and the long term survival of mice treated with P10 over controls ($p<0.002$). Three out of 9 treated mice were alive and disease free by 120 days, while all control mice died within 50 days. Dead animals showed metastatic tumour growth, involving mainly adrenals, kidney, ovary, liver and bone marrow. Similar results were obtained with the human NB cells Htla-230. No toxicity took place up to 600 mg/kg. Although the mechanisms of action of P10 deserve further investigation, our data suggest that P10 holds promise as an anti-cancer compound, independent of its drug carrier suitability.

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Ref ID: 063 **#259**

The histone deacetylase inhibitor valproic acid activates a differentiation program in neuroblastoma cells

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BACKGROUND: Spontaneous differentiation is one of the biological hallmarks of childhood neuroblastomas. The degree of differentiation towards ganglionic cells is recognized as the principal morphological feature to be of prognostic importance. Furthermore, certain drugs like retinoic acid are able to differentiate neuroblastoma cells into a ganglionic phenotype. We hypothesized that neuroblastoma cells contain a differentiation program which can be activated either pharmacologically or by unknown endogeneous mechanisms. In order to define such a differentiation program, we analysed an in-vitro differentiation model of neuroblastoma cells as well as tumor-samples.
RESULTS: The histone deacetylase (HDAC) inhibitor valproic acid (VPA) induces morphological features of ganglionic differentiation in 3 neuroblastoma cell lines and neuroblastoma cells isolated from a patient with stage IV disease. VPA inhibited HDACs in all cell lines studied. Using cDNA-microarray experiments, we found that VPA causes a shift of the transcriptome prior to morphological differentiation on correspondence analysis. Most of the up-regulated genes belong to gene ontology annotations associated with neurogenesis, cell differentiation, cytoskeletal-binding proteins, cell adhesion and immune function. Comparison of these in-vitro experiments with patient samples from neuroblastomas with favourable histology (ganglioneuroblastoma) versus unfavorable histology (poorly differentiated neuroblastoma) revealed striking similarities in gene expression profiles. Using a combined chromatin-immunoprecipitation/microarray approach, we found that only a limited number of genes become associated with hyperacetylated histone proteins following short-time treatment of cells with VPA. These genes encode forkhead transcription factors and other DNA-binding proteins which possibly control a differentiation program.
CONCLUSION: Our data suggest that the HDAC-inhibitor valproic acid is able to activate a differentiation program in neuroblastoma cells that converts the malignant phenotype towards a benign condition. This differentiation-process involves transcriptional activation of a very limited number of primary target genes which might control a ganglionic-differentiation program.

Ref ID: 103.1 **#261**

c-KIT expression identifies a subset of aggressive neuroblastomas

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We showed recently that c-Kit is preferentially expressed in MYCN-amplified neuroblastomas and its signaling promotes in vitro neuroblastoma cell proliferation that can be selectively inhibited by STI571 (imatinib mesylate, Gleevec). This study aimed at further investigating in 168 neuroblastic tumors the clinico-biological characteristics of the subset that utilizes the SCF/c-Kit pathway and may be responsive to selective inhibitors. Expression of mRNA (by Northern blot) and protein (by immunohistochemistry) for c-Kit was detected in 22% and 13% of tumors, respectively; the latter was confined to neoplastic neuroblasts. Expression of mRNA and protein for SCF was documented in 31% and 28% of tumors, respectively, with 66% of the c-Kit-positive tumors also expressing SCF. Mutations in exon 11 of the c-kit gene were not found in the 9 c-Kit-positive and 9 c-Kit-negative tumors that were analysed. Expression of c-Kit correlated with advanced stage (3 and 4), MYCN amplification and 1p36 allelic loss ($p<0.001$); expression of SCF with adrenal primary ($p<0.05$), MYCN amplification and 1p36 allelic loss ($p<0.001$). Overall survival (OS) probability was 17% in c-Kit-positive cases vs. 68% in c-Kit-negative, 43% in SCF-positive cases vs. 78% in SCF-negative ($p<0.001$). Using Cox multiple regression analysis neither c-Kit nor SCF expression were independently associated with a shorter OS. The SCF/c-Kit pathway is expressed in a subset of particularly aggressive neuroblastomas for which selective therapeutic targeting is needed.

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Ref ID: 202.3 **#262**

P2X7 receptor expression and function in human neuroblastoma

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The purinergic P2X receptors are ligand-gated ion channels activated by ATP. One member of P2X receptor family, P2X7 receptor (P2X7R), is expressed in hematopoietic cells and it is able to allow passage of cations upon a brief stimulation, whereas a long-term activation by ATP can induce apoptosis. Neuroblastoma is a common extracranial pediatric tumor with low response to conventional therapy in patients with advanced stage disease. Novel strategies have emerged to circumvent such treatment failures and much work has focused on the possibility of triggering apoptotic pathways. We analyzed the expression of P2X7 in primary NB lesions and cell lines. Paraffin-embedded tissue sections of NB showed a strong staining for P2X7 with a reactivity unrelated to the stage of disease. Western blot analysis showed high expression of P2X7 protein in whole cell extracts from different NB cell lines. We also investigated functional responses coupled to P2X7R activation. Challenge with ATP, or the P2X-selective agonist benzoyl ATP, triggered a massive increase in cytoplasmic Ca. The nucleotide dose-dependency was biphasic, suggesting the activation of two different classes of P2 receptors, one with high affinity (ATP Km in the 10 _M range), and a second one with high affinity (ATP Km of about 1 mM). The high Km of the second class of receptors is typical of low affinity P2X7R. Future studies will evaluate the biological effect induced by P2X7-ligands in order to understand if P2X7 could be an useful target for antitumor therapy of NB.

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Ref ID: 386.2 **#263**

Internalization and mitochondria targeting are involved in the cytotoxic activity against MYCN-amplified neuroblastoma cells by the petide LfcinB

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BACKGROUND AND AIMS: Neuroblastoma is a tumor arsing from the primordial neural crest cells and is the most common solid tumor in children. MYCN-amplification is a genetic abnormality linked to the neuroblastoma and is strongly associated with the presence of metastatic disease and poor prognosis in spite of aggressvie multimodal therapy. Since antimicrobial peptides are known to induce cell death in cancer cells, we investigated the cytotoxic activity of the antimicrobial peptide LfcinB against several neuroblastoma cell lines (NB).
METODOLOGY AND RESULTS: LfcinB induced cell death in both MYCN-amplified and non MYCN-amplified NB cell lines. Normal fibroblasts were much less sensitive to LfcinB exposure than the NB cell lines. A direct interaction with the cytoplasmic membrane was found. In addition, LfcinB translocated into the cytoplasm in NB cells. Immunostained- and fluorescent-labelled LfcinB was shown to colocalize with the mitochondria. The direct targeting of the mitochondria by LfcinB induced a depolarization of the mitochondria membrane potential and irreversible changes in the mitochondria morphology. Even though caspases were activated, specific and broad spectrum caspase inhibitors did not inhibit LfcinB-induced cell death.
CONCLUSION: It seems that LfcinB act via dual targets in inducing cell death in NB cells since both the cytoplasmic membrane and the mitochondria membrane are disrupted by LfcinB exposure in a concentration-dependent manner.

