Annual meeting EuroBoNeT

21-23 January 2009

Valencia, Spain

Location:
Hotel Medium Valencia, General Urrutia, 48, 46013 Valencia
and
Colegio Oficial de Médicos de Valencia, Avda de la Plata, 20

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Meeting programme

Location: Colegio Oficial de Médicos de Valencia, Avda de la Plata, 20, 46013 Valencia, right next to Hotel Medium.

day 1 - Wednesday, 21st January

10:00 - 15:00 Steering Committee meeting

14:30 *poster mounting*

15:00 Start of the meeting
15:00 - 15:15 organisational matters (P. Hogendoorn)
15:15 - 16:00 staff exchange experiences (3*12 min + 3 min questions)
A.S. Martins 'Study of the involvement and significance of clathrin-dependent/ independent endocytosis on 1GF1R signalling pathway in ES
G. De Boeck 'A TMA-based osteoclast expression profile study in giant cell containing lesions'
A. Neumann 'Expression array analysis of osteosarcoma cell lines to determine candidate genes for invasiveness'
16:00 - 16:15 virtual BioBank (P Riegman)
16:15 - 16:30 15 min poster summary RL1 by RL leader
16:30 - 17:00 tea poster viewing
17:00 - 18:30 talks, 6*12 min + 3 min questions RL1 (1.30 hours)
K.H. Hallor 'Genomic profiling of chondrosarcoma: implications for the distinction between central and peripheral tumors'
T.Pansuriya 'Identifying candidate genes for Ollier disease using SNP array'
J. Diaz-Romeo 'Immunophenotypic profiling of flow cytometry data via cluster analysis: application to the study of chondrosarcomas'
J.V.M.G. Bovée 'Kinome profiling of chondrosarcoma reveals Src-pathway activity and dasatinib as option for treatment'
I. Jennes 'Characterization of the exostosin-1 (EXT1) promoter'
C.E. de Andrea 'Primary cilia organization in growth plate and osteochondroma'
18:30 - 19:30 **I. Stamenkovic** (45 min talk, 15 min discussion)

19:30 - 21.30 Bioinformatics session for those interested

20:00 - 21:30 Welcome party by invitation of the local sponsors (IVO Foundation, AECC, and Colegio de Medicos)

day 2 - Thursday, 22nd January

09:00 - 09:30 technology platforms
A.M. Cleton: Progress and plans Technology platforms (10 min)
E. de Alava: TP Proteomics and mass spectrometry (10 min)
A. Llombart-Bosch: TP Xenografts (10 min)
09:30 - 10:00 bioinformatics review and plans (E. Korsching)
10:00 - 10:45 talks, 3*12 min + 3 min questions RL2 (45 min)
S.L.J. Verbeke 'Haemangiopericytoma of bone: real or imagined?'
I. Machado 'Histopathology, immunohistochemistry and xenotransplant in osteosarcomas: a tissue microarray study'
H.M. Namløs 'Integrated analysis of miRNA, gene expression and copy number variation in osteosarcoma cell lines'
10:45 -11:00 15 min poster summary RL2 by RL leader
11:00 - 11:30 coffee poster viewing

11:30 - 12:00 talks, 2*12 min + 3 min questions RL2 (30 min)

M. Debiec-Rychter 'Tyrosine kinases as possible therapeutic targets in chordoma'

E.P. Buddingh' 'Chemoresistant osteosarcoma cells are sensitive to DNAM-1 and NKG2D dependent NK cell cytotoxicity'

12:00 - 13:15 talks, 5*12 min + 3 min questions RL3 (1.15 hours)

R.G. Forsyth 'Scarring areas in giant cell tumour of bone: of biological importance?'

H. Knowles 'Acute hypoxia and osteoclast activity: a balance between enhanced resorption and increased apoptosis'

M. Balke 'Establishment of the chick chorio-allantoic membrane assay for giant cell tumour of bone'

A. Conti 'Global molecular profile in giant cell tumours of bone'

W. Van Hul 'Involvement of TNFRSF11A polymorphisms in the development of sporadic Paget's disease of bone'

13:15 - 13.30 15 min poster summary RL3 by RL leader

13:30 - 15:00 lunch poster viewing

15:00 - 16:00 **P. Sorensen** 'Targeting kinase pathways in bone cancers' (45 min talk, 15 min discussion)

16:00 - 16:50 Enrique De Alava, Overview of WP4.1 (including poster summary)

Arjan Lankester, Overview of WP4.2 (including poster summary), limited time because of 2 talks in following session.

Katia Scotlandi: Overview of WP4.3 (including poster summary)

16:50 - 17:20 tea poster viewing

17:20 - 18:00 talks, 4*10 min including questions RL4 (40 min)

C. Guerzoni 'Triggering of CD99 induces apoptosis of Ewing's sarcoma cells through re-activation of P53 functions'

D. Berghuis 'High expression of chemokine genes by Ewing sarcoma tumours predicts survival'

S. Savola 'Inflammatory gene profiling of Ewing sarcoma family of tumours reveals potential target molecules for drug therapy'

D. Osuna 'Assessment of human mesenchymal stem cells as a valid cellular model for Ewing sarcoma pathogenesis'

18:00 - 18:10 company presentation (Z. Holló, SOLVO)

18:10 - 18:30 discussion + questions

21:00 - 24:00 Dinner in Astoria Palace restaurant, near Plaza del Ayuntamiento (transfer by bus at 20.30 from the hotel)

day 3 - Friday, 23rd January

09:00 - 09:30 The new online reporting system (L Rozeman)

09:30 - 10:00 General Assembly voting and conclusion of the general meeting

10:00 start of the RL/WP meetings

*11:00 - 11:*30 tea

	Room 1			Room 2		
Time	**WP**	**RL Leader**	**WP Leader**	**WP**	**RL Leader**	**WP Leader**
1000-1100	RL4	P Picci		WP2.1	H Bűrger	O Myklebost
1100-1200	WP3.1	N Athanasou	R Forsyth	WP2.2	H Bűrger	M Serra
1200-1300	WP3.2	N Athanasou	N Athanasou	WP1.1	P Hogendoorn	J Bovée
1300-1330	WP3.3	N Athanasou	W van Hul	WP1.3	P Hogendoorn	W Wuyts
1330-1500		Lunch break			Lunch break	
1500-1600	WP2.3	H Bűrger	F Mertens	WP1.4	P Hogendoorn	P Mainil-Varlet
1600-1700				WP1.2	P Hogendoorn	P Hogendoorn

Abstract Prof Sorensen

TARGETING KINASE PATHWAYS IN BONE CANCERS

Poul Sorensen, MD, PhD, Department of Molecular Oncology, BC Cancer Research Centre, Vancouver, BC, Canada (psor@interchange.ubc.ca)

Understanding how tumor cells respond to extra- or intracellular signals provides unique insights into the signal transduction pathways that are specific to tumor cells. Targeting these pathways can then be used as a treatment strategy, while minimizing effects on normal cells. Central to cellular signaling pathways are protein kinases, which enzymatically link phosphate groups to tyrosine, serine, or threonine residues of substrate proteins. There is great interest in assessing the expression and activation states of kinases and phosphatases, which dephosphorylate proteins, in human cancer. It is now well established that receptor protein tyrosine kinases (PTKs) can be activated by mutations or over-expression in human tumors. Activated receptor PTKs such as EGFR, ERBB2/HER2, and IGF1R can then be potentially targeted therapeutically, although additional mutations selected for during treatment may lead to kinase inhibitor resistance. Similar activation of non-receptor tyrosine kinases (e.g. BCR-ABL and Src family kinases) as well as serine/threonine (e.g. AKT and mTOR) and lipid kinases (e.g. phosphatidyl inositol 3-kinase, or PI3K) has also been identified in human neoplasia. Moreover, mutations in phosphatases have also been documented in human tumors, although translation of this information to the development of phosphatase inhibitors is less well established than for kinases. Many other kinase pathways, such as the energy-sensing AMPK cascade, have only recently been studied in tumor cells. In spite of these advances, systematic evaluation of the expression and activation status of the kinome (and the phosphatome) in bone cancers has not been reported. During this lecture, our approaches to kinase evaluation in bone tumors will be highlighted, including our efforts to systematically catalogue and validate kinases that are consistently up-regulated in bone sarcomas through gene expression profiling of primary tumor samples and siRNA high content screening of tumor cell lines.

Abstract Prof Stamenkovic

MESENCHYMAL STEM CELLS AND ORIGINS OF SARCOMA

Abstracts staff exchange experiences

EXPRESSION ARRAY ANALYSIS OF OSTEOSARCOMA CELL LINES TO DETERMINE CANDIDATE GENES FOR INVASIVENESS

A. Neumann[1], E. Korsching[1], A.M. Cleton-Jansen[3], R. Duim[3], H. Bürger[2], K. Agelopoulos[1]

[1]*Institut für Pathologie, Universitätsklinikum Münster – EuroBoNeT;* [2]*Institut für Pathologie, Paderborn;* [3]*Department of Pathology, Leiden University Medical Centre, NL – EuroBoNeT*

Aims: Osteosarcoma (OS) is the most common primary malignancy of bone, with up to 80% of patients suffering from metastatic or micrometastatic disease at the time of diagnosis. For the metastatic potential of tumours invasiveness plays an important role. This study intends to determine new candidate genes for cell invasiveness.

Methods: Eight OS cell lines (MNNG, HOS, MG63, SJSA1, OST, ZK58, U2OS, SAOS) were analysed using a modified Boyden Chamber Assay to separate invasive and non-invasive cells. Total RNA isolation and Illumina hybridisation Arrays (V3 bead arrays) were performed for both fractions.

Results: Out of the eight cell lines, five (MNNG, HOS, MG63, SJSA1, OST) displayed an invasive fraction between 1.76 and 0.02%, which proved sufficient for subsequent RNA analysis. Pair wise comparison yielded 161 differently expressed genes between invasive and non-invasive cells. These are involved in important pathways such as cell motility, cell communication or signal transduction.

Conclusions: The generated new candidate genes might play an important role in metastasis of OS. Their functional characterization has been started combining knock-down experiments (RNAi) with the invasion assay. Validation will be done by RT-PCR and immunohistochemistry on a larger sample using OS-TMAs. Determined genes and pathways will be correlated with clinical parameters like metastasis, survival and chemotherapy sensitivity in order to improve understanding of the biology of OS.

SETTING UP A TMA-BASED OSTEOCLAST EXPRESSION PROFILE STUDY IN GIANT CELL CONTAINING LESIONS

Gitte De Boeck

PhD-student, N. Goormaghtigh Institute of Pathology, Ghent University Hospital, Belgium

Recently, it was reported that osteoclasts in GCTB are CD33+ / CD14-, indicating their initial bone marrow origin. Further fusion with CD14+/RANK+ macrophages is suggested to become large osteoclasts as seen in this tumor, but also in chondroblastomas and Paget's disease (JBMR, Jan.09).

The aim of this study is to determine osteoclast membrane markers in different giant cell containing lesions in order to get a better sight on the mechanisms and origin (blood / marrow / neoplastic) of reactive and tumoural osteoclastogenesis.

Therefore, 24 giant cell containing lesions out of the archive of the Ghent University Pathology department were selected according to the intra-laesional presence of giant cells (n=165). From each case 2 regions of interest were selected.

Integrating activity was set up between Semmelweis University (SEM) and APD-UZG regarding tissues, TMA constructing and analyzing facilities. A PhD transfer from APD-UZG to SEM had been taken place for two weeks in order to fulfill all technical procedures together. Also used procedures of both institutes were compared with each other. After one week of work, results were discussed and new planning for the next week were made by the staff of both institutes.

In SEM the regions of interest were subjected to TMA-construction leading to the availability of giant cell containing lesion TMA slides for the EuroBoNeT-consortium. In SEM immunohistochemical procedures were updated to create an ideal (double chromogenic and fluorescent) signal intensity for scanning later on. After testing, immunohistochemistry and -fluorescency was performed against osteoclast membrane markers and subsequent digitalized (chromogeneous / fluorescent scanning) using the Mirax facilities. Analysis of the stained TMA's is ongoing, again using the facilities of SEM.

In summary, a study was set up using TMA-based technology. I learned techniques and procedures to create TMA's; we discussed and compared immunohistochemical and –fluorescent procedures in order to get the best results with our material on the scanner of SEM; special attention was paid to double immunohistochemistry. As result, TMA-slides of (rare) giant cell containing lesions are available for the whole consortium as result of exchange of knowledge and people, and finally as part of further integration activity.