Ref ID: 398.1 **#265**

Cellular distribution of autoantigens and functional activity of autoimmune sera from pediatric patients with opsoclonus-myoclonus-syndrome (OMS)

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As a rare neurological disease, the paraneoplastic form of OMS in children is almost exclusively associated with neuroblastoma and characterized by symptoms of conjugated, saccadic eye movement disturbances and ataxia. Since paraneoplastic syndromes in adults are suspected to be autoimmune, we investigated 16 sera from pediatric OMS patients for their reactivity with SK-N-SH, SH-SY5Y, NMB-P5 and CHP100 cells to localize respective autoantigens and functional effects on different neuroblastoma cell lines. Using indirect immunofluorescence microscopy, the majority (12/16) of patients´ immunoglobulin G autoantibodies revealed strong cytoplasmic staining with striking nuclear sparing, whereas a few (3/16) patients´ autoantibodies indicated marked immunoreactivity to nuclear antigens and in one case (1/16), a punctuated fluorescence pattern localized on the plasma membrane was observed. In control experiments with untreated neuroblastoma cells und such incubated with healthy donors´ sera either no fluorescence of cells or no distinct fluorescence pattern was observed. Sera and IgG fractions from pediatric OMS patients exhibited a significant anti-proliferative effect in cultured human neuroblastoma cell lines without signs of increase in cell death or cytotoxicity, in comparison to untreated cells and neuroblastoma cells cultured with sera and purified IgG from healthy donors ($P<0.05$). These findings indicate that the neuronal dysfunction observed in OMS patients can be due to direct (or indirect) interactions between autoantibodies and their target antigens eliciting the inhibitory effects on neuronal cell growth. We conclude that in paraneoplastic childhood OMS an antibody mediated suppression of cell proliferation against neurones may contribute to the pathogenesis of this rare neurological syndrome.

Ref ID: 202.4 **#264**

Involvement of CXCR4 in the development of neuroblastoma metastases

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Stage 4 neuroblastoma often presents with metastatic dissemination in bone marrow. The mechanism responsible for this process is still undefined. Stromal-Derived Factor-1, secreted by bone marrow stromal cells, which binds CXCR4, has been postulated to be a driving force in homing of NB cells to this site. Expression of the CXCR4 was evaluated in a panel of human and murine NB cell lines as well as in 20 NB primary tumors by RT-PCR, flow cytometry and immunohistochemistry. CXCR4 mRNA was found in most NB cell lines tested, whereas the CXCR4 protein was expressed on the surface of only 2 cell lines. All paraffin-embedded NB tumors examined were found to express CXCR4, regardless of stage and presence of metastasis. In order to evaluate the potential role of CXCR4 in NB spreading, we tested the effect of the specific CXCR4 inhibitor AMD 3100 in a xenogenic metastatic model of NB. After establishing MTD for i.v. injected AMD 3100 at 2.5 mg/Kg, nude mice, that had been inoculated 4 or 24 hours before with 5x106 HTLA-230 human NB cell line, were treated with 5 daily doses of AMD 3100 at MTD. Following either schedule, AMD 3100 did not show any antitumor activity, and all the dead animals displayed extensive metastatic tumour growth, involving mainly bone marrow, adrenal gland, kidney, liver and ovary. Since AMD3100 is a partial antagonist of CXCR4, these results do not allow to exclude a role of this receptor in human NB metastasis. Experiments with a monoclonal antibody to CXCR4 have now been started in the same murine model.

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Polyamine Biosynthetic Pathway as a Drug Target for Neuroblastoma Therapy

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BACKGROUND AND AIMS: Patients who have neuroblastomas (NBs) with high MYCN gene amplification tend to have a very poor prognosis due to the extreme tumor invasiveness and the formation of distant metastases. MYCN protein induces ornithine decarboxylase (ODC), one of the key enzymes in polyamine biosynthesis. It is well established that increases in ODC activity play a significant role in tumor development and therefore, selective pharmacological interference with the polyamine biosynthetic pathway presents an attractive way to reduce tumor cell growth. Specific inhibitors of the polyamine biosynthetic enzymes ODC and S-adenosylmethionine decarboxylase (SAMDC) reduce the growth of many cancer cells in vitro and in vivo, and inhibitor combinations exhibit more pronounced effects, presumably due to the near total depletion of intracellular polyamine pools. Since relatively little is known about the effects of such drugs on human NB cells, we tested the clinically established ODC inhibitor DFMO and SAMDC inhibitor SAM486A on MYCN-amplified or MYCN single copy human NB cells.
METHODOLOGY: Cell cultures, flow cytometry, HPLC, and Western blotting techniques were used to perform these experiments.
RESULTS: Treatment of human NB cells with either DFMO or SAM486A reduced cell growth in a dose-dependent manner. However, only drug-treated MYCN-amplified NB cells showed significant morphological changes and distinct (p53-independent) G1 arrest. The latter was more profound with combination treatments and was associated with a reduction of cyclin-dependent kinase(cdk) 4 as well as a decrease in total polyamine pools.
CONCLUSIONS: Based on our observations, we propose that polyamine biosynthesis inhibitors may be particularly effective against MYCN-amplified NB tumors, which can be highly malignant and respond poorly to conventional therapy.

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NF-kB signalling in neuroblastoma cell survival

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Nuclear Factor-kappaB (NF-kB) includes a family of dimeric transcription factors involved in transcriptional regulation in response to cytokines and cellular stresses. NF-kB has a key role in apoptosis and cell division, and is therefore a possible target in cancer therapy. The prognosis for children with metastatic neuroblastoma remains poor. A method for enhancing the apoptotic response of neuroblastoma cells to cytotoxic agents could therefore be very valuable. We found that drug-induced or genetic inhibition of NF-kB signalling can induce cell death in neuroblastoma cell lines (e.g. SK-N-AS). Co-treatment with etoposide and NF-kB inhibitors led to much more rapid cell death (100% within 3-4 h) implying synergy between these two treatments. We also studied the dynamics of NF-kB signalling in response to treatment with TNFa or etoposide by imaging the dynamics of degradation of IkBa-EGFP and the movement of NF-kB (p65-dsRed) into the nucleus. Long time-course experiments demonstrated that p65-dsRed had regular oscillations between the cytoplasm and nucleus, with a period of around 100 minutes. The time-period over which these oscillations continued (following TNFa or 20uM etoposide treatment) was far longer in SK-N-AS cells than in HeLa cells and this correlated with longer lasting transcriptional up-regulation (measured by real-time luciferase imaging in living cells) in the SK-N-AS cells compared to HeLa cells. We therefore propose that manipulation of the level and kinetics of NF-kB signalling may be a useful adjunct to cytotoxic therapy in neuroblastoma treatment.

Ref ID: 280.1 **#268**

Glutathione S-transferase polymorphism, genetic susceptibility and outcome in neuroblastoma

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The glutathione S-transferases (GSTs) activities are involved in the metabolism of carcinogens and of some anticancer drugs and may also confer resistance to them. The GSTT1 and GSTM1 genes exhibit a deletion polymorphism, which in case of homozygosity leads to absence of the proteins (null genotype), whereas the GSTP1 gene displays a polymorphism (Ile105Val) which confers a different catalytic activity. Subjects with a modified ability to metabolize carcinogens are at increased risk of cancer. Furthermore, in case of cytotoxic treatment, the low enzymatic activity, reducing the detoxification of anticancer drugs, may increase the cytotoxic effect of them. In our study we hypothesized that GSTs genotype may have a role in the susceptibility and outcome of neuroblastoma. We compared GSTs genotypes of 264 children with neuroblastoma with those of 392 normal subjects. Within the neuroblastoma group we further analyzed if any particular GSTs genotype was correlated to different risk factors or to disease outcome. No significant differences of allele frequencies were found between neuroblastoma patients and controls (GSTT1 null 19% vs. 18%, p=0.68; GSTM1 null 52% vs. 53%, p=0.81; GSTT1 null + GSTM1 null 9% vs. 10%, p=0.81; and GSTP1 Ile/Ile 47% vs. 53%, Ile/Val 46% vs. 38%, Val/Val 7% vs. 9%, p=0.14). We did not detect any particular association in the analysis of the relationship of GSTM1, GSTT1, and GSTP1 genotypes, and age at diagnosis, sex, primary site and stage of the disease, levels of VMA, HVA, LDH, Ferritine, NSE, MYCN amplification, 1p36 deletion and 1p36 imbalance. No correlation with outcome was observed. Our data do not support an important effect of GSTs genotype on neuroblastoma susceptibility.

CLINICAL

Ref ID: 239.3 **#99 bis**

Ibuprofen interferes with the capillary gas-chromatography determination of urinary HVA and VMA in Neuroblastoma diagnosis

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Neuroblastoma (NB) is the first cause of disease-related death in pre-school age children, affecting the nervous cells of the sympathetic nerve centre. The large majority of neuroblastomas produces catecholamines that are detected as metabolites in urine: the omovanillic acid (HVA) and the vanilmandelic acid (VMA) are the most important prognostic markers. We analyzed these biogenic amines by capillary gaschromatography (GC). The method provides the extraction of HVA and VMA with ethyl acetate, the conversion of the compounds to their trimethylsilyl esters (TMS) and the quantitation by GC with Flam Ionisation Detector (FID). During the diagnostic workup, using GC-FID, we found in two urinary samples a significant increase of biogenic amines. Thus HVA and VMA were assayed by the confirmatory thin-layer chromatography method. In this case samples were negative. The analysis of patients' clinical data revealed that they were treated with Ibuprofen [(+/-)-2-(p-isobutylphenyl)propionic acid], an analgesic and anti-rheumatic drug. To verify how Ibuprofen or its metabolites may interfere with this method, we analysed by capillary gaschromatography- mass spectrometry (GC-MS) the following samples: 1- Ibuprofen dissolved in saline; 2- two urine samples from patients assuming the drug; 3- a negative urine control and 4- the same negative urine enriched with the drug. Our results showed in samples 1, 2 and 4 the presence of a peak identified as TMS-ester of hydroxy Ibuprofen having the same retention time than VMA. GC-MS analysis, using selected ion monitoring, can, of course, overcome the problem. These experimental data confirm that Ibuprofen interferes with the determination of cathecolamines using GC-FID and underline the need to assay this biogenic amines by GC-MS if any anti-inflammatory therapy is contemporary administrated.

Abstracts

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Ref ID: 358.1

Transdifferentiation underlies the development of cellular heterogeneity in human neuroblastoma cell lines

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Human neuroblastoma cell lines comprise cell phenotypes which reflect the neural crest origin of this cancer. We and others have shown that three predominant cell types- neuroblastic (N-type), non-neuronal (S-type), and stem cell (I-type) are present in uncloned cell lines as well as in tumors. We have proposed previously that this cell heterogeneity arises through a process of differentiation and/or transdifferentiation and have provided biochemical and cytogenetic data to support this conclusion. Recently, a second hypothesis has been advanced that clonal selection alone and not transdifferentiation accounts for the cell diversity in SK-N-SH cell variants. While clonal expansion plays a major role in propagation of phenotypic variants, evidence from multiple cell lines indicates that it is the very rare transdifferentiating cell, not a clonal contaminant, that is selected for. For example, the non- neuronal clonal cell line LA1-5s gave rise to a neuroblastic LA1-55n cells. Similarly, an N-type LA1-19n clone gave rise to cells with an S cell phenotype (LA1-19Bs). In both pairs, cytogenetic markers confirm the origin of one phenotype from the other. In the second cell family, both the cloned N-type BE(2)-M and its subclone BE(2)-M17 spontaneously gave rise to S-type cells. Finally, subcloning of the S-type SH-EP cell line yielded three neuroblastic clones as well as S-type clones. All clones, irrespective of cell phenotype, retain the isochromosome 1q marker characteristic of SH-EP but not seen in any other SK-N-SH clonal lines. Thus, whereas clonal selection may be one mechanism by which the extent of cell diversity is attained in neuroblastoma, it is the mechanism of transdifferentiation [and/or differentiation (N - I - S)] by which this heterogeneity arises.

Ref ID: 140.2

Flt-3 expression in neural crest-derived tissues and tumor cells

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Flt-3 is a class III tyrosine kinase receptor that plays with its ligand FL a central role in proliferation and survival of human hematopoietic progenitors and immature thymocytes. We have previously demonstrated that flt-3 is expressed in human neural crest-derived tumor cell lines and that FL promotes their survival and proliferation, suggesting an autocrine loop. We have also observed by RT-PCR Flt-3 expression in neuroblastoma (NB) biopsies, while there are no data about flt-3 expression in normal human neural crest-derived tissues. To investigate if flt-3 expression is a tumor-related phenomenon, in the present work we have studied by immunocytochemistry flt-3 expression in the biopsies of 10 NB with various differentiation patterns, 8 Ewing Sarcoma (ES) and 3 normal adrenal glands. The rabbit polyclonal sc-479 anti-flt-3 antibody was utilized with an avidin-biotin-peroxidase detection system. All ES and NB biopsies showed a cytoplasmic positivity (++/+++) for flt-3 in the 75-95% of tumor cells, unrelated to differentiation pattern. Adrenal medulla was also flt-3 positive, even if with a lower intensity (+/++). The study of a larger series of NB biopsies is ongoing, in order to correlate flt-3 expression with staging and clinical course. The preliminary results suggest that flt-3 expression in neural crest-derived tumors is related to the embryologic origin rather than to the tumorigenesis. However, the higher flt-3 expression in tumors and the still poor prognosis of advaced stadies don't rule out a possible use of flt-3 inhibitors in selected cases of neuroectodermal tumors.

Ref ID: 406.1

Neuroblastoma in Denmark. A 20 years population based study

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All patient charts from patients below 15 years of age diagnosed with neuroblastoma (NBL), ganglioneuroblastoma (GNBL) and ganglioneuroma (GNR) between 1981 and 2000 were reviewed retrospectively. 160 children (88 boys and 72 girls) were diagnosed. The median follow-up was 118 months (range 32-270 months). 50/160 (31%) were below 12 months at diagnosis, 22 (14%) were between 1 and 2 years of age and 87 (55%) were 2 years or older at the time of diagnosis. The stage distribution was as follows, st 1: 7%, st. 2: 15%, st. 3: 17%, st. 4: 52%, st. 4s: 6%. The overall 5 year progression free survival was 0,42 at 5 years. There was a significant increase in the 5-year PFS between the four 5-year periods from 0,28 in 1981-1985 to 0,61 in 1996-2000 (p=0.007). Also when analysing the results of children with stage 4 disease the PFS increased significantly between the two 10-year periods (0,14 from 1981-1990 and 0,29 in 1991-2000; P=0,017). Age was a significant prognostic factor both when analysing the entire population (p< 0,001) and also when considering only st. 4 (p= 0,02). Stage was a significant prognostic factor, the 5-year survival was: st. 1: 1,00; st. 2: 0,86; st 3: 0,42; st 4: 0,22 and st 4s: 0,50. Also the localisation of the primary tumor had prognostic significance (p=0,012).
This observational study could detect any significant impact of autologous stemcell transplantation.

Ref ID: 387.2

Neuroblastoma in the first year of life. Analysis of one Institution in the period of 41 years

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INTRODUCTION: Favorable age period, as a second most important, irrespective of stage of advancement prognostic factor, needs the explanation at the epidemiological and basic science level. Epidemiological, biochemical, cytogenetic, histology data were investigated. Detailed analysis of the demised children were carried out.
MATERIAL: Between 1962 and 2003, 97 children at the age 0 to 12 months with the diagnosis ganglioneuroma (GN), gangloneuroblastoma and neuroblastoma, were treated. Neuroblastoma stage I, II, IVS, III and IV were found in 2, 11, 17, 19, 19, 29 children, respectively. From the survival analysis 21 children were excluded due to loss from observation. Localization of primaries were: adrenal-41(44%); abdominal sympathetic ganglia-18(19%);mediastinum-17(18%); paraganglion of Zuckerkandl- 11 (11%); unknown-6(6%) and neck-2 (1%).
RESULTS: In the favorable stage group (I,II,IVS) comprised of 40 patients, 33 children survived (82%). The death in stage I was treatment related. In the stage III of moderate prognosis, 11/16 (69%) of children were cured. In the group of 18 stage IV children only 6 (33%) survived. Three out of six children have short observation period.
CONCLUSIONS: In neonates the diagnosis of ganglioneuroma is extremely rare, however neuronal differentiation is possible. The frequency of favorable stages -49% (I, II, IVS) equals moderate stage III (20%) and dismal stage IV (30%). The prognosis of stage IV neonates is still far from satisfactory. Analysis of factors decisive of survival in neonates is indispensable.**Ref ID:**

Ref ID: 284.1

Monoclonal antibody NB84 expression in the normal fetal sympathetic nervous system development and its relation with other neuroendocrine immunomarkers