STUDY OF THE INVOLVEMENT AND SIGNIFICANCE OF CLATHRIN-DEPENDENT/INDEPENDENT ENDOCYTOSIS ON IGF1R SIGNALLING PATHWAY IN ES

Ana Sofia Martins[1], Maria Debiec-Rychter[2], Frans Prins[3], Pancras Hogendoorn[3] and Enrique de Álava[1]

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Background: Receptor tyrosin kinases (RTK) are involved in countless intracellular signalling pathways related with proliferation, apoptosis, cell cycle, differentiation, among others, and that their deregulation is implicated in various diseases, including cancer. These signalling cascades have been extensively elucidated, nevertheless, there remain important gaps in knowledge with respect to RTK function and activation centered in receptor internalization and trafficking.
Lately it has been reported that IGF1R endocytosis is triggered by ligand binding which causes IGF-IR internalization via clathrin-coated vesicles [1] and that IGF1R internalization and recycling mediate the sustained phosphorylation of Akt [2]. Tirado et at. [3] showed that Cav1 is a necessary target of EWS/FLI1 for EWS tumourigenicity and that it is determinant for the oncogenic phenotype and tumourigenicity of Ewing's sarcoma cells. Recently, Baserga and colleagues [4] demonstrated as well a strict correlation between Cav1 and IRS-1 (insulin receptor substrate-1) protein levels, being the expression of IRS-1 down-regulated in mouse embryo fibroblasts (MEFs) with a deletion of cav1 genes (KO cells), suggesting that this mechanisms of RTK endocytosis may also be implicated in IGF1R signalling.
Based on these evidences, we started a study of clathrin/cav1 involvement in IGF1R signalling, in which we have studied IGF1R internalization and co-localization with clathrin/cav1 upon ligand binding in normal and interfered situations, as well as IGF1R pathway status in the same conditions and the cellular proliferation and apoptosis of the interfered/inhibited ES cells.

Methods and results: We observed that in the native conditions (unstimulated), p-IGF1R was in the membrane surface and that after IGF1 treatment it was, at least partially, being internalized, co-localizing with Cav1/clathrin. In the interfered/treated conditions, even after IGF1 treatment, p-IGF1R stayed in the membrane, being unable to be internalized, only signalling from its "membrane position". With WB/p-array studies we demonstrated that Cav1/Clathrin interference and especially the cholesterol depletion with CPMZ inhibited IGF1R signalling, inhibiting AKT and MAPK phosphorylation, reaching levels of more then 60% of reduction. We observed that Cav1/Clathrin interference markedly reduced ES cells proliferation, with IC50 around 10µM, being the results obtained with the apoptosis induction very analogous to this ones. We are also at the present moment characterizing the combination of usual IGF1R-TK inhibitors (AEW742) with the CPMZ and/or MBCD at the level of proliferation inhibition, apoptosis induction and pathway inhibition. Preliminary results indicate that this combination would result in a more efficient inhibition of IGF1R signalling pathway.

Conclusion: Based on these results we assume that by inhibiting IGF1R internalization, both by clathrin dependent or independent mechanisms, we are inhibiting part of IGF1R signalization only allowing it to signal through it's "membrane state" and not allowing it to signal through its "caveolae/CCV position" and that to achieve a better receptor inhibition we would have to consider to combine the usual RTK inhibitors with the endocytic inhibitors.

References:
[1]. Carelli S, et al. J Cell Physiol. 2006;208:354-62. *[2]. Romanelli RJ, et al. J Biol Chem. 2007;282:22513-24.*

[3]. Tirado OM, et al. Cancer Res 2006;66:9937-47. *[4] Chen J, et al. J Cell Physiol 2008;217:281-9.*

Abstracts technology platforms

BIOBANKING MODEL: SUCCESSIVE XENOGRAFTS OF SARCOMAS AND SARCOMAS CELL-LINES IN NUDE MICE: AN ANALYSIS OF 438 CASES

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Introduction: Biological and molecular studies of human sarcomas require the availability of fresh tumour tissue, cell lines or frozen tissue banks in order to offer access to an adequate material. However, such material is not always easily available, and a shortage of tissue is frequent.

Nude mice xenografts of human sarcomas is an alternative method for obtaining large amounts of tissue, over an indefinite period, in so far as the tumour can be transferred in vivo throughout generations, maintaining the histological and genetic particularities.

Since 1985 *(Supported by several FISS grants from the Spanish government, from the FIVO Cancer Center, Spain, and from EUROBONET),* we have produced a xenograft tissue bank of sarcomas on nude mice (Balb-c, nu nu) storing available tissue in vivo for several passages and frozen in tissue banks. A total of 502 human tumours have been kept in vivo throughout several generations (up to 40). At present 48 tumours are maintained in vivo by tissue transfer.

Material and Methods: Tumour tissue is transferred into the animal (two for each experience) within two hours of surgery, under sterile conditions (1 – 2 mm^3 of tumour tissue), being inoculated subcutaneously in the back of the animal. The tumour is followed until it reaches a size of 1 – 2 cm in diameter, and transferred to new nude mice for several generations. Material obtained from each transplant is kept for histology, cell culture, EM, and frozen.

Results: Table 1 compiles the cases transferred (438 cases) into nude mice and the number of positives taken. Special emphasis is given to Osteosarcoma, Chondrosarcoma and Es/PNET. Frozen tissue is available for almost all cases. 61 tumours with diagnosis of Es/PNET (48 solid tumours and 13 cell lines) were xenografted, 33.3% and 69.2% of which grew respectively, with a mean transfer time of 78.7 and 56.2 days. Regarding Osteosarcomas, 65 solid tumours and 4 cell lines were inoculated in mice, the growth rate was slightly higher in cell lines (50%) than original tumours with similar transfer time (100 days). Likewise, 27 Chondrosarcomas as well as 4 cell lines were xenografted. However, unlike Os, the percentage growth in cell lines was greater than in the solid tumours, with a mean growth time for cell lines of almost half that of the solid tumour.

Conclusion: Nude mice xenograft of sarcomas provides an alternative way to store large amounts of tumour tissue. When transferred into nude mice, the percentage and the growth rate of Es/PNET, Osteosarcomas and Chondrosarcoma cell lines is higher than the original neoplasm. Additionally, this methodology provides a number of advantages regarding the histology and biological analysis of the tumours, in as far as they reproduce the phenotype and maintain the genetic hallmarks of the original sarcoma.

Abstracts Research Line 1 talks

GENOMIC PROFILING OF CHONDROSARCOMA: IMPLICATIONS FOR THE DISTINCTION BETWEEN CENTRAL AND PERIPHERAL TUMOURS

Karolin H. Hallor[1], Johan Staaf[2], Judith V.M.G. Bovée[3], Pancras C.W. Hogendoorn[3], Anne-Marie Cleton-Jansen[3], Sakari Knuutila[4], Suvi Savola[4], Otte Brosjö[5], Henrik C.F. Bauer[5], Fredrik Vult von Steyern[6], Kjell Jonsson[7], Mikael Skorpil[8], Nils Mandahl[1], Fredrik Mertens[1]

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Background: Histological grade is currently the best predictor of clinical course in chondrosarcoma patients. Grading suffers, however, from extensive interobserver variability and new objective markers are needed. Hence, we have investigated DNA copy numbers in chondrosarcomas with the purpose of identifying markers useful for prognosis and sub classification.

Methods: The overall pattern of genomic imbalances was assessed in a series of 67 chondrosarcomas using array comparative genomic hybridization. Statistical analyses were applied to evaluate the significance of alterations detected in subgroups based on clinical data, morphology, grade, tumour size and karyotypic features. Also, the global gene expression profiles were obtained in a subset of the tumours.

Results: Genomic imbalances, in most tumours affecting large regions of the genome, were found in 90% of the cases. Several apparently distinctive aberrations affecting conventional central and peripheral tumours, respectively, were identified. Although rare, recurrent amplifications were found at 8q24.21-q24.22 and 11q22.1-q22.3, and homozygous deletions of loci previously implicated in chondrosarcoma development affected the *CDKN2A*, *EXT1* and *EXT2* genes. The chromosomal imbalances in two distinct groups of predominantly nearhaploid and near-triploid tumours, respectively, support the notion that polyploidization of an initially hyperhaploid/hypodiploid cell population is a common mechanism of chondrosarcoma progression. Increasing patient age as well as tumour grade were associated with adverse outcome, but no copy number imbalance affected metastasis development or tumour-associated death.

Conclusion: Despite similarities in the overall genomic patterns, the present findings suggest that some regions are specifically altered in conventional central and peripheral tumours, respectively.

IDENTIFYING CANDIDATE GENES FOR OLLIER DISEASE USING SNP ARRAY

Twinkal Pansuriya[1], Jan Oosting[1], Karoly Szuhai[1], Luca Sangiorgi[2], Raf Sciot[3], Pancras Hogendoorn[1] and Judith Bovee[1]

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Background: Ollier disease is a rare skeletal disorder. It is characterized by the occurrence of multiple enchondromas with a marked unilateral predominance mainly affecting medulla of the metaphyses and diaphyses of the short and long tubular bones of the limbs, especially the hands and feet. The risk of malignant transformation is increased up to 35%. We hypothesize that Ollier disease is a mosaic condition as it is polyostotic. Here we aimed to identify molecular defects in Ollier disease related enchondromas and chondrosarcomas using SNP array approach.

Methods: Affymetrix SNP 6.0 was performed on 48 samples which include 10 blood samples and 3 matched blood-saliva samples as a control; 7 tumour samples from 7 Ollier patients and 14 matched tumour-normal samples for paired comparison from 11 Ollier patients. In total, 7 enchondromas and 14 chondrosarcomas from 16 Ollier patients were used.

Results: All samples were divided into three groups: normal, enchondroma and chondrosarcoma. The number of numerical genomic changes in the chromosomes were not different for the enchondromas ($p<0.27$) while large genomic aberrations were seen in chondrosarcomas as compared to normal samples ($p<0.01$). Copy number variation (CNV) analysis showed a 4K deletion at 2q14, a 1M deletion at 9p11 and a 20K deletion at 14q11 in 4 out of 7 enchondromas containing several genes. A deletion at 12p11 which does not contain any known genes was found in 5 out of 8 normal samples from patients. Paired loss of heterozygosity (LOH) analysis failed to show LOH in enchondromas. LOH was observed at 5p, 6q, 7q and 9p in high grade chondrosarcomas associated with loss of chromosomes.

Conclusion: Results of CNV analysis showed deletion at 2q14, 9p11 and 14q11 in enchondromas. We were unable to detect LOH in enchondromas at 1Mb resolution containing approximately 500 SNP probes. High grade chondrosarcomas showed LOH at different chromosomes. In future, we will study LOH and CNV changes at gene level. Allele specific copy number changes and unpaired LOH analysis will be done on larger series.

KINOME PROFILING OF CHONDROSARCOMA REVEALS SRC-PATHWAY ACTIVITY AND DASATINIB AS OPTION FOR TREATMENT

Yvonne M. Schrage[1], Inge H. Briaire-de Bruijn[1], Noel F.C.C. de Miranda[1], Antonie H.M. Taminiau[2], Tom van Wezel[1], Pancras C.W. Hogendoorn[1], Judith V.M.G.Bovée[1].

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Background: Chondrosarcomas are notorious for their resistance to conventional chemo- and radiotherapy, indicating there are no curative treatment possibilities for patients with inoperable or metastatic disease. We therefore explored the existence of molecular targets for medical therapy of chondrosarcoma using kinome profiling.

Method: Peptide array was performed for 4 chondrosarcoma cell lines and 9 primary chondrosarcoma cultures. Activity of kinases was verified using western blot and active Src- and PDGFR signalling were further explored *in vitro* using imatinib and dasatinib on chondrosarcoma.

Results: The AKT1/GSK3β pathway was clearly active in chondrosarcoma. In addition, the PDGFR pathway and the Src kinase family were active. PDGFR and Src kinases can be inhibited by imatinib and dasatinib respectively. While imatinib did not show any effect on chondrosarcoma cell cultures, dasatinib showed a decrease in cell viability at nanomolar concentrations in 3 out of 5 chondrosarcoma cultures. Whereas inhibition of phosphorylated Src (Y419) was found both in responsive and non-responsive cells, caspase-3 related apoptosis was found only in cell line GIST882, suggesting that the mechanism of decreased cell viability in chondrosarcoma by dasatinib is caspase-3 independent.

Conclusion: Using kinome profiling we found the Src pathway to be active in chondrosarcoma. Moreover, we showed *in vitro*, that the inhibitor of the Src pathway, dasatinib, may provide a potential therapeutic benefit for chondrosarcoma patients which are not eligible for surgery.

PRIMARY CILIA ORGANIZATION IN GROWTH PLATE AND OSTEOCHONDROMA

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Background: Primary cilia (PC) are microtubule-based organelles projecting from the mother centriole into the extracellular matrix. They function as sensory units coordinating a variety of signal transduction pathways: including Hedgehog, Wnt, and platelet-derived growth factor. The transduction of these pathways as well as the assembly and maintenance of PC, are mediated by several motor proteins responsible for the intraflagellar transport. Altered cilia formations have been related to defects in endochondral bone formation and, consequently, in the growth plate (GP). GP is a cartilaginous template of the long bones, divided in three zones with distinct patterns of proliferation, differentiation and cell morphology. Chondrocytes are aligned in parallel columns, with less differentiated cells at one end (resting zone) and terminally differentiated chondrocytes at the other end (hypertrophic zone). Many GP disorders are caused by failure in the mechanisms regulating this organization. Osteochondromas (OC) are the most common benign bone tumours, arising at the external surface of bones preformed by endochondral ossification and consisting of a bony stalk covered by a cartilage cap. Histologically, OC resemble GP, with cells resembling chondrocytes from different zones of GP and focally arranged in parallel columns. We hypothesized that PC might play an important role in this cellular organization and evaluated PC organization in GP and OC.

Methods: Primary cilia and the intraflagellar transport were detected using antibodies to acetylated alpha-tubulin and Kif3A, respectively. Thick GP and OC sections were imaged at high magnification using confocal laser scanning microscope. The images analyses, including the orientation, the length of PC and the sub-cellular co localization of motor and skeletal proteins, were performed using ImageJ software (NIH Image, Bethesda, MD, http://rsb.info.nih.gov/ij/).

Results: PC were present in all the three zones of the GP and also in OC. They were more often observed in the morphologically less differentiated cells. The lengths of PC were variable and the intraflagellar transport protein, Kif3A, was detected in both shorter and longer cilia. In GP, PC were preferentially found in the apical or basal region of the chondrocyte, at the centre of the columnar cells forming a line along the longitudinal axis of the bone. In OC, PC were randomly located in the apical, basal or lateral region of the neoplastic cells, pointing to different directions or far from each other; however, when columnar organization was present, PC were aligned along a median axis parallel to the growing axis of the OC.

Conclusions: In GP, PC show often polarization while in OC, they assume mainly a random distribution. PC are likely to play an important role in the columnar formation which might be disrupted in OC.

CHARACTERIZATION OF THE EXOSTOSIN-1 (EXT1) PROMOTER

Ivy Jennes[1], Monia Zuntini[2], Luca Sangiorgi[2] and Wim Wuyts[1]

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Introduction: Multiple osteochondromas (MO) is an autosomal dominant skeletal disorder characterized by the formation of multiple cartilage-capped protuberances. MO is genetically heterogeneous and is associated with a mutation in the *EXT1* or *EXT2* tumour suppressor genes. Both genes are ubiquitously expressed and encode proteins that function as glycosyltransferases in the biosynthesis of heparan sulphate. At present, very little is known about the transcriptional regulation of the *EXT* genes.
To elucidate transcriptional regulation of *EXT1*, we isolated and characterized the *EXT1* promoter.

Methods, results and conclusion: Theoretical analysis of the 10 kb upstream of the *EXT1* start codon was performed with promoter prediction programs TSSG, TSSW, FPROM, BDGP, Promoter 2.0 Prediction Server and Web Promoter Scan. These programs showed presence of a CpG island containing CG and CAAT boxes but no TATA box, which is characteristic for a housekeeping gene. Two potential functional promoter regions were identified, located respectively ~2.650 bp and ~900 bp upstream of the start codon.
To confirm the correct promoter region experimentally, overlapping PCR fragments of the 10 kb putative EXT1 promoter region were generated and cloned in the pGL4.72 Luciferase Reporter Vector. Promoter activity was subsequently determined by luciferase assays after transfection in Human Embryonic Kidney (HEK) cells. Our luciferase assay located the actual core promoter within the 560 bp fragment containing the predicted promoter sequence ~900 bp upstream of the start codon.
Further fine mapping located the minimal core promoter within in a fragment of 350 kb.
The identified promoter region was additionally analyzed for protein binding capacities with transcription binding prediction programs AliBaBa2, Cister, TFsearch, TFSiteScan and TESS. This analysis revealed various putative transcription factor binding sites.
Finally, the 560 bp fragment containing the core promoter was analysed for the presence of SNP’s that might affect its promoter activity. Two polymorphic G/C SNP’s were identified. Subsequently, we designed new luciferase assays to test whether these two SNP’s might indeed influence the EXT1 promoter activity. The assay revealed that the promoter activity was lower in CC genotypes compared to GG genotypes. Moreover, for both SNP locations, the presence of a cytosine destroys the binding site of a transcription factor predicted by the transcription binding prediction programs. Consequently, we identified both SNP’s as primary modifiers that might explain part of the clinical variation observed in MO patients. To test this hypothesis, both SNP’s are currently being characterised in a large set of MO patients.

IMMUNOPHENOTYPIC PROFILING OF FLOW CYTOMETRY DATA VIA CLUSTER ANALYSIS: APPLICATION TO THE STUDY OF CHONDROSARCOMAS

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Background: Chondrosarcoma classification relies upon a combination of tissue morphology and clinical features. Analysis of cell membrane proteins may help to better define disease subtypes or uncover clinically and/or therapeutically relevant biomarkers. In this study we have combined flow cytometry with cluster analysis to compare the immunophenotypic profile of chondrosarcoma cells from primary tumours (CS), chondrosarcoma cell lines (CCL), bone-marrow derived mesenchymal stem cells (MSC), normal articular chondrocytes (HAC), and primary fibroblasts (FIB).

Methods: Human CS, CCL (OUMS27, SW1353, and CH2879), MSC, HAC and FIB were cultured in monolayer until passage 3 in medium containing 10% FCS, 5 ng/ml FGF-2, and 1 ng/ml TGF-β1, a combination of growth factors stimulating proliferation of mesenchymal cell types. After culture, cells were analyzed by flow cytometry for the expression of 20 surface markers chosen on the basis of our previously published results. For comparison purposes, 3 carcinoma cell lines (Caski, HELA, and SW48) were also included in the study. FACS data were expressed as mean fluorescence intensity (MFI) ratio between MFI of a given marker over the respective isotype control, and clustered with wCLUTO, a web-enabled data clustering application designed for the clustering and data-analysis requirements of gene-expression datasets (http://cluto.ccgb.umn.edu/cgi-bin/wCluto/wCluto.cgi).

Results: Selection of a panel comprising 9 surface markers allowed separation of HAC, MSC, and FIB into three well differentiated clusters. CS clustered mainly with either MSC or HAC, with the exception of L2338, a grade II CS, which cluster together with the tumour cell lines. CD14, a co-receptor for LPS generally considered as a marker for monocytes/ macrophages, was selectively expressed on HAC, and CCL CH2879 was the only other analyzed cell type showing significant expression of CD14. The three CCL analyzed clustered together with the carcinoma cell lines, and showed decreased or lost expression of the mesenchymal marker CD90 (Thy-1), aberrant expression of β-4 integrin, normally associated with cells of epithelial origin, and increased expression of CD221, the receptor for insulin-like growth factors IGF-I and IGF-II, which was especially prominent in the case of CH2879.

Conclusion: Flow cytometry combined with cluster analysis provides a tool to identify optimal combinations of surface markers allowing differentiation between closely related cell lineages. Preliminary data with primary chondrosarcoma cells suggest the possibility of using the present approach to discriminate between different chondrosarcoma types based on their degree of proximity to MSC or HAC clusters. The gained knowledge is relevant for further understanding of these neoplasms.

Abstracts Research Line 1 posters

BALANCED RE-ARRANGEMENT OF CHROMOSOME 5 AND 17 POINTS TO A ROLE FOR CA10 AND SEX STEROIDS HORMONES IN CHONDROBLASTOMA PATHOGENESIS

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Background: Chondroblastoma is a benign cartilaginous tumour of bone predominantly affecting the epiphysis of young males. Conventional cytogenetics has shown no recurrent chromosomal re-arrangements so far. We have identified an index-case with a balanced translocation and further investigated the involvement of candidate regions/genes in a cohort of chondroblastomas.

Design: COBRA-FISH karyotyping followed by FISH mapping of the breakpoints using BAC/fosmid clones were performed on the index case. Tumour DNA was isolated and hybridized on BAC array-CGH. Candidate regions were subsequently investigated in other 14 chondroblastomas by breakpoint specific FISH probes. Expression of candidate genes was verified by immunohistochemistry in 14 extra cases.

Result: A translocation t(5,17)(p15,q22-23) was found. By array-CGH no additional copy number changes were observed in this index-case confirming the balanced nature of the observed translocation. By using breakpoint specific interphase FISH, re-arrangement was found in mononucleated cells in the index case and not in multinucleated giant cells thereby present. Re-arrangement was not observed in other 14 chondroblastomas. The breakpoint in 5p15 was proximal to steroid reductase 5 α1 (*SRD5A1*) gene region. Diffuse expression of the protein, as identified by immunohistochemistry, was found both in the index case as well as in all the chondroblastomas tested. Similar pattern of expression was found also for the other sex-steroid signalling-related molecules: estrogen receptor alpha, aromatase and androgen receptor. The breakpoint in 17q22-23 was proximal to the carbonic anhydrase X (*CA10*) gene region with a possible involvement of gene transcription regulatory element. Down-regulation of the CA10 protein, as identified by immunohistochemistry, was found in the index-case only.

Conclusion: We report a novel t(5,17)(p15,q22-23) in chondroblastoma without alterations of any of the two chromosome regions in other cases. Diminished expression of the CA10 protein – a member of the carbonic anhydrase family possibly involved in calcification and bone resorption was observed in the index case. Impairment of this gene is hypothesized to be relevant for chondroblastoma pathogenesis. Diffuse expression of SRD5A1 and sex-steroid-signalling related molecules confirms the role of this pathway in cartilaginous tumours. Multinucleated giant cells in chondroblastoma are most likely reactive.

BALANCED AND UNBALANCED REARRANGEMENT OF CHROMOSOME ARM 6q IN CHONDROMYXOID FIBROMA (CMF): DELINEATION OF BREAKPOINTS AND ANALYSIS OF CANDIDATE TARGET GENES

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Background: CMF is a benign cartilaginous tumour of bone mainly occurring in the second decade of life in long bones. Recurrent rearrangements of chromosomal bands 6p25, 6p23-25, 6q12-15 or 6q23-27 are reported. We aimed to refine location and functional consequences of these rearrangements.

Design: Structural chromosomal aberrations were studied in 7 cases, including 5 not previously reported, by CoBRA-FISH and conventional G-banding. Breakpoints FISH mapping was performed in case L1788. Array CGH was performed on 15 cases to identify additional copy number changes. On the basis of the results 14 cases were further evaluated by interphase FISH. The expression level of candidate genes close to the breakpoints was studied by immunohistochemistry and Q-PCR in 25 and 15 cases, respectively.

Result: The karyotypes in the 5 new cases were:
46,XX,t(6;17)(q23;p13) (L1788),
46,XY,der(6)(6qter->6q2?::6p2?->6q13::6q2?->6q2?::6q13->6qter),del(6)(q13),der(22)t(6;22)(q24;p11) (L2367),
46,XY,del(6)(q2?1),-13,der(?)t(?;1)(?;q12) (L2499),
46,XY,del(6)(q15q23),del(6)(q1?3q2?5),add(11)(q2?5) (L2514),
46,XY,der(6)t(6;10)(p25;p11)t(6;11)(q24;p12),del(6)(q12),der(10)t(6;10)(q12;p11),der(11)t(6;11)(q24;p12) (L2515).
The other two showed:
46,XY,del(6)(q15),der(6)t(6;6)(q15;q27)inv(6)(p25q13) (L1787);
46,XX,del(6)(?q21?q23),add(7)(q21) (L1789).
No copy number changes were found in 12 cases, including L1788, L2367, L1787 and L2515. A small overlapping deletion on 6q24, in the region of UTRN, was found in 4 cases, including L1789 and L2499. FISH mapping showed possible involvement of BCLAF1 on 6q23. No significant expression impairment of these two tumour suppressor genes was found in the evaluated series.