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Neuroblastoma (NB) is an embryonal, sympathetic nervous system (SNS)-derived tumor that may originate at any site where SNS tissue is located. The NB84 monoclonal antibody, raised to NB cells, is a sensitive marker for NB. A model of tumorigenesis based upon immunophenotypic diversity in the normal fetal SNS histogenesis might indicate tumor progenitor status and define biologic and clinical behaviour. Immunohistochemistry was used to examine a panel of cellular markers shown to be expressed during SNS development (tyrosine hidroxylase, chromogranin, N-CAM and HNK-1) and NB84 in the normal human fetal SNS from 6 to 36 weeks' gestational age. The sympathoadrenal (SA) lineage develops in the trunk region from the neural crest cells that aggregate at the dorsal aorta to form the primary sympathetic anlagen with expression of TH, N-CAM and HNK-1. SA cells subsequently re-migrate to their final destinations, the sympathetic ganglia or the adrenal medulla and locations of extra-adrenal chromaffin tissue. Immature migratory SNS cell types at 8 week's gestational age expressed all the markers analyzed. TH and N-CAM were expressed in all SNS cell types, the former with intensity increasing with increasing gestational age. Chromogranin was abundantly expressed in adrenal chromaffin, small
intensely fluorescent (SIF) and paraganglia cells. HNK-1, was detected in maturing ganglion cells and adrenal neuroblastic cells. NB84, was noticeably absent in SIF and paraganglia cells and was detected in both adrenal neuroblastic and ganglionic SNS cells. These marker profiles may provide an explanation for the putative progenitor cell types of NB.

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Ref ID: 387.1

Analysis of neuroblastoma treatment results of children between 1 and 2 years old in the aspect of moleculare, hystopathological and biochemical diagnostic markers

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PURPOSE: At present it is agreed that the diagnosis of NBL after completion of 1st year of life changes the prognostics and has a meaningful impact on the treatment procedures. Literature data concerning children in the age 1 - 2 years old is minimal. The purpose of the research is a trial to analyse the NBL treatment results among children in the age of 1 – 2 years old in the aspect of genetic, histopa-thological and biochemical factors.
MATERIALS AND METHODS: The study included 74 children with diagnosed neuroblastoma in the age between 12 and 24 months, treated in the Clinic during the years 1962 - 2003. NBL was diagnosed based on histopathological test of the primary tumour or metastases. The stage of advancement was determined in accordance to Ewans' classification and recently based on INSS. 3,12,1,20 and 38 chil-dren were respectfully classified into stages I, II, IVS, III, IV. Paraffin material underwent cytogenetic analysis. The biochemical blood test and urine analysis was conducted in accordance with the gener-ally approved standards.
RESULTS: The frequency of failures as well as the number of load factors would increase with age growth. Children in stage I, II, IVS made up for a total of 16, and 58 children belonged to stage III and IV. 23 children of the studied group died, in that 17 in IV stage of advancement. All of the children in stage I, II, IVS are alive and are under observation.
CONCLUSIONS: In the analysed material, children up to 2 years old would make up for a rather good prognostic group. Age cannot be the only prognostic exponent during diagnosis. The analysis of the prognostic factors should have a decisive meaning in making decisions as to the therapy.

Ref ID: 200.1

Peculiar presentation of infant neuroblastoma: stage 4 or 4s?

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Stage 4S is a unique subtype of neuroblastoma, accounting for less than 10% of all NB cases. SIOP (International Society of Pediatric Oncology) Europe Infant Protocol has recently parted from the classical INSS stage definitions, including among stage 4S also cases with tumors crossing the midline and further subdividing Stage 4, with the introduction of a new stage definition with a favourable prognosis and receiving no treatment other than observation. We report a case of a little patient presented at the age of 11 months with a well conditions, mass in right flank, subcutaneous nodule on the abdominal wall. Radiolabel MIBG scintigraphy disclosed tracer uptake both within the abdominal primary lesion and the left femur. MDP Tc-99m was negative for bone involvement. Bone marrow aspirate was negative both morphologically and by immunocytology; trephine biopsy was also negative. Biopsy of the primary tumor showed a favourable biologic markers. Even if patient was older than 1 year at diagnosis, it was decided to follow the SIOP protocol, with observation only. Staging and therefore protocol assignment was controversial because a discrepancies: between extent of disease and clinical condition, age at clinical diagnosis and age at time of biopsy and finally histology. We decide for \"wait and see\" strategy. The choice of therapeutic strategy was based on the following data: not with standing the huge abdominal mass, the Philadelphia score was 0, even if urinary catecholamines were elevated and the patient was clinically well; biological prognostic factors (including MYCN, 1p deletion, LDH and histology) were all favourable. At the 6 months follow up we disclosed a partial remission of primary mass and a steadily decreasing urinary catecholamines.

Ref ID: 272.1

Megachemotherapy with auto HSCT in children with advanced neuroblastoma

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Haematological recovery, postransplant morbidity and clinical outcome in children with advanced neuroblastoma who underwent megachemotherapy followed by HSCT were investigated. In the study 10 children with advanced neuroblastoma treated in our Department were analysed. Median age of children was 5 years. 5/10 children were transplanted in I CR, 1/10 in II CR, 4/10 in PR. Pheresis was possible for 8/10 patients with an average 3,35 x 106 CD34 cell/kg and was not possible in 2/10 children. In these children bone marrow was collected followed by purging of residual neuroblastoma cells, with an average 1,3 x 106 CD34 cell/kg. Reinfusion of CD34 cells followed Busulfan / Treosulfan + Melfalan myeloablative chemotherapy in 8/10 patients; Thiotepa + CTX + Carbo in 1/10 patient and Melfalan + VP16 + Carbo in 1/10 patient. Median days to achieve leucocytes >1,0 x 109/l, platelets > 20,0 x 109 and reticulocytes were 12,5; 17 and 13 respectively. 6/10 children died due to disease progression, four of them were transplanted in II PR, one in I PR and two in I CR. 1/10 patient died due to early postransplant complications on day +40. 3/10 children are alive with median follow up 11 months, two of them were transplanted in II CR and one in ICR. Conclusions: Outcome of the treatment with megachemotherapy followed by autoHSCT in patients with advanced neuroblastoma after relapse is still poor. Children with advanced neuroblastoma who undergone megachemotherapy with auto HSCT in CR seem to have more chances to be cured. Delayed platelet recovery were observed in children who received less than 2 x 106 CD34 cell/kg.

Published-only Abstracts

Ref ID: 022.1

Double megatherapy (from auto-auto to auto-allo) for high-risk neuroblastoma

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BACKGROUND: We have performed double megatherapy (DM) for stage 4 neuroblastoma with bone metastases and/or multiple copies of MYCN, as well as poor responders for current chemotherapy.
METHODS: 21 patients were eligible for DM. Median age was 1 year 11 months ranging from 5 months to 9 years old. Autologous stem cells for double graft were harvested after 2 to 3 courses of chemotherapy. DM (1st: IFO+LPAM, 2nd: BU+TEPA) was carried out after several courses of chemotherapy, and the interval of DM was set at three months. The extirpation of primary tumor was carried out before the second megatherapy. In case of patients whose autologous stem cells were not available, allogeneic transplant was performed.
RESULTS: Four out of 9 patients who underwent auto-auto DM are alive and three patients are free of disease (8 years to 10 years 3 months). Ten patients received allo-graft for the second transplant, and 2 patients received double allo-graft from the same donors. Six out of 12 patients who received allo-graft are continuing remission (7 months to 5 years 9 months). Four patients of these 6 received a HLA mismatched graft, resulting in four out of seven patients who received HLA mismatched graft (CD34 positive cell transplant from father: 3, HLA mismatched unrelated cord blood transplant: 1) achieved continuous remission. One patient died soon after the second transplant, therefore treatment related mortality rate was 5% in the present setting.
DISCUSSION: DM can be safely performed without severe complications even in the setting of allo-graft. The preliminary results of HLA mismatched allo-graft instead of HLA matched allo-graft seem to be encouraging.