Conclusion: The present study confirmed the non-random involvement of chromosome arm 6q in CMF. Both balanced and unbalanced recurrent rearrangements were detected and candidate genes were studied. These data indicate that multiple genes in chromosome 6 are involved in the pathogenesis of CMF.

ZEBRAFISH MODEL FOR STUDIES ON THE SKELETAL DEFECTS IN MULTIPLE OSTEOCHONDROMAS

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Background: Multiple Osteochondromas (MO) previously known as Hereditary Multiple Exostosis (HME) is caused by a mutation in *Exostosins (EXT) genes, EXT1* or *EXT2*. MO is characterized by the formation of multiple cartilaginous bone outgrowths near growth plates. However, it seems that MO phenotype is not restricted to osteochondromas. In order to find more information about MO, we use zebrafish mutant called *dackel (dak)* that is mutated in a homologue of the *EXT2* gene. *dak/ext2* mutant is the only available vertebrate that completes embryo development when homozygous for mutation in an *ext* and displays various phenotypical changes e.g. similar chondrocyte organization as this in osteochondromas. We have recently shown that mutation in *dak/ext2* does not affect cartilage differentiation but it disrupts cartilage morphogenesis and bone development (Clement *et al*., 2008). As not much is know about the dental and bone condition in MO, we examined skeletal changes in homozygote larvae lacking functional *dak/ext2* gene and in juvenile and adult skeletons heterozygous for mutation in dak/ext2(+/-).

Methods: Zebrafish *dak/ext2* mutant and its siblings were fixed in 4% PFA at different time points. 6-day-old larvae were used for *in situ* detection of *osterix* and *collangen X* mRNA transcripts. Juvenile and adult *dak/ext2(+/-)* mutant and its siblings were subjected to TRAP, Alizarin red and Alcian blue staining. Skeletons were analyzed under dissecting-scope. Each group had minimum 10 fish. Experiments were repeated two times with similar results.

Result: In 20% of *dak/ext2* (+/-) mutant deformities similar to those that are well recognized in MO patients i.e. osteochondromas-like outgrowths, bone curving and bone shortening/thickening were found. Similar deformities were also observed in dermal bones in zebrafish *dak/ext2* (+/-) mutant. TRAP staining indicated altered osteoclast function in *dak/ext2* mutants severe tooth defects were observed in 100% of *dak/ext2 (-/-)* larvae. At 6 days post fertilisation (pdf) 100% of homozygote *dak/ext2* mutants had one and malformed tooth instead of three teeth on each 5th pharyngeal arch. Deformities such as spitted crowns and enamel lesions were also found in 20% of heterozygote *dak/ext2* adults.

Conclusion: Our findings from zebrafish model indicate that MO patients can have general dental and skeletal problems. Deformities of endochondral bones are more likely to result from malformations of cartilage templates whereas the presence of defects in intra-membranous bones and teeth are most likely due to dysfunction in skeleton remodelling.

INDUCTION OF CHONDROGENESIS IN HUMAN MESENCHYMAL STEM CELLS OF MULTIPLE OSTEOCHONDROMAS PATIENTS

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Background: Patients with Multiple Osteochondromas (MO) have a variable number of benign cartilage capped bony outgrowths located at the surface of long bones. Germline mutations in the tumour suppressor genes, EXT1 or EXT2, are found in almost 90% of the MO patients. We previously demonstrated for EXT1 that both copies of the gene are inactivated in the cartilaginous cap. However the exact role of the EXT proteins in the formation of normal cartilage and the consequences of EXT inactivation in this process are not yet known. The EXT1/2 complex elongates the heparan sulphate chains onto the proteoglycan core proteins in the Golgi lumen. In this study we investigated if human mesenchymal stem cells (hMSCs) derived from bone marrow of MO patients (one inactivated copy of the EXT gene) have chondrogenic capacities. In addition, the cells, derived from the cartilage cap (two inactivated copies of the EXT gene), were cultured in the same 3D-pellet *in vitro* model.

Methods: Bone marrow cells of an MO patient were harvested during the removal of an osteochondroma after informed consent. Subsequently, the MSCs and cells of the cartilage cap of the same patient were expanded in a monolayer and subsequently collected for differentiation. A 3D-pellet model was used to induce chondrogenesis. Pellets were harvested at 2, 4 and 6 weeks, followed by analysis.

Results: The bone marrow hMSCs derived from MO patients showed a large increase in the production of sulphated glycosaminoglycans (GAGs) during time. The cells originating from the cartilage cap were producing a lower amount of sulphated GAGs during the 6- weeks-period.

Conclusion: hMSCs derived from the bone marrow of an MO patient have the capacity to induce chondrogenesis. This 3D-pellet model is excellent to study the downstream pathways of the EXT genes in order to unravel the function of EXT in normal chondrogenesis and to better understand osteochondroma formation.

NO ROLE FOR CELECOXIB TREATMENT OF CHONDROSARCOMA

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Background: In both Enchondromatosis (EC) and Multiple Osteochondromas (MO), multiple benign cartilaginous tumours occur, which have an increased risk of malignant progression. Preventing new tumour formation and malignant progression would benefit the prognosis of these patients. A protective effect of selective Cox-2 inhibitor, celecoxib, has been suggested against development and growth of colorectal cancer in familial syndromes.

Methods: We studied Cox-2 protein expression by immunohistochemistry (IHC) in a group of 37 central and 39 peripheral cartilaginous tumours, including 12 EC and 19 MO related cases. mRNA expression was measured by quantitative RT-PCR in 34 independent samples, including 16 EC cases. Four chondrosarcoma cell lines (OUMS27, CH2879, SW1353 and CS3248 (EC derived)), were treated with celecoxib. Cox-2 activity was measured by prostaglandin E_2 (PGE_2) ELISA and a proliferation assay was performed to evaluate cell survival *in vitro*. The (prophylactic) effect of celecoxib on chondrosarcoma growth was assessed *in vivo* using a xenograft model of immunoincompetent nude mice which were injected with cell line CH2879 subcutaneously. Tumour volume was measured during 8 weeks. Celecoxib serum levels were determined by HPLC. Expression of proliferation marker Ki67 and cox-2 was assessed by IHC.

Results: Ten of 27 (37%) central chondrosarcomas were Cox-2 positive, of which 6 were related to EC. 18 out of 39 peripheral chondrosarcomas were Cox-2 positive, 4 related to MO. mRNA levels of EC related tumours were slightly higher than the solitary tumours. 72 hours of celecoxib treatment of the chondrosarcoma cell lines, which all expressed Cox-2, resulted in a 3 fold decrease of PGE_2 levels already at 5 µM. A significant decrease in proliferation was found at 10 µM in OUMS27 and at 25 µM in SW1353 and CH2879 compared to DMSO controls. Our *in vivo* results also showed a beneficial effect of prophylactic celecoxib treatment. Tumour volumes were negatively correlated with celecoxib serum levels (r^2=0.152). However, at the end of pubertal growth of the mice, a catch-up tumour growth was observed, resulting in the absence of differences in tumour volume between control and treatment groups. Accordingly, proliferation marker Ki67 was higher expressed in the treated groups at sacrifice.

Conclusion: In conclusion, despite an effect on tumour cell proliferation *in vitro*, our *in vivo* data suggest that there is no role for celecoxib in the treatment of adult chondrosarcoma patients. Celecoxib treatment of pre-pubertal patients, especially to prevent formation of new tumours in EC and OC patients, might be beneficial and should be further investigated.

MMP AND TIMP EXPRESSION IN CONVENTIONAL CHONDROSARCOMA

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Background: Disassembly of the extracellular matrix plays an important part in the invasive growth of chondrosarcoma. As matrix metalloproteinases (MMPs) are capable of degrading extracellular matrix proteins under physiological conditions they could be a central element of chondrosarcoma invasiveness. Therefore quantitative analysis of MMP expression as well as their specific inhibitors, the tissue inhibitors of metalloproteinases (TIMPs), could help elucidate the molecular background of chondrosarcoma invasion.

Methods: Quantitative real-time PCR assays for nine MMPs as wells as four TIMPs were established. mRNA expression of eleven conventional chondrosarcoma (n=4 grade I, n=5 grade II, n=4 grade III) and three normal cartilage specimens was measured. Obtained results were evaluated correlating expression level to invasiveness and compared to data from other partners.

Results: Significantly higher mRNA expression in chondrosarcoma in comparison to normal cartilage could be observed for MMP9 ($p=0.04$), MMP13 ($p<0.001$), MMP14 ($p=0.02$), and MMP15 (0.04). Elevated expression could also be observed for MMP1, while MMP3 demonstrates decreased expression in the tumours. TIMP2 as wells as TIMP4 both show decreased expression in the majority of analysed chondrosarcoma. Significant in- or decrease with tumor grade could not be noted. Regarding level of expression we found a generally high expression (>10% of GAPDH) of MMP3, MMP13, MMP14, as well as TIMP1 and TIMP3, a moderate expression of MMP1, MMP2, MMP9, TIMP2 and TIMP4 and only a low expression (<1% of GAPDH) of MMP8 and MMP15.

Conclusion: Although we can find significant differences in expression of several MMPs comparing normal and chondrosarcoma specimens, so far we cannot find any correlation of expression level and tumour grade/invasiveness. With uniform grading still ongoing, final results might give better conclusions. Also current results don’t resemble data obtained from microarray analysis performed by other partners. However comparison of data is also dependent on consistent grading and therefore still ongoing as well.

SEX-STEROID SIGNALLING AS A TARGET FOR CHONDROSARCOMA TREATMENT

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Background: Chondrosarcomas are malignant cartilage-forming tumours which are highly resistant to conventional chemotherapy and radiotherapy. The only effective treatment is often-mutilating surgery. For irresectable or metastatic tumours no curative options are available at all. Therefore, it is important to identify new treatment strategies. One of the potential targets for therapy is the sex steroid-signalling pathway. Estrogen signalling is known to play an important role in proliferation and differentiation of chondrocytes. In addition, estrogen and the steroid precursor androstenedione have been shown to enhance proliferation of chondrosarcoma cells *in vitro*. Our experiments focus on unravelling the role of sex steroids in the regulation of neoplastic cartilage growth and on inhibiting progression of chondrosarcomas *in vitro* and *in vivo* using known anti-steroidal drugs.

Methods: Using Illumina HumanWG-6 v3.0 expression arrays and quantitative RT-PCR we measured the mRNA levels of the estrogen receptors alpha (ESR1) and beta (ESR2), androgen receptor (AR), and the aromatase gene CYP19A1 in tumour specimens and (primary) chondrosarcoma cultures respectively. Furthermore, chondrosarcoma cells were cultured in the presence of a range of doses of 17β-estradiol, androstenedione or dihydroxytestosterone. After several days, dose-dependent growth stimulation was determined by cell counting and/or WST-1 cell viability assay. To enable accurate monitoring of chondrosarcomas *in vivo*, chondrosarcoma cell lines stably expressing various reporter constructs were generated using lentiviral infection. Functionality of the constructs was verified using luciferase assays and fluorescence activated cell sorting.

Results: We showed mRNA expression of ESR1, AR and CYP19A1, but no expression of ESR2 was detected. In addition, the dose-response assays with various sex steroids showed no effect on cell proliferation *in vitro*. We have generated chondrosarcoma cell lines with various functional reporter constructs and the cells will be characterized further to select the most potent line for *in vivo* studies.