Ref ID: 084.1

Possible graft vs. neuroblastoma effect after partially matched related hematopoietic transplantation

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We treated 2 cases of relapsed stage IV NB (age 6 and 7 years) with a stem cell transplant from a haploidentical family donor. Both patients lacked a matched sibling. Conditioning consisted of thiotepa 400 mg/m2, fludarabine 90 mg/m2, ATG and melphan 140 mg/m2 followed by purified CD34+ peripheral blood stem cells (CliniMACS, Miltenyi Biotec, Bergish Gladbach, Germany). No further GVHD prophylaxis was administered. Hematological recovery was prompt in both cases with >98% persistent donor engraftment. Post-trasplant serial marrow (BM) and blood (PB) residual disease was assessed by anti-GD2 immunocytology (sensitivity 10-5-10-6). In patient n.1 evaluable disease was limited to BM. On haematological recovery form haploidentical transplantation MRD evaluation showed persistent BM positivity and detectable circulating NB cells. This finding prompted the administration on day +26 of 6 x104 donor CD3+ cells/kg followed by a second infusion of 12 x 104 donor CD3+ cells/kg on day +57 since no GVHD developed after the first DLI, he developed grade III intestinal GVHD on day +71. This patient died 5 months post trasplant with decreasing MRD for a deep-sited fungal infection. Patient n.2 had both local (mediastinum) and bone marrow relapse. He was treated with an initial course of thiotepa and melphalan (same doses as the conditioning regimen) and autologous stem cell rescue with a partial response. Marrow disease became undetectable following haploidentical transplantation while local mediastinal disease regressed 5 months post transplant. This patient is alive and well with no evidence of disease, complete chimerism, at 10 months from trasplant. Both cases lacked an NK alloreactivity setup as predicted by HLA mismatch. In conclusion haploidentical transplantation may provide a graft vs.
neuroblastoma effect.

Ref ID: 165.1

Docosahexaenoic acid potentiates the cytotoxic effect of chemotherapeutic drugs in neuroblastoma cells

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BACKGROUND: Dietary omega-3 polyunsaturated fatty acids (PUFAs), such as decosahexaenoic acid (DHA) have been implicated in the inhibition of tumour development and progression.
METHODS: We analysed the effect of DHA alone and in combination with chemotherapeutic drugs, non-steroidal anti-inflammatory drugs (NSAIDs) and inorganic arsenic trioxide (As(2)O(3)) on neuroblastoma in vitro.
RESULTS: Treatment with DHA inhibited cell proliferation of both MYCN amplified and non-amplified cell lines in a dose-dependent manner. DHA used in combination with sub-lethal doses of cisplatin potentiated the cytotoxic effects in non-amplified cells but had limited effect on MYCN amplified cells. Addition of the pan-COX inhibitor diclofenac, or the COX-2 specific inhibitor celecoxib, together with DHA resulted in enhanced cytotoxicity in both MYCN amplified and non-amplified cells. Arsenic trioxide induces remission in refractory acute promyelocytic leukaemia (APL) and induces apoptosis of APL cells as well as in solid tumour cell lines, including neuroblastoma. Hence, we investigated the effect of low concentrations of As(2)O(3) in combination with DHA on neuroblastoma cell lines. This combination resulted in increased cytotoxicity in all cell lines tested.
CONCLUSION: These findings indicate that intake of dietary DHA may potentiate the effect of cytostatic drugs in the treatment of children with neuroblastoma.

181.1

Overexpression of embryonal transcription factor OCT-3/4 in poor risk neuroblastoma

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Background: Oct-3/4 transcription factor is a key regulator of pluripotency in the earliest stages of mammalian development. Oct-3/4 expression is down-regulated in embryonal carcinoma and embryonal stem cells that are treated with retinoic acid. Oct-3/4 overexpression in mouse teratomas resulted in primitive neural tissue proliferation, histological similar to human neuroblastomas. We therefore studied the role of Oct-3/4 expression in archival biopsies of 24 patients(pts) with neuroblastomas and 4pts with ganglioneuroblastomas.
Methods: All pts were treated at Hadassah University Hospital between years 1988-2000. Formalin fixed paraffin embedded tissue sections were stained by immunohistochemistry. Polyclonal rabbit anti-sera against human Oct-3/4 proteins were raised in rabbits. Correlation between Oct-3/4 expression, age at diagnosis, primary site, disease stage and outcome were tested by Fisher's exact test. Statistical analyses performed using SPSS (version 10).
Results: Oct-3/4 was expressed in 11(39%) out of 28 biopsies: 7(54%) out of 13pts with stage IV, 4(44%) out of 9pts with stage III and none out of 6pts with stage I, II or IVs disease. Twelve (43%) out of the 28pts are alive and free of disease with follow-up time between 1-150months (median-120months), 1(9%) out of 11pts with positive Oct-3/4 expression compared to 11(65%) out of 17pts with negative Oct-3/4 expression (p=0.006, Fisher's exact test). The correlation between Oct3/4 expression and disease free survival is statistically significant independent of age, primary site and stage of disease.
Conclusions: Although small in number, our study suggest that Oct-3/4 may serve as a prognostic factor in neuroblastoma, however, a large prospective multivariate analyses is needed to confirm this preliminary finding.

Ref ID: 141.1

Neutropenia and fever in children with neuroblastoma treated with the European High-Risk Protocol. A mono-institutional experience

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BACKGROUND AND AIM: To report on frequency and severity of neutropenia and infections in children treated with the European HR-NBL-1 Protocol [highly intensive COJEC regimen, followed by megatherapy with autologous peripheral blood staminal cells reinfusion (aPBSCR)].
PATIENTS: In the period January 2002 – January 2003 57 children with neuroblastoma received antineoplastic chemotherapy. Among them 20 (35%) were treated according with the European HR-NBL-1 Protocol.
RESULTS: During the COJEC phase 166 episodes of neutropenia (absolute granulocyte count <1000/cmm) were documented, with a mean of 8.3 episodes/patient, lasting a total of 2271 days at risk. Development of fever (T> 38°C) was observed during 42 episodes (25%) of neutropenia, with an incidence rate of 18.5 episodes/1000 days at risk. The diagnosis of infectious episodes was fever of unknown origin in 38 cases, bacteremia in 3, and clinically documented infection of skin and soft tissues in 1 case. After aPBSCR, 34 episodes of neutropenia were documented for a total of 402 days. Development of fever was observed in 18 cases (53%), with an incidence rate of 44.8 episodes/1000 days at risk. The diagnosis of infectious episodes was fever of unknown origin in 15 cases, bacteremia in 2 and microbiologically documented infection without bacteremia (UTI) in one case. No patient died due to infectious complications in any phase of treatment.
CONCLUSIONS: In children with neuroblastoma treated with the European HR-NBL-1 Protocol febrile neutropenia is not a frequent complication during the highly intensive COJEC regimen while it represents a more common problem after high-dose chemotherapy.

Ref ID: 067.1

Randomization and Informed Consent in Pediatric Oncology

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Despite improvements in the outcome of most childhood cancers over the last 30 years, stage IV neuroblastoma (NB) still harbors a poor prognosis. The lack of significant progress in NB survival rate curves begs new clinical strategies, which most often entail experimental protocols that include randomization (Rm). However, parents asked to give their written consent to the Rm protocol after detailed briefings are often unprepared to grasp, let alone accept, the implications of this scientifically crucial but seemingly unfair mechanism whereby a computer and not the physician chooses the treatment. True, physicians are present to answer questions, and families may also entreat their general practitioner, relatives, or others for suggestions. Communication and the new relationship that develops among the family, under-aged patient, and physician are trying and emotionally taxing, and parents' coping-adaptation becomes a balancing act influenced by risk and resistance factors. Ultimately, parents' unwillingness to accept the Rm process may jeopardize enrollment in and validation of trials; case in point is seen in UK parents' refusal of Rm included in the SIOP-MMT-95 protocol for rhabdomyosarcoma (1). The ethical question that arises is whether it is necessary to ask for consent a second time at Rm. We view consent as a shared cognitive process involving the physician, parents and the under-aged patients (2) that must be requested only at recruitment into the whole trial, even if it includes Rm. Continuous earnest communication is pivotal to achieving all-inclusive consent to a clinical trial.

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2. Massimo LM, Wiley TJ, Casari EF. From Informed Consent to Shared Consent: a Developing Process in Paediatric Oncology. Lancet Oncology, 2004; in press.

Ref ID: 412.1

Challenges in management of Advanced Neuroblastoma: Experience at Tata Memorial Hospital, Mumbai, India

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Background: Poor response in advanced neuroblastoma prompted to implement TMH-NB-1 & 2 since 1987 and TMH-NB-3 since 1996 with the aim to improve survival with manageable toxicity.
Methods: 128 cases from 1987 to 2000 were analyzed. In infants (n = 29) median age at presentation 9 months, duration of symptoms 60 days, M: F: 1.6: 1. Stage III (17), IV (1) received 6 # of Adriamycin & cyclophosphamide or 1 -2 # of Adriamycin, cyclophosphamide, Cisplatin & Etoposide. Surgery considered after 3/6 # or 1/2# of chemotherapy respectively. In children > l year (n=99) median age at presentation 48 months, duration of symptoms 60 days, M: F: 1.3: 1. Stage IIB (3), III (30), IV (60) received1cycle of TMH NB-2 as induction followed by either 2# of TMH NB-2 or 4 # TMH NB-3 as consolidation. In Stage III surgery after induction, whereas in stage IV at the end of consolidation. Radiotherapy or MIBG treatment was given where surgery not feasible or in R1 resection. ABMT was offered for gross residual & 13 Cis -Retinoic acid for minimal residual disease in stageIV.
Results: In Infantile group 62% constituted intermediate risk (stage III, IV). Overall response to therapy was 97%. OS is 75.06%. 58% are alive & disease free at median follow-up of 42.9 months. In children>1year 94% constituted high risk (IIB, III, IV). 74% showed response to therapy. OS is 54.31%. 18.6 % are alive & disease free at a median follow up of 14.9 months.
Conclusions: High-risk patients remain an area of concern. Proper risk stratification has not been possible at our center & remains the main constraint in tailoring therapy.

Ref ID: 006.1

Large cell neuroblastoma: A distinct type of neuroblastoma with aggressive clinical behavior

Tamàs Tornóczky[1], Tibor Nyári[2], Andrew DJ Pearson[3], Julian Board[3], Hiroyuki Shimada[4]

Universities of Pécs[1], and Szeged[2], Hungary; University of Newcastle upon Tyne[3], UK; Children's Hospital Los Angeles4, CA, USA.

BACKGROUND: Among cases of undifferentiated and poorly differentiated neuroblastomas, the authors histologically identified a group of rare tumors known as large cell neuroblastomas (LCNs) that are composed of large cells with sharp nuclear membrane and prominent nucleoli.
METHODS: Histologic and immunohistochemical features of LCN were characterized. Morphologic characteristics, clinical features and MYCN status were compared between LCNs and conventional neuroblastoma cases documented in the files of two European Centers.
RESULTS: Of 92 peripheral neuroblastic tumors (pNTs including neuroblastoma 81 cases, ganglioneuroblastoma, intermixed 6 cases, ganglioneuroblastoma, nodular 5 cases), 7 cases (7.6%) qualified as LCN. All were classified as unfavorable histology according to the International Neuroblastoma Pathology Classification. The LCNs were composed of monomorphous undifferentiated neuroblasts, and shared certain histologic features, such as a high incidence of high mitosis-karyorrhexis index and a low incidence of calcification, with other neuroblastomas in the conventional unfavorable histology group (c-UH). These features were significantly different from those of the conventional favorable histology (c-FH) group. The LCN tumor cells were positive for NSE, PGP9.5, synaptophysin, tyrosine hydroxylase and NB84, and negative for CD99. The LCN patients had similar clinical features (regarding the age, and occurence of metastasis) to the c-UH patients. The clinical features also were significantly different from those of c-FH patients. LCN group was significantly different from both the c-UH and c-FH groups with respect to MYCN status (MYCN amplification: 4/5 vs. 3/17 in c-FH and 8/17 respectively: p=0.023) and survival rate(4-year expected survival, 0% vs. 71% vs 17% respectively p<0.01).

CONCLUSION: Because of its unique clinicopathological features, the authors propose to that LCN be recognized as a distinct entity within the neuroblastoma category.

Ref ID: 057.1

Role of Bmi1 in neuroblastoma

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We recently identified the MYCN oncogene as a tissue-specific target gene of E2F-1 in neuroblastomas (Strieder et al., 2003; Kramps et al., 2004). A search for additional oncogenic target genes of E2F-1 using human neuroblastoma cells expressing a 4-hydroxy-tamoxifen (4-OHT) regulated E2F-1-ER fusion protein and cDNA microarray analysis identified the polycomb group gene BMI1. Real-time RT-PCR and Western-blotting confirmed the induction of BMI1 by E2F-1. Cycloheximide did not inhibit induction of BMI1 by E2F-1-ER suggesting that BMI1 is a direct target of E2F-1. Indeed, the human BMI1 promoter contains a putative E2F binding site that is conserved in the mouse. In transient assays, this binding site was required for the activation of a BMI1-driven reporter construct by E2F-1. In addition, chromatin-immunoprecipitation revealed binding of E2F-1 to the BMI1 promoter in vivo. These data establish BMI1 as a direct E2F target gene. Bmi1 in turn, via repression of p16INK4A, can stimulate E2F-activity suggesting a positive feedback loop between E2F-1 and Bmi1. BMI1 was recently shown to be essential for the self-renewal of both hematopoietic and neuronal stem cells and to act as an immortalizing oncogene early in tumorigenesis. Moreover, Bmi1 co-operates with c-Myc in the pathogenesis of lymphomas by blocking Myc-induced apoptosis. Thus, a failure to down-regulate BMI1 may be a critical event in the initiation of neuroblastomas. On the one hand, Bmi1 can promote immortalization, on the other hand Bmi1 may pave the way for MYCN amplification by simultaneously stimulating E2F-activity and blocking Myc-induced apoptosis. Consistent with a role of Bmi1 in neuroblastoma, BMI1 expression was detected in all primary neuroblastomas analyzed. We will present results of loss-of-function and gain-of-function studies addressing the role of Bmi1 in neuroblastoma cells.

Ref ID: 416.1

Spinal Neuroblastoma in Stage 2/3 Disease – Experience From One Institution

J Begent, P Brock, J Laddie, D Saunders, D Thompson, K Phipps and G Levitt

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Spinal cord compression secondary to neuroblastoma can be considered an emergency. There is no treatment consensus for symptomatic presentation. Aim: to analyse management and outcome of children with stage 2/3 neuroblastoma with spinal involvement treated at Great Ormond Street Hospital (GOSH) over 20 years. Data was acquired by retrospective note review.

Results: 387 children had neuroblastoma, 115 had stage 2/3 disease. 22 patients had evidence of an intraspinal component. 16 presented at <1 year of age (mean 0.74 years, range 0 to 1.8 years); 14/22 had stage 3 disease, 8/22 had stage 2 disease. 9/22 were thoracic tumours and 7/22 were pelvic.

Presentation: time from first symptoms to GOSH - 24 hrs to 3 months (mean 18 days). 11 children had limb neurology, 4 had bladder / bowel symptoms and 7 non-neurological symptoms.

Treatment: 5 had steroids, 21 had chemotherapy; 7 underwent laminectomy (3 at presentation, 4 after chemotherapy); 3 required further surgery to primary disease; 15 children underwent surgery on the intra thoracic/abdominal component of the primary; 2 received radiotherapy.

Follow up: the interval is wide (1 to 20 years; mean 9.3 years); all but one of these patients are alive, one has possible localised disease progression.6 patients cannot walk, all presented with limb neurology. 12 have urinary incontinence / bowel control problems. 6 children have no significant sequelae. Overall survival from disease is 100% however morbidity free survival is 27%.

Conclusion: 19% of children with stage 2/3 neuroblastoma had spinal involvement. Management of stage 2/3 neuroblastoma involving the spinal cord has not been consistent; prompt treatment may improve outcome. Although prognosis from this disease is good, long-term morbidity is high. We need to recognise this early and investigate prospectively which treatment best reduces long term sequelae.