Conclusion: The presence of ESR1, AR and CYP19A1 in chondrosarcoma tumours and primary cultures supports a possible role of sex steroids in the regulation of chondrosarcoma proliferation. However, our *in vitro* studies have shown no effect of steroids on the proliferation of chondrosarcoma cell lines so far. Additional studies *in vitro* and *in vivo* are necessary to further investigate the role of sex steroids in chondrosarcoma development and the therapeutic potential of anti-steroidal drugs for cartilaginous tumours.
Future perspectives: Immunohistochemistry will be performed on approximately 100 chondrosarcomas to measure protein levels of ESR1, ESR2, AR and aromatase. Furthermore, dose-response curves will be generated of primary chondrosarcoma cells cultured in the presence of various anti-steroidal drugs. In addition, chondrosarcoma cell lines containing reporter constructs will be injected into the tibia of mice to generate a model for *in vivo* monitoring of chondrosarcoma behaviour in the presence of steroids and anti-steroidal drugs.

MOLECULAR HETEROGENEITY IN TISSUE FROM SECONDARY PERIPHERAL CHONDROSARCOMA

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Background: Osteochondroma is the most common of the benign tumour of the bone. It could present as a single lesion called solitary osteochondroma (SO), whereas it occurs as multiple lesions (MO) in multiple osteochondroma. Malignant transformation of osteochondroma in secondary peripheral chondrosarcoma (CSP) happens in 1-5% of patients. The aim of this study was to evaluate the molecular mechanism involved in the formation of osteochondromas and their malignant progression, considering also a possible molecular analogy between solitary and multiple lesions.

Methods: We investigated 44 tissue sample, including 24 osteochondromas (19 MO and 5 SO) and 19 peripheral chondrosarcomas (13 derived from MO and 6 from SO) with 2 recurrences, for the presence of mutations in either EXT1 (8q24) or EXT2 (11p11-12) genes, using DHPLC analysis, subsequent direct sequencing, MLPA analysis and RealTime PCR. Except for SO samples, the analysis was performed also on corresponding constitutional blood sample. When a sufficient quantity of tissue was available we analyzed different areas of the same resections in order to evaluate the genetic homogeneity of the sample.

Results: 2 out of 5 SO samples showed the presence of a mutation in EXT1/EXT2. The heterozygous germ line mutation was found at homozygous status in 1 MO tissue, 1 CSP derived from MO and 2 recurrences from a primary CSP; in 2 CS samples the heterozygous mutation detected in the primary tumour was found at homozygous status in the recurrences. In 3 CSP the mutation detected in HE status in lymphocytes and MO related tissue was found both in HO and in HE in different areas of the same tumour specimen. 1 CSP samples presented 3 different genetic profile in different areas.

Discussion: The presence of EXT1/2 mutations in SO tissue samples reveals molecular analogy between solitary and multiple lesions, whereas the absence of a second hit mutation in MO samples (except than for 1 case) suggests that MO growth may not necessarily require two genetic alterations in EXT1/2 genes. Taken together, results obtained from peripheral chondrosarcomas analyses suggest that a progressive variation of EXT1/2 genes from wild-type genetic status is involved in the malignant transformation of osteochondromas.

ENHANCED SYNDECAN 3 AND HIF1α EXPRESSION IN HIGH-GRADE CENTRAL CHONDROSARCOMA

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Background. The molecular processes responsible for the regulation and control of the development and progression of central chondrosarcoma are so far poorly understood. Pathways involved in the regulation of normal growth plate are abnormally regulated in these tumours, but other mechanisms may be relevant for their development and progression as well. The aim of this project was to identify genes involved in the development and progression of central chondrosarcoma through the comparison of gene expression of chondrosarcoma and normal hyaline cartilage and through the correlation of expression profiles to histological grade.

Methods. The mRNA of 19 chondrosarcomas with different histological grades and of 8 normal cartilage samples was analysed. Gene expression profiles were assessed on a customised cDNA array including 230 genes with a focus on cartilage- and stem cell-relevant genes. After normalisation the data were analysed using the Significance Analysis of Microarrays software. Results were confirmed by real-time RT-PCR and further, central and peripheral tumour samples were analyzed by immunohistochemistry for the expression of syndecan 3, galectin 1, HIF1α and carbonic anhydrase IX.

Results. Thirteen genes higher expressed in neoplastic compared to normal cartilage were identified. Among others these included genes involved in the regulation of normal growth plate such as the receptor PTHR1 and the heparin sulphate proteoglycan syndecan 3. For syndecan 3, immunohistochemical analysis revealed significant higher expression in high-grade central chondrosarcoma. Among the genes differentially expressed between tumours of different grade, the hypoxia-induced molecule galectin 1 was found. Expression of the protein galectin 1 was detected only in a low amount of tumour cells, but immunohistochemical analysis of markers for hypoxia revealed significantly higher nuclear expression of HIF1α in high-grade central chondrosarcoma.

Conclusion. Syndecan 3 is a modulator of proliferation and differentiation in normal growth plate and its expression pattern indicates that it may be involved in the development and progression of central chondrosarcoma. Its expression may in particular be implicated in the maintenance of high-grade chondrosarcoma cells in a dedifferentiated state. Our results further suggest that high-grade central chondrosarcoma display a hypoxic phenotype which may have played a role in malignant progression of the tumours.

EXPRESSION PROFILING OF BONE MORPHOGENIC PROTEINS AND THEIR RECEPTORS IN CENTRAL CHONDROSARCOMA

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Background: The temporal and local coordination of the expression of bone morphogenic proteins (BMP) and their receptors determine numerous processes in the homeostasis of the normal growth plate. Parallels between the development of central chondrosarcoma and differentiation stages of chondrocytes in the growth plate suggest that BMPs may be involved in the development of these tumours. The aim of this project was to perform a systematic quantitative study of the expression of BMPs and BMP receptors in central chondrosarcoma of different histological grades.

Methods: Fourteen chondrosarcoma samples including 5 grade I, 6 grade II and 3 grade III were included in this study. mRNA was extracted and cDNA synthesised. Quantitative RT-PCR analysis was performed for the genes BMP2, 4, 6, 7, TGFB1, TGFB3, BMPR1A, BMPR1B, BMPR2, ACVR1, ACVRL1, TGFBR1 and TGFBR2. The identity of PCR products was confirmed by sequencing. The expression of the housekeeping genes SRPR, CPSF6, CAPNS1 and HNRPH1 was used for normalisation.

Results: Expression of the BMPs and BMP receptors was found in almost all tumour samples, however at very low levels for all genes. Highest expression levels were detected for the genes for transforming growth factor beta 1 (TGFB1) and for the receptor ACVRL1. Especially in high-grade chondrosarcoma expression levels were very heterogeneous and no significant differences between low-grade and high-grade tumours were found for any of these genes. TGFB1 was slightly higher expressed in low-grade chondrosarcoma while for TGFB3, BMP2 and the receptors ACVR1, ACVRL1 and TGFBR1, a trend for higher expression in high-grade tumours was found.

Conclusion: BMPs and BMP receptors are expressed by chondrosarcoma cells but no evidence for a transcriptional regulation during progression was found. A determination of protein expression levels does not appear promising. In opposition to other regulators of the growth plate, and especially to the PTHrP signalling pathway, BMPs, thus, do not appear to be major players in the progression of central chondrosarcoma.

IDENTIFICATION AND STRUCTURAL ANALYSIS OF GAG IN CARTILAGINOUS TISSUES

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Background: EXT1/EXT2 genes encode for two Golgi transmembrane enzymes which specific activity is the polymerisation of the Heparan Sulphate side chains of extracellular matrix proteoglycans and their post-translational modifications play an essential role in all regulation systems that control endochondral ossification. Moreover, heparan sulphate side chains are key players in the organisation of morphogens (IHH and PTHrP) gradients and growth factors signalling throughout cartilage anlagen. Unfortunately we currently lack a comprehensive structural and molecular analysis of glycosaminoglycans side chains in MO patients.
Aim of the study is to fulfil the lack of this information by a refined structural analysis of GAGs, and to compare the glycosaminoglycans structure of samples collected from MO affected individuals to reference samples (healthy articular cartilage) and to growth plate cartilage.

Methods and results: We are proposing NMR (both in solid state and in D_2O solution) and Mass Spectrometry techniques for this study to obtain an accurate indication of the real mass of the polymers (by Mass Spectrometry analysis) and a precise indication of the structure by mean of NMR studies.
In order to set up the NMR spectroscopy and Mass Spectrometry analysis method for identification and structural profiling of heparan sulphates and the related proteoglycans in cartilaginous tissues, we will perform a study comparing rabbit articular and growth plate cartilage samples. Rabbits aged 2/3 months will be utilized to obtain growth plate cartilage before skeletal maturation (4–6 months of age). After setting the methods on the animal model, cartilaginous tissues from osteochondromas excised from 12 MO patients selected by mean of their clinical classification will be included in a pilot study; as reference control human growth plate and articular cartilage will be used. Same analyses will be performed also on peripheral chondrosarcomas tissue samples in order to evaluate alterations involved in the malignant transformation process.

Conclusions: Comparing samples from patients, harbouring different EXT1/EXT2 mutations, we expect to find a difference in the degree of elongation of the chain due to the different residual activity of the mutated EXT1/EXT2 enzyme dimer; this will probably affect the subsequent activities of chain modification like N-de-acetylation, sulphation and epimerization. This information will be correlated to clinical and phenotypical data of more than 400 patients carrying EXT1/EXT2 mutations previously clinically and genetically characterized and data obtained from the study will be used to perform correlation studies with highly statistical significance in order to find useful indications for unravel both pathogenesis and patient treatment.

GENOTYPE-PHENOTYPE CORRELATION IN 540 PATIENTS WITH MULTIPLE OSTEOCHONDROMA

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Background: Multiple Osteochondroma (MO) is a disorder characterized by a wide spectrum of clinical manifestation and a broad mutational heterogeneity. To evaluate any relationship between genetic background and both the severity of disease and the risk of malignant transformation we performed a genotype-phenotype correlation study analysing the larger MO case study ever collected; clinical parameters were evaluated with a new classification based on deformity and functional limitations caused by the lesions.

Methods: The study was performed in 540 patients (sourcing from two different case-studies: 408 from the Genetic Unit of Bologna, IOR, and 132 from University Hospital of Antwerp) with clinical and radiographic diagnosis of MO. The presence of mutations in either EXT1 (8q24) or EXT2 (11p11-12) genes was investigated using a DHPLC-MLPA combined screening protocol and/or direct sequencing. The case study is composed by 274 males and 264 females, representing 348 probands with their affected relatives; 148 patients were sporadic cases whereas 363 had a positive family history (in 29 cases familiarity could not be ascertained).

Results: Mutational analysis showed 345 mutations in EXT1 and 133 in EXT2; in 62 patients no disease-causing mutation was found.
Disaggregating 408 patients from IOR we observed a predominant role of EXT1 in defining a severe phenotype; according with this observation patients with EXT1 mutations have a reduced stature (compared to control population) and are characterized by a higher number of sites with osteochondromas. Adverse clinical presentation is associated with sporadic case and male gender is linked to a progression of ingravescence in the disease (compared with their parents). Interestingly, EXT negative patients are strongly associated with mild phenotypes and a stature equal or closely approximating to the average percentile.
Results obtained analysing Antwerp patients are similar to previously described IOR correlation study except for a light tendency of linking EXT negative patients towards more severe clinical presentation.
Analysing all 540 patients, the results confirmed what observed in IOR case study providing interesting significative information about MO disease. Malignant transformation was observed in 28 patients (19 with a MO positive family history, 3 sporadic cases and 6 unknown); no association between chondrosarcoma occurrence and EXT1 mutations and/or more severe clinical presentations was fond.

Conclusions: The comprehensive study, beside the intrinsic limitations of multicentre revisions, shows that trends observed in the IOR has been confirmed in the Antwerp patients corroborating the constancy and substantiate the statistical significance of the data. These observations could be considered a constant feature of MO and therefore could play a relevant role in the clinical approach to MO patients (providing information to be offer during genetic counselling, personalizing follow-up and helping in defining appropriate clinical treatment) except for prevention of malignant transformation where a regular screening is still highly recommended for MO patients. Moreover the study could offer homogeneous sub cohort of MO patients (EXT1/2 negative patients, for instance) where appropriate studies could be performed in order to better characterize the pathogenesis of the disease.

MODELLING PROTEIN STRUCTURE PREDICTION OF EXT1/EXT2

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Background: Multiple Osteochondromas is generally related to mutations on EXT1 / EXT2 genes. These two genes encode for two transmembrane proteins EXT1 and EXT2, belonging to glycosyltransferase family that are normally present in Golgi apparatus. The specific activity of these proteins, whose three dimensional structure has not been resolved yet, is the polymerisation of the Heparan Sulfate side chains of matrix proteoglycans.

Methods: The aim of these studies is the structure analysis of EXT1 and EXT2 proteins. So wild-type and mutant proteins were modelled by homology and then we made a deep computational analysis of all structures (wild-type and mutant models) in order to investigate how disease-associated mutations disturb structural features affecting enzymatic activity.
Several techniques have been developed for protein structure prediction starting from the primary sequence[1]. However, it turns out that this is a very difficult task since the solution space is exceedingly large (due to the number of possible conformations) and because protein folding dynamics are not yet fully understood (the mechanisms guiding the transition to an energetically favourable conformation).
A class of methods, known as “homology modelling”, have been profitably employed to generate 3D atomic-resolution models of (target) proteins from homologous[2] proteins with known structure (template). The observation that the 3D structure of supposedly homologous proteins tends to be better conserved than their primary sequence has also been used to design models of proteins from homologues; such models are generated by taking into account a finite number of inveterate folding traits.
Analysis to search for similarity sequence will be conducted using the HHpred server. (Söding J, Biegert A, and Lupas AN. (2005) The HHpred interactive server for protein homology detection and structure prediction. Nucleic Acids Research 33, W244--W248 (Web Server issue). doi:10.1093/nar/gki40)
HHpred's sensitivity is competitive with the most powerful servers for structure prediction currently available and is the first server that is based on the pair wise comparison of profile hidden Markov models (HMMs).
The preliminary studies of the EXT1 protein structure showed similarities with the two proteins of known structure: EXTL2 and MurG of Eschirichia coli. From all this it was possible to build a reliable model for only a small central region which corresponds to an area involved in the bond dell'UDP-sugar and the C-terminal Region relevant to the site of catalysis of UDP-N-acetyl glucosamine. It can therefore infer that the EXT1 is a protein anchored to the membrane with a peptide of 20 amino acids.
Genetic data that are used come from the study of genotype phenotype correlation on 400 patients and the results will be compared with the clinical data to find information on clinical impact of structural change of protein.

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[1]The performance of these methods are assessed in the CASP experiments every two years.
[2]The generally accepted meaning of the term homology is “having a common evolutionary origin”; this is a qualitative relationship among biological entities corroborated by sequence or structure similarity. It is unfeasible to derive the exact evolutionary history of any species since we are lacking the genetic material of extinct species (evolution takes place on geological time scales). For this reason we resort to sequence similarity of extant species to infer evolutionary relationships among proteins.

EXT1 - EXT2 GENE COPY NUMBER ANALYSIS IN A LARGE MO PATIENT COHORT BY MLPA AND REAL TIME PCR

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Background: Multiple Osteochondromas (MO) is a dominant autosomal disorder associated to mutations on EXT1 (8q.24.1) and EXT2 (11p12-11) genes.
Our molecular screening protocol for MO patients includes dHPLC coupled by direct sequencing of samples that present a chromatographic profile diverging from wt. After running these analyses, 13% of 400 samples screened did not show point mutations or small deletions/insertions; they are therefore supposed to harbour big insertions, deletions or rearrangements that cannot be tested with these techniques.
The most common technique used to quantify the genes copy number is Real Time PCR, a robust and reliable screening method. The disadvantages of the qPCR are the expensive costs and the long reaction time needed to obtain large fragments amplification. The MLPA (Multiple Ligation Probe Amplification) screening offers the possibility to run copy number analyses with cheaper costs and high possibilities of multiplexing. Aim of this study was to analyse 53 MO patients negative for point mutations detectable with dHPLC using MLPA and subsequently confirm the results by qPCR.

Methods: Genomic DNA was isolated from peripheral blood following standard procedures. MLPA has been performed using SALSA EXT1/2 kit (MRC-Holland, Probemix P215) and the results have been validated with qPCR with an Intercalating Dye chemistry (RT^2 SYBR Green qPCR Master Mix, SuperArray) using β-actin as endogenous calibrator.

Results and conclusions: Big gene rearrangements were detected in 17 out of 53 patients; 10 deletions and one duplication are located on EXT1 gene and 6 deletions on EXT2. In 36 MO patients, including 15 sporadic cases and 1 patient treated with TBI, any abnormality was present. For 26 of these negative patients complete clinical data are available and about 65% of them (17/26) have a mild clinical presentation of the disease (class I in IOR classification).
These results, obtained with MLPA, were all confirmed by qPCR technique confirming a complete accordance between the two methods of analysis. We can therefore state that combining MLPA protocol with qPCR verification of positive and doubtful results in EXT genes analysis is a sensitive and cheaper implementation of the dHPLC and direct sequencing first screening.

GENETIC SCREENING OF INDIAN HEDGEHOG (IHH) IN MULTIPLE OSTEOCHONDROMAS PATIENTS WITHOUT EXT1/EXT2 MUTATIONS

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Background: Multiple Osteochondromas (MO) is one of the most common skeletal disorders characterized by the formation of multiple cartilage-capped, benign bone tumours (exostoses). This genetically heterogeneous, autosomal dominant disorder is, in most cases, associated with germline mutations in the EXT1 or EXT2 genes, which encode for glycosyltransferases involved in the heparan sulphate polymerization. EXT and heparan sulphate proteoglycans (HSPGs) are implicated in regulating the diffusion and receptor binding of several signalling molecules and growth factors during endochondral bone formation including Indian Hedgehog (IHH). Moreover, it has been proposed that IHH signalling impairment may be involved in the exostosis development in MO. Aim of this study was therefore to screen a group of MO patients negative for mutations in the EXT genes to investigate whether their phenotypes could correlate with mutations involving IHH.

Methods: Genomic DNA of 34 probands and 20 relatives (13 non affected and 7 affected) was isolated from peripheral blood following standard procedures. The screening for point mutations in the 3 exons of IHH was performed by DHPLC followed by direct sequencing of samples showing an abnormal elution profile; possible big deletions/duplications were analyzed by quantitative Real-Time PCR. For one family with peculiar clinical characteristics total RNA was also extracted to analyze IHH expression by RT-PCR and Real Time PCR.
Results: The DHPLC-based screening of all 54 samples showed the presence in three unrelated probands of an intronic polymorphism [(IVS1)+8, C>T] never described previously. Since these patients show similar mild phenotypes, characterized by the presence of a small number of osteochondromas in the hands, the possible functional effects of this polymorphism will be evaluated. No deletions/duplications were identified by Real-Time PCR analysis.
The expression analysis of IHH performed in the family whose proband is characterized by the presence of two exostoses with uncommon shape showed, by both RT-PCR and Real Time PCR, very low levels for the proband compared to healthy controls and parents.

Conclusions: Taken together, the results obtained from IHH mutation screening and expression analysis performed on EXT1/2 negative MO patients indicate a possible correlation of the detected polymorphism or of low IHH transcript levels with mild and morphologically defined MO phenotypes. Therefore, our findings suggest a role for IHH signalling impairment as an alternative molecular mechanism in osteochondromas onset; additional studies are needed to further validate this hypothesis.

PROTOCOL SET UP OF A MOLECULAR REFINED ANALYSIS METHOD FOR GLYCOSAMMINOGLYCANS EXTRACTED FROM MO PATIENTS SAMPLES

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Background: Glycosaminoglycans (GAG) are linear polysaccharides that constitute the main post-translational modification of proteoglycans.
These cartilage E.C.M. glycoproteins play a key role in structural and biochemical organisation of growth plate cartilage; in particular they control water content, the diffusion of nutrients and oxygen and the morphogen gradient formation. Moreover, GAG are important players also in the ligand-receptor interaction of FGF-FGFR family.
MO is an autosomal dominant disorder in which EXT1 and EXT2 genes are involved; they encode for Golgi transmembrane heparan sulphate polymerases.
Mutations in these two genes are supposed to modify the activity of HS polymerases thus leading to the production of different chains from the wt status.

Aim of the study: To evaluate the presence of all types of disaccharides and their different degree of modification (N-acetylation, sulphation and epimerisation) trying to define biological markers able to distinguish between resting and growing cartilaginous caps of osteochondromas and peripheral chondrosarcomas.

Methods: GAGs will be isolated from urines and plasma of healthy and affected patients by mean of ion exchange chromatography and afterwards degraded to unsaturated disaccharides by specific eliminases.
After fluorescent labelling the disaccharides will be analysed by fluorescent electrophoresis.

Expected results and conclusions: We expect different length, specific amount of epimerized glucuronic acid and, subsequently, different degrees of sulphation for each genotype. Furthermore these data will be compared with a genotype-phenotype correlation study on 400 MO affected patients: Relationship between secreted GAGs (and the similar chemical species present in cartilage) and correlation between the molecular profile of GAGs, the effective genotype status and the clinical phenotype will provide an easy way for the monitoring of the metabolism of cartilage (including the MO malignant progression). This will provide biomarkers for the biochemistry of cartilage that can play a predictive role in the progression of MO.

Abstracts Research Line 2 talks

HAEMANGIOPERICYTOMA OF BONE: REAL OR IMAGINED?

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Background: Haemangiopericytoma (HPC) was first described by Murray and Stout as a soft tissue neoplasm with distinct morphologic features, presumably composed of pericytes. Over the years, it became clear that many tumours could mimic a HPC-like pattern. These days, it is accepted that in soft tissue most lesions diagnosed as HPC in the past are actually solitary fibrous tumours (SFT), synovial sarcomas (SS) or myofibromatoses. It has been unclear whether the very rare HPC of bone is a true entity, or that the HPC-like vessels are non-specific and part of other, different entities.

Design: We collected 10 primary HPC of bone from 4 institutions diagnosed between 1952 and 2002. All data were reviewed. Immunohistochemistry was performed for CD31, CD34, factor VIII, SMA, keratin AE1/AE3 and EMA. Staining was evaluated as focal positive, diffuse positive or negative.

Result: There were 5 female and 5 male patients between 21 and 73 years of age (mean 45.3 y). All tumours were located within bone. The primary site of the tumour was the femur in two patients, humerus in one, fibula in one, sacrum in two and vertebra in three. All tumours showed the presence of prominent thin-walled branching vessels surrounded by more undifferentiated spindle or round cells. However these cells showed some variation in their morphologic pattern: 5 tumours showed a pattern-less architecture and varying cellularity, consistent with SFT. Three tumours showed more densely packed sheets of poorly differentiated cells, similar to SS, and 1 case each represented paraganglioma and PEComa, possibly metastatic. Tumours resembling SFT showed usually focal to diffuse staining for CD34. All tumours were negative for SMA. Two tumours more similar to SS showed focal positive staining for keratin AE1/AE3 or EMA (66%). Some tumours showed severe decalcification artefact. None of the 10 tumours show CD31 and factor VIII expression. FISH is performed to study SYT rearrangements.

Conclusion: Our retrospective review of tumours diagnosed as HPC of bone in the past revealed the absence of true pericytic differentiation and the existence of both SFT of bone and SS of bone. Therefore, as in soft tissue tumours, HPC-like features are non specific. Diffuse CD34 staining is helpful to diagnose SFT of bone, whereas keratin/ EMA staining is suggestive for SS of bone.

HISTOPATHOLOGY, IMMUNOHISTOCHEMISTRY AND XENOTRANSPLANT IN OSTEOSARCOMAS: A TISSUE MICROARRAY STUDY

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Background: Osteosarcoma (OS) is the most common primary malignancy of bone in children and adolescents. OS is an aggressive neoplasm composed of spindle, round, or epithelioid cells producing osteoid in most cases. The most frequent histological subtypes include osteoblastic, chondroblastic, fibroblastic and parosteal OS.

Design: A total of 58 cases with diagnosis of OS (primary tumours, recurrences and/or metastasis and xenotransplanted OS) with formalin-fixed paraffin block (FFPB) were collected. Thirteen Tissue Microarrays (TMAs) were performed and all the cases were distributed in the following groups: a) only FFPB available from primary/recurrence and/or metastatic tumours; n=20 (2 TMAs), b) FFPB from primary/recurrence and/or metastatic tumours available as well as from the corresponding xenograft; n=30 (8 TMAs); c) only FFPB available from xenotransplanted OS; n=8. Several xenograft tumours from different passages were selected from initial passages (1-15), middle passages (16-30) and late passages (31-68). A reclassification of all the cases according to the new criteria for OS diagnosis was performed; in addition conventional haematoxylin eosin staining and immunohistochemistry (Osteonectin, Osteocalcin, S-100, SOX-9, Ki-67, Bcl-2, p53, p16 Survivin, CD99 and Caveolin) were performed and analyzed in TMAs.