Abstracts

Opsoclonus Myoclonus

Opsoclonus Myoclonus

Ref ID: 370.2

Neuroblastoma-associated Opsoclonus, Myoclonus, Ataxia syndrome: A Clinical and Biologic Dilemma

Katherine K Matthay

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Opsoclonus-myoclonus ataxia syndrome (OMA), also called “dancing eyes-dancing feet”, is a rare paraneoplastic disorder that occurs in less than 2-4% of patients with neuroblastoma. This syndrome is characterized by neurologic findings including the presence of involuntary, conjugate, chaotic eye movements and myoclonic limb jerking. These symptoms are often accompanied by significant mood and sleep disturbance. Although OMS may also occur in association with infections approximately 50% of children with OMS are diagnosed with neuroblastoma. The majority of children present with localized, favorable biology tumors. Most children present between age 1-4, with a median of 2 years. Data from patient tumors and sera suggest an immune- mediated process, with an excess of lymphoid infiltrates in the tumors and a high percent of children with anti-neuronal antibodies in sera at diagnosis. MRI examinations over time show progressive cerebellar atrophy. Although the outcome from the standpoint of the malignancy tends to be favorable, with greater than 90% survival, 70% of children with OMA inevitably develop significant neurologic sequelae and developmental and speech delay. Recent approaches to treatment have focused on intensive immunosuppression rather than specific anti-tumor therapy. The current management, usually successful in arresting the acute symptoms, is tumor resection, followed by steroid or ACTH therapy and often other immunosuppression, either cyclophosphamide or intravenous gammaglobulin. Newer approaches being tested include rituximab. Future efforts must include further investigation of the pathogenesis of this disorder, and careful assessment of the effectiveness of such therapies on prevention of the late neurologic and developmental deficiencies resulting from OMA.

Ref ID: 429.1

Longitudinal neurodevelopmental evaluation of children with opsoclonus-ataxia

Wendy G. Mitchell, Virdette L. Brumm, Colleen Azen, Kirsten E. Patterson, Jenny Rodriguez

We previously reported upon children with opsoclonus-ataxia due to neuroblastoma and found pervasive neurodevelopmental deficits, years after onset, without clear relationship to treatment modality or timing of treatment. A significant negative correlation of functional status with age at testing raised a question of whether OA is a progressive encephalopathy, which we attempted to clarify with repeated testing.
METHODS: Thirteen of 17 children previously reported1 were reevaluated a second time 2-4 years later. In addition, 5 new subjects (2 with NB, 3 without NB) were enrolled, and were evaluated twice at a minimum interval of one year between sessions. Intercurrent medical course was recorded, emphasizing medication (ACTH, oral steroids, IVIg, other immunosuppressants) and relapse history. Cognitive, adaptive behavior, speech and motor abilities were assessed.
RESULTS: Generally, younger subjects' cognitive and adaptive behavior scores improved while older subjects' scores were stable or dropped slightly. Only four children are currently functioning in the average range with FSIQ of 90 or above. All four were unusual, in that despite severe initial symptoms, each appeared to have a monophasic course of gradual improvement on steroids or ACTH. None experienced a relapse upon medication taper or with intercurrent illnesses. These four subjects were the only subjects without any relapses.
The results continue to be raise concern that opsoclonus-ataxia represents a progressive encephalopathy, at least in some children. It appears that some children have a monophasic course. Despite initial severity of symptoms, these children may be biologically different, with a more benign prognosis. There is some evidence that aggressive initial treatment may improve overall outcome, but this does not reach statistical significance in this observational study. A well-organized randomized clinical trial comparing different modalities of immune suppression of opsoclonus ataxia is needed.

References: 1. Mitchell WG, Davalos-Gonzalez Y, Brumm VL, Aller SK, Burger E, Turkel SB, Borchert MS, Hollar S, Padilla S. Opsoclonus-ataxia caused by childhood neuroblastoma: Developmental and neurological sequelae. Pediatrics, 2002;109:86-98.

Acknowledgements: Initial testing was supported by a grant from Aventis-Behring (formerly Centeon Pharmaceuticals); The second phase was supported in part by the NIH NCRR GCRC grant MO1 RR-43 and was performed at the GCRC at Childrens Hospital Los Angeles.

Ref ID: 131.2

Immunosuppression with cyclophosphamide in the treatment of patients with opsomyoclonus syndrome

Barbara Hero[1], Dominique Plantaz[2]

Children's hospital, University of Cologne, Germany; Children's hospital, University of Grenoble, France.

Treatment with corticosteroids or ACTH is well etablished in the therapy of patients with opsomyoclonus syndrome, but in many patients the acute symptoms do not respond well to this treatment and long term developmental deficites may occur. Analysis of the literature for long term outcome of patients with opsomyoclonus syndrome showed advantage for patients with chemotherapeutic treatment indicating a potential positive side effect of the immunosuppression caused by the chemotherapeutic treatment. Cyclophosphamide in immunosuppressive dosage has been used for many years e.g. in children with nephrotic syndrome, autoimmune nephritis or rheumatoide arthritis. The acute side effects of treatment with low dose cyclophosphamide appear moderate, but the extent of long term side effects with those low doses is not well investigated. For a treatment protocol of patients with opsomyoclonus syndrome, the risk of late side effects caused by cyclophosphamide has to be balanced against the potential positive influence of cyclophosphamide and against the opportunity to reduce the total amount of corticoidsteroids with their considerable acute and chronic toxicity.

Ref ID: 410.2

COG Therapeutic trial in OMS: A neurologist’s view

Dr Michael Pike MA, MD, FRCPCH, Consultant Paediatric Neurologist

John Radcliffe Hospital, Oxford, UK

The COG therapeutic trial is a welcome attempt to evaluate the role of treatment options, particularly intra-venous immunoglobulin, in the management of this distressing disorder. From the perspective of a paediatric neurologist the following issues deserve discussion:

- Inclusion criteria specifically in relation to children without associated neuroblastoma.
- Age range – particularly in relation to the inclusion of young adults.
- The acceptability of cyclophosphamide to families whose children do not have neuroblastoma and to treating clinicians.
- The difficulties of OMS evaluation resulting from (a) the intermittent nature of opsoclonus in some children, (b) The natural fluctuation of all symptoms of OMS in relation to a variety of factors including intercurrent infections in many children.

These and related issues will be discussed with colleagues.

Ref ID: 152.1

Lymphoid infiltration in neuroblastoma-associated Opsoclonus Myoclonus Syndrome (OMS)

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Laboratories of Oncology and Pathology, G. Gaslini Institute, Genoa, and Laboratory of Pathology, Bambin Gesù Institute, Rome, Italy

OMS is an autoimmune disorder of the central nervous system that may affect children with neuroblastoma. This association is observed in 1.3% of neuroblastoma cases in the Italian population. Distinctive features of OMS-related neuroblastoma are the predominant ganglioneuroblastoma histology, the lack of MYCN amplification and the presence of abundant lymphoid infiltrates. These are often organized in lymphoid follicles with a well defined network of follicular dendritic cells, a mantle and a germinal centre, indicating the occurrence of a process of lymphoid neogenesis.. B cells are concentrated in the follicles, whereas T cells, mainly with a CD8+ immunophenotype, are found in extrafollicular location. Similar features may be observed in OMS-unrelated ganglioneuroblastoma, in which less infiltrating lymphoid cells are detected. Absence of HLA class I from the surface of neuroblasts in OMS-associated tumors militates against the hypothesis that T cell recruitment to the tumor tissue is antigen driven. Key players in lymphoid neogenesis are the CCL19, CCL21, CXCL12 and CXCL13 chemokines, that interact with their cognate receptors, i.e. CCR7, CXCR4 and CXCR5. In preliminary experiments we have investigated expression of these receptors in OMS-related tumors by immunohistochemistry. CCR7, CXCR4 and CXCR5 were found to be expressed on the majority of tumor cells, with higher intensity on the more differentiated gangliar component. CXCR5, but not CXCR4 or CCR7, was detected on infiltrating lymphoid cells. Studies are now in progress to investigate expression of the ligands of these receptors in tumor tissue.