Results: The distribution of cases according to the histopathological tumour pattern was as follows: thirty-two osteogenic OS; seven chondrogenic OS, six parosteal OS, three small cell/ microcellular OS, one telangiectatic OS, two poorly differentiated OS, one dedifferentiated OS, two pleomorphic-like MFH-OS and four mixed OS with a different pattern. Osteonectin and osteocalcin were the most expressed antibodies with no significant variation in immunohistochemical expression during subsequent passages. CK, Bcl2 and CD99 revealed low expression. Caveolin and survivin showed immunoreactivity in the majority of the tumours. p16 and p53 displayed heterogenic expression and the proliferation index was not significant although the aggressive histopathology pattern of the tumour.

Conclusions: TMA studies are very helpful for the diagnosis and study of a large cohort of OS material. Xenotransplanted TMA studies improve knowledge related to the evolution of histopathology and immunohistochemical expression in OS during subsequent passages. TMAs are available for testing new antibodies and for carrying out genetic studies in paraffin that could improve the therapeutic and diagnostic methods for these tumours.

INTEGRATED ANALYSIS OF MIRNA, GENE EXPRESSION AND COPY NUMBER VARIATION IN OSTEOSARCOMA CELL LINES

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Background: Increasing evidence shows that expression of miRNAs is deregulated in human cancer. Subsets of specific miRNAs have been shown to be regulated during osteogenic differentiation, and may play important roles in tumourigenesis.

Methods: In the present study, we have done miRNA profiling on the EuroBoNeT panel of 20 osteosarcoma cell lines using Agilent Human miRNA Microarrays. Two different strategies will be used to identify interesting miRNAs in these samples. First, we will compare the expression pattern of the cell lines with that of osteoblasts and human normal bone samples with the aim to identify miRNAs that are differentially expressed between tumour and normal progenitor and/or bone samples. As a second strategy for this project we will identify regions with gain or loss in the OS cell lines by use of Affymetrix Genome-Wide Human SNP Array 6.0, and do an integrated analysis where we identify the miRNAs that are transcribed/not transcribed in these regions. Each miRNA can regulate a number of target mRNAs, and following the identification of interesting miRNAs we will finally integrate the miRNA data with the gene expression data from the same samples, facilitating identification of differentially expressed target genes that may be regulated by miRNAs in the OS cell lines.

TYROSINE KINASES AS POSSIBLE THERAPEUTIC TARGETS IN CHORDOMA

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Objectives: Chordomas are rare, slowly growing but potentially aggressive bone tumours, originating from remnants of the foetal notochord. The current treatment for chordoma is surgery, followed by radiotherapy. Five and 10 year survival rates are of 68% and 40%, respectively. Preliminary clinical data suggest the moderate benefit from the treatment with imanitib mesylate in a subset of chordoma patients. The goal of this study was to explore the role of tyrosine kinases (TKs) in the pathogenesis of chordoma and their significance as possible targets for the TK receptor directed therapy.

Material and Methods: Histopathological features of 43 chordomas (11 primary and 32 advanced), were assessed by H&E staining and immunohistochemistry. 31 cases were studied by FISH using *KIT*, *PDGFRB*, *CSF1R* and *EGFR* probes. Nine cases with sufficient frozen material (3 primary, 6 advanced tumours) were analyzed by the whole-genome 4.5K CGH-array, the semi-quantitative 96-well-plate TKs RT-PCR assay, Western immunoblotting and the proteome profiler phospho-TK array. The study was supplemented by direct sequencing of *KIT*, *PDGFRB*, *CSF1R* and *EGFR*.

Results: By TKs RT-PCR assay, several TKs were expressed, with high expression of EGFR, CSF1R and IGF1R in all examined cases. Notably, PDGFRs, KIT or ABL, which are considered the main targets of imatinib, were not significantly expressed. By immunohistochemistry, moderate to high expression of CSF1R and EGFR was confirmed in respectively 70% and 50%, while KIT was expressed only in sporadic cases. FISH analysis revealed EGFR polysomy and low level EGFR amplification in 30% and 2% of cases, respectively. No copy number changes or rearrangements of *KIT*, *PDGFRB* or *CSF1R* were found. By CGH-array analysis, frequent losses of chromosome regions 1p, 3p, 3q, 9p, and 22q were present. Gains of chromosome 7 were disclosed in 35% of cases. High level amplifications were not detected. By sequencing, no mutations of *KIT*, *PDGFRB*, *CSF1R* or *EGFR* were found. By Western immunoblotting, expression of PDGFRB was found in almost all chordomas, but activation of this receptor was seen only sporadically. KIT was neither generally expressed nor phosphorylated. Notably, expression and activation of EGFR was demonstrated in 50% of cases. The constant activation of CSF1R was confirmed by phospho-TK array analysis.

Conclusions: We have demonstrated that PDGFRB, CSF1R and EGFR are activated in a subset of chordomas. Lack of activating mutations, rearrangements or amplification of these genes suggests activation by autocrine/paracrine ligand stimulation. All can be possible targets for the therapy using specific TK inhibitors.

CHEMORESISTANT OSTEOSARCOMA CELLS ARE SENSITIVE TO DNAM-1 AND NKG2D DEPENDENT NK CELL CYTOTOXICITY

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Background: High grade osteosarcoma (OS) is the most common primary malignant bone sarcoma, occurring primarily in adolescents. To explore the feasibility of natural killer (NK) cell-mediated immunotherapy for patients with advanced-stage OS, we investigated the susceptibility of OS cell lines to NK cell-mediated cytotoxicity and identified molecular mechanisms involved in this process.

Methods: Expression of ligands for inhibitory and activating NK cell receptors was evaluated in nine OS cell lines and three chemotherapy resistant variant cell lines of SAOS-2 and U2-OS (resistant to doxorubicin, methotrexate or cisplatinum). Cytotoxicity was determined in chromium release assays, using resting and interleukin (IL)-15 activated NK cells obtained from healthy donors and newly diagnosed osteosarcoma patients. Blocking antibodies against specific ligands/receptors were used to study the contribution of these molecules.

Results: All cell lines tested were sensitive to NK cell mediated killing, albeit at different levels, consistent with the differential expression of ligands for the activating NK cell receptors DNAM-1 and NKG2D. Blocking of these receptors resulted in complete abrogation of cytolysis of HOS, demonstrating their essential role in NK cell mediated lysis of OS. The level of HLA class I expression on the OS cell lines was negatively correlated with sensitivity to NK cell mediated lysis. Blocking of HLA class I resulted in increased killing of SJSA-1, a cell line with high HLA class I expression. Chemoresistant variants of U2-OS and the original cell line were equally sensitive to NK cell-mediated cytolysis. In contrast, the SAOS-2 cisplatinum variant was less efficiently lysed, which correlated with a lower CD95 expression. NK cells of either newly diagnosed OS patients (n=18) or healthy donors (n=10) were equally effective in lysing two osteosarcoma cell lines (SJSA-1 and HOS). Culturing of donor and patient NK cells in IL-15 potentiated their cytolytic effect towards all tested OS cell lines. Phenotypic analysis using flow cytometry for NKG2D, DNAM-1, perforin and granzyme expression on NK cells did not differ between healthy controls (n=17) and osteosarcoma patients (n=22).

Conclusion: Osteosarcoma cell lines are susceptible to NK cell cytotoxicity which is dependent on the expression of activating NK cell receptor ligands. Conversely, high HLA class I expression is associated with decreased sensitivity to cytolysis. NK cells from newly diagnosed osteosarcoma patients and healthy donors are equally effective, supporting the use of autologous NK cells in immunotherapeutic strategies. The use of IL-15 cultured NK cells greatly potentiated cytolysis to both chemosensitive and chemoresistant cell lines.

Abstracts Research Line 2 posters

FUNCTIONAL EVALUATION OF CRITICAL ANGIOGENESIS-RELATED FACTORS IN OSTEOSARCOMA

P Kunz

Ortho Heidelberg

Introduction: Angiogenesis is essential for growth and metastasis of malignancies. Besides the identification of angiogenic factors crucially involved in the formation of new vessels within a certain tumour entity, functional analysis of their in vivo effects are a prerequisite for a successful approach towards an entity-specific anti-angiogenic therapy. In osteosarcoma, very few angiogenic factors have been identified so far, functional in vivo investigations of their effects on osteosarcoma growth and metastasis are almost completely absent.
Our aim is the identification of crucial angiogenic factors in human osteosarcoma kryo-samples and to develop an osteosarcoma animal model suitable for functional dynamic PET-measurements. After inoculation of angiogenic factor- transfected cell lines, functional dynamic PET measurements are conducted. Radioactive tracers like H_20^{15}, Gallium-albumin and RGD-peptides give insight of the effects of the transfected factor on angiogenesis-related aspects like perfusion, blood pooling and neo-angiogenesis of the tumour.

Methods:
qPCR Kryo / low-density arrays angiogenic factors
UMR-106 osteosarcoma cell lines – transfection with/overexpression of angiogenic factors
Rat animal model-established
PET-measurements FDG, evtl. RGD and Albumin

Results (so far):
- certain factors identified (-DELIVERABLE)
- stable transfection of cell lines
- reliable animal model (take rate 100%) and suitable for PET (moderate osteoid production-FDG –uptake in vivo) (-DELIVERABLE)
- tumour growth of stable transfected UMR-osteosarcomas compared to WT (hopefully finished `till Valencia)

Conclusions: will follow

IMPACT OF C-MYC IN DRUG RESISTANCE OF HUMAN OSTEOSARCOMA

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Background: Aim of this study was to validate the previously identified methotrexate (MTX) resistance-associated genetic changes involving the *dihydrofolate reductase (DHFR)* and *C-MYC* genes in human osteosarcoma (OS) cell lines and clinical samples.

Patients and Methods: The involvement of *C-MYC* in MTX resistance of human OS cell lines was validated with an antisense approach. C-MYC and DHFR protein levels at diagnosis were assessed by immunohistochemistry on series of patients treated with either a MTX-based protocol (IOR/OS-1; 72 patients) or with a standard four-drugs regimen (ISG/SSG 1; 61 patients).

Results: Down-regulation of *C-MYC* significantly decreased the MTX resistance level of OS cells, demonstrating its causal involvement in this phenomenon. In clinical samples, a worse outcome was associated with increased levels of DHFR and CMYC at diagnosis in the IOR/OS-1 patients and of CMYC only in the ISG/SSG 1 patients.

Conclusions: The assessment of C-MYC and DHFR at diagnosis, together with that of other known prognostic markers, can be considered for an early identification of subgroups of OS patients with higher risk of adverse outcome. Moreover, meanwhile the adverse clinical impact of *DHFR* overexpression appeared to be closely related to the relevance of MTX in the chemotherapeutic protocol that of *C-MYC* overexpression was more general and not strictly drug-related. Further studies have clarified that C-MYC may exert its role on drug resistance in human OS cells through the modulation of ABC transporters expression. Ongoing studies are focused on the identification and validation of the ABC transporters which are mostly relevant for osteosarcoma treatment response and prognosis.

TARGETING GLUTATHIONE-S-TRANSFERASE AS A NOVEL THERAPEUTIC STRATEGY TO OVERCOME DRUG RESISTANCE IN OSTEOSARCOMA

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Background: The increase of both intracellular level and enzymatic activity of glutathione-S transferase (GST) isoenzymes contributes to chemoresistance in many human cancers. In human osteosarcoma (OS) cell lines, we have shown that GST-P1 is responsible for the acquisition of resistance to cisplatinum (cis-diamminedichloroplatinum, CDDP) and may be also involved in the development of resistance against methotrexate (MTX) and doxorubicin (DX), the other two drugs which are most commonly included in the osteosarcoma chemotherapy protocols.
On the basis of these findings, we evaluated the clinical impact of GSTP1 in a series of 34 high-grade OS patients and we found that the increased expression of GSTP1 gene at diagnosis was associated with a significantly higher relapse rate and a worse clinical outcome. All these indications prompted us to assess the in vitro effectiveness of 6-(7-nitro-2,1,3-benzoxadiazol-4-ylthio)hexanol (NBDHEX), a new promising anticancer agent which is a very efficient inhibitor of GSTP1.