Ref ID: 413.1

Immunopathogenesis of Opsoclonus-Myoclonus-Syndrome

Franz Blaes

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Opsoclonus-myoclonus syndrome (OMS), also known as Dancing eye syndrome or Kinsbourne´s syndrome is characterized by chaotic, omnidirectional synchronous eye movements (opsoclonus), ataxia and myoclonus. In children, additional symptoms like irritability, behavioural disturbances and language difficulties may also occur. As a paraneoplastic syndrome, about 50% of the childhood OMS are associated with neuroblastoma and vice versa, 1-3% of neuroblastoma patients have OMS. Most OMS patients respond to immunosuppressive or immunomodulatory drugs, which led to the suspicion of an autoimmune pathogenesis. Additional features support this hypothesis: Inflammation: Some OMS patients have been reported to have lymphocytic pleocytosis or oligoclonal bands in the CSF. Relapses and deteriorations in OMS often occur in association with infections. Tumour immunology: Neuroblastoma of OMS patients have more lymphocytic infiltration than neuroblastoma without OMS and children with OMS have a better prognosis of the tumour disease, suggesting a (partially) effective antitumour immunity. Autoantibodies: Different autoantibodies have been described in children with paraneoplastic OMS. Some of them have anti-Hu antibodies, which are directed against a group of RNA-binding proteins expressed in nervous system and tumour tissue. Other patients exhibit autoantibodies against the cytoplasm of cerebellar Purkinje cells. However, no common autoantigen was described yet. Recently we found a 55 kD reactivity against cerebellar and neuroblastoma proteins in 40% of our patients. Taken together, these findings support the idea of an autoimmune pathogenesis of paraneoplastic OMS, which may result from a cross-reactive immunity between neuroblastoma and nervous system tissue.

Ref ID: 410.1

Opsoclonus-myoclonus (OMS): Diagnostic criteria and Neurological evaluation

Dr Michael Pike MA, MD, FRCPCH, Consultant Paediatric Neurologist

John Radcliffe Hospital, Oxford, UK

OMS, also called the Dancing Eye Syndrome, is a rare condition usually starting in the second or third year of life and sometimes, but not invariably, associated with an underlying neuroblastoma. It is characterised by opsoclonus (bursts of rapid multi-directional conjugate eye movements), myoclonus-ataxia (a jerky unsteadiness of posture and movement) and behavioural change usually consisting of irritability which may be extreme.
There is no diagnostic test and the diagnosis, which depends on recognition of the clinical features, may not always be obvious. This is particularly the case if the opsoclonus is intermittent and fleeting or if the onset is insidious. The diagnosis is important for a number of reasons – first, it should prompt a search for an underlying neuroblastoma; second, the symptoms respond to immunomodulatory treatment such as steroids and intra-venous immunoglobulin and third the neurological disorder carries a substantial risk of long-term motor and cognitive disability and a need for continuing neurodevelopmental supervision.
A video will be shown and suggested diagnostic criteria discussed.

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Social Programme

Opening Ceremony & Welcome Reception

Date: Thursday, June 17, 2004
Time: 7:00 - 09:30 PM
Location: Magazzini del Cotone Congress Centre and Italian Navy's Amerigo Vespucci
Fee: Included in the **Senior, Young A, Young B** and **Accompanying person** fee.

The Welcome Reception will be held on the "Molo Vecchio" Pier (the Congress venue's Pier in the Old Harbour) and aboard the spectacular Amerigo Vespucci tall ship, the only square-rigged three-decker sailing ship still in existence and one of the largest that is active nowadays.
The pride of the Italian Navy, she is a full ship-rigged steel hull (and masts) frigate built during 1930-31 in the former Royal Shipyard at Castellammare di Stabia (Naples) as a sail-training ship. Inspired by the big 19th Century frigates, with high freeboard, stern gallery and white-painted strakes, her bow and stern decorated with intricate gilt carvings, the Vespucci is one of the grandest of the tall ships and a spectacular sight under full sail. The ship is employed for the annual Italian Naval Academy training cruise.

Gala Dinner

Date: Friday, June 18, 2004
Time: 8:30 - 11:00 PM
Location: Aquarium of Genoa
Fee: € 100,00 (included in the **Senior, Young A** and **Accompanying person** fee).

The Gala Dinner will take place inside the spectacular marine environments of the Aquarium of Genoa, the largest aquarium in Europe and one of the most frequently visited cultural sites in Italy, thanks to its dimensions of 10.000 square metres and 71 tanks. Most tables will be arranged along two 25-metre long tanks (1.300.000 litres of water), face to face with the world's most feared and beloved marine creatures, so that the sharks' elegant gait and the dolphin's contagious curiosity will set the scene for a most extraordinary evening, unique and unforgettable, in the only such setting in the world, which will leave you speechless.

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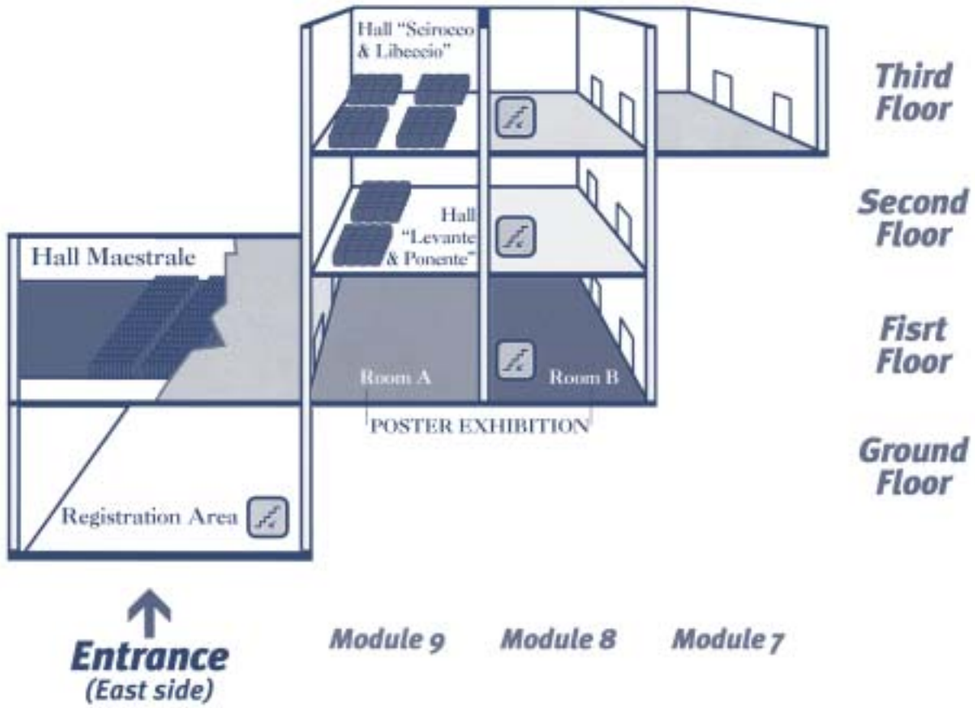
Floorplan Congress Venue

"Magazzini del Cotone" Congress Centre



Hall "Maestrale"
Auditorium, First Floor

Workshop on Microarray Technology (June 16)
Plenary Session (June 17-18-19)
Parallel Session E: Molecular Biology - Translational (June 19)

Hall "Scirocco & Libeccio"
Module 9 - Third Floor

Parallel Session A: Translational (June 17)
Parallel Session C: Biology and Genetics (June 18)

Hall "Levante & Ponente"
Module 9 - Second Floor

Parallel Session B, D, F: Clinical (June 17-18-19)
Workshop on Spinal Cord Compression (June 19) Hall "Levante"
Workshop on Opsoclonus Myoclonus (June 19) Hall "Ponente"

Posters Exhibition
Room A *Biology - Clinical - Genetics 1*
Room B *Genetics 2 - Molecular Biology - Translational*

Maps of Genova

"Magazzini del Cotone" Congress Centre

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