Methods: NBDHEX was tested on a panel of 10 human OS cell lines and 20 variants of the U-2OS or Saos-2 cell lines, which were resistant to CDDP, MTX or DX.

Results: NBDHEX resulted to be very active on the vast majority of these cell lines, including the drug-resistant variants with higher GSTP1 enzymatic activities. Drug combination studies showed that NBDHEX can also be associated with CDDP and provided useful indications concerning the best modality of administration when these two drugs are used together. On the other side, the combined use of NBDHEX with MTX or DX must be carefully planned on the basis of pre-clinical in vitro evidence.

Conclusion: In conclusion, we showed that GSTP1 has a relevant impact for both drug resistance and clinical outcome of high-grade OS. Moreover, our findings indicated that targeting GSTP1 with NBDHEX may be considered a new promising therapeutic possibility for high-grade OS patients, which are unresponsive to conventional chemotherapy.

HIGH-GRADE ANGIOSARCOMA OF BONE: A CLINICOPATHOLOGICAL STUDY OF 64 CASES

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Introduction: High-grade angiosarcoma (HGAS) of bone are rare and represent less than 1% of the primary malignant bone tumours. Because of their rareness little is known. Clinically, it is accepted that they are extremely aggressive. Due to the lack of uniform terminology and accepted histological criteria, terminology and classification of primary malignant vascular tumours of bone has been highly controversial. Today, angiosarcoma is the most accepted term for high-grade primary vascular tumour of bone, recognized by the 2002 WHO Classification. However, distinct histological hallmarks to define a HGAS of bone are not clear.

Method: We collected 64 HGAS of bone diagnosed between 1964 and 2007 from the files of the departments of pathology, Leiden University Medical Center (Leiden), Rizzoli Institute (Bologna) and University Hospitals (Leuven). All clinical, radiological, and pathological data were reviewed and different histological criteria were scored. A tissue micro-array was constructed containing 57 HGAS of bone. To confirm the vascular origin of all lesions and to investigate the diagnostic value of commonly used markers, immunohistochemistry was performed for CD31, CD34, Factor VIII, and keratin AE1/AE3. Staining was evaluated positive or negative.

Results: Among 64 patients with HGAS of bone, there are 41 males and 23 females. There is a wide age distribution, with a nearly equal distribution from the second to the sixth decade. The solitary cases are mostly located in the extremities (66%) followed by trunk (12,8%), axial/central location (10,6%) and pelvis (10,6%). 17 cases (73%) have multifocal bone lesions. HGAS of bone showed variable histological patterns. Association with clinical outcome (chi-square test) revealed that there was a significant poor survival when the tumour had 3 or more mitoses (p=0,001), a macronucleoli (p=0,011) or there was an absence of an eosinophilic infiltrate (p=0,023). The HGAS of bone are positive for CD31 in 53/55 (96%), CD34 in 33/57 (58%), Factor VIII in 47/55 (86%), and keratin in 40/57 (70%). Only 15 out of 40 (38%) keratin positive angiosarcomas, showed an epithelioid phenotype at classical morphology. All tumours with an epithelioid phenotype are keratin positive.

Conclusions: Although HGAS of bone in general have a poor outcome, histological criteria such as three or more mitoses, the presence of a macronucleolus and the absence of an eosinophilic infiltrate can be useful to predict a more aggressive course, consistent with the clinical behaviour of a high-grade angiosarcoma.
CD31 and Factor VIII are the best diagnostic markers for HGAS of bone. It is striking that keratin positivity is seen in the majority of cases, and is independent of epithelioid morphology. Pathologists should be aware of this to avoid misinterpretation as metastatic carcinoma.

INACTIVE WNT/β-CATENIN SIGNALLING IN HIGH-GRADE OSTEOSARCOMA

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Background: Osteosarcoma (OS) is the most frequent primary malignant bone tumor. High-grade central OS frequently affects children and young adolescents. The prognosis has changed dramatically after the introduction of neoadjuvant chemotherapy, but the overall survival has reached a plateau. Frequent locations of OS at metaphysis of long bones as well as the age of the patients during the most rapid bone growth suggest that the pathogenesis of OS may be closely related to high bone turnover. Recent research has demonstrated that canonical Wnt pathway is indispensable for osteogenic linage differentiation. Active Wnt signalling has been shown to play a role in several types of tumours development; however, its role in OS is poorly understood.

Methods: A canonical Wnt signalling luciferase reporter construct was transfected in 4 OS cell lines.β-catenin expression was determined in 4 OS cell lines and on a tissue array containing 144 OS samples from 88 patients .The effect of Wnt signalling modulation on cell proliferation as well as osteogenic differentiation capability was assessed.

Result: Canonical Wnt signalling was not active in 4 OS cell lines as determined by the luciferase reporter assay. Negative nuclear β-catenin staining was found in all 4 OS cell lines and 87% of cases in our OS tissue array. GIN (a GSK3β-inhibitor) can stimulate canonical Wnt signalling activity determined by luciferase reporter assay and nuclear β-catenin translocation. In addition, an increase of Axin2 mRNA expression, a downstream target gene of Wnt pathway, was shown in 4 OS cell lines after GIN treatment. However, Wnt3a failed to upregulate canonical Wnt signalling activity, suggesting that an upstream part of Wnt signalling is impaired. Around 50% decrease of cell proliferation was found in MG-63 and U-2-OS but not in SJSA and HOS cell lines after GIN treatment. SJSA and HOS cell lines retain the capability toward osteogenic differentiation. Increase of both ALP (alkaline phosphatase) activity and mineralization were observed in SJSA and HOS upon stimulation of Wnt signalling by GIN. MG-63 and U-2-OS cell lines can not differentiate towards the osteogenic linage and restoration of Wnt signalling could not rescue this phenotype.

Conclusion: Canonical Wnt signalling is shown to be inactive in OS. Restoration of canonical Wnt signalling in OS cell lines promotes osteogenic differentiation or inhibits proliferation. Activation of Wnt signalling has been shown to promote carcinogenesis in several tumour types, however our data demonstrate an opposite effect of Wnt signalling in OS development.

TUMOURIGENIC MURINE MESENCHYMAL STEM CELLS AS A MODEL TO STUDY OSTEOSARCOMA

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Background: Previously we identified murine mesenchymal stem cells (mMSCs) that formed aggressive osteosarcoma (OS) like lesions after injection in mice (Tolar et al. STEM CELLS 2007; 25; 371-379). Here we aimed to recapitulate this tumourigenic transformation and study these cells in a step-wise manner to identify early events in OS genesis since OS progenitor cells are still elusive.

Methods: Previously reported tumourigenic MSCs (TmMSCs) and tumours derived from these cells (T1 and RT2) as well as two new bone marrow derived MSCs cultures from C57BL/6 and BALB/c mice (B6-mMSCs and Bc-mMSCs respectively) were cultured. To characterize the cells FACS analysis and multi-lineage differentiation analysis were performed. Genetic alterations were identified using genome wide high resolution arrayCGH (all 7 samples) and COBRA-FISH (for 5 samples) and validated at the gene expression level by qRT-PCR. To look for the clinical relevance of the findings an OS tissue array containing human OS samples from 88 well-characterized patients was constructed and stained for the relevant proteins by IHC. Correlations with the survival of the patient and other characteristics were analyzed by using SPSS software.

Results: A homozygous deletion on the q-arm of mouse chromosome 4 was identified in all cell cultures except B6-mMSCs at low passage (p12). This region contains the *CDKN2A/p16 Arf/p14 and CDKN2B/p15* genes. Next to this loss and overall aneuploidization many other genetic aberrations were detected, but none as consistent as the chromosome 4 deletion. Subsequently in clinical human OS, CDKN2A/p16 protein expression significantly correlated with the survival of the patients (LogRank $p<0.000$).

Conclusion: Our findings provide substantial evidence that MSCs are the progenitor cells of OS, moreover we define the complete loss of CDKN2 as an important event responsible for malignant transformation of MSCs. Finally we show clinical relevance of CDKN2A/p16 loss by significant association between *CDKN2A/p16* protein expression in OS and patient's survival, providing a prognostic marker that is superior to the current standard, which is the response to chemotherapy.

NOVEL FUNCTIONAL SP1 BINDING SITES IN EGFR GENE INTRON 1

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Aims: Tumourigenesis is often accompanied by transcriptional deregulation of oncogenes, such as the Epidermal Growth Factor Receptor (EGFR). Transcriptional activation of a gene requires the binding of transcription factors (TF) to regulatory DNA elements at specific transcription binding sites (TFBS). A better understanding of these interactions and regulation mechanisms is essential for the development of improved therapeutic applications.

Methods: ChIP was carried out to prove the existence of four new SP1 binding sites within intron 1 of the *EGFR* gene. Site-directed Mutagenesis was performed on plasmids carrying the regulative sequence of the *EGFR* gene in order to alter these binding sites. Activity of these sites and their influence on the transcriptional regulation were analysed by in vitro transcription and quantification using Ribonuclease Protection Assay (RPA) and qRT PCR.

Results: Using ChIP, four novel SP1 binding sites could be confirmed to be active at the *EGFR* gene intron 1 locus. Expression of the *EGFR* gene was found to be highly dependent of these sites. Consequently, their mutation led to a 50% decrease of the transcriptional activity of the *EGFR* gene.

Conclusions: The four new SP1 binding sites in the *EGFR* intron 1 have a functional role in the *EGFR* gene regulation, leading to a higher transcription rate. As so far only little is known about *EGFR* gene activation, more TFs and TFBSs have to be analysed in order to gain a comprehensive understanding about the regulation of this important oncogene.

DNA COPY NUMBER PROFILING IN FIBROSARCOMA OF BONE

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Background: Very little is understood regarding the genetics of fibrosarcoma of bone. In this study, array comparative genomic hybridization (aCGH) was used to identify genes or chromosomal regions with potential importance in this tumour.

Methods: Analysis was carried in thirteen fresh frozen tissue specimens from eleven patients with fibrosarcoma of bone (nine primary tumours and three local recurrences). Only samples with tumour proportion over 80% were included in the analysis. DNA was extracted and hybridisations were performed on Agilent 244K CGH oligo-arrays. The data was analysed using Agilent DNA Analytics software.

Results: The number of changes per patient ranged from zero to 105. Numerous regions with recurrent aberrations were identified. The most frequent gains were seen at chromosomal regions 1q, 4q, 5p, 8q, 12p, 16q, 17q, 20q, 22q and Xp. Seven high level amplifications were detected, two of them at 4q12, including the *KIT*, *PDGFRA* and *VEGFR2* genes, all of which play important role in many tumour types. *KIT* and *PDGFRA* are frequently activated by mutations in gastrointestinal stromal tumour, in which the genes are targets of Imatinib therapy. No activating mutations in *KIT*, *PDGFRA* or *VEGFR2* were found in any of our samples in a screening analysis. A small minimal common region of gains (6/11) at 1q12 includes *CHD1L*, a novel candidate oncogene in hepatocellular carcinoma.
Losses were most commonly detected at the regions 6q, 8p, 9p, 10, 13q, 15q and 20p. The tumour suppressor locus *CDKN2A* at 9p21.3 was homozygous deleted in six patients. The *p14ARF* and *p16INK* genes encoded by the locus were both hyper-methylated in one of sixteen patients studied. Interestingly, the *CCND1* (Cyclin D1) gene, was gained in three of the four recurrences, emphasizing the important role of the RB1 pathway for cell cycle checkpoint in this tumour.

Conclusion: Our aCGH pinpointed several chromosomal regions and genes with potential importance in development or progression of fibrosarcoma of bone. The candidate genes include *CDKN2A*, *CCND1*, *KIT*, *PDGFRA*, *VEGFR2* and *CHD1L*. The results open up a good starting point for hunting diagnostic and prognostic markers, as well as potential targets of therapy in this rare tumour type.

GENOME-WIDE GENETIC AND EPIGENETIC CHANGES IN HUMAN OSTEOSARCOMAS

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Background: Genetic and epigenetic alterations are frequently seen in cancer, and are responsible for the deregulation of differentiation and proliferation programs.

Methods: Using different microarray technologies, we have analysed genetic and epigenetic changes genome-wide in the EuroBoNeT panel of 20 human osteosarcoma cell lines. DNA copy number changes have been mapped at high resolution using the Affymetrix Genome-Wide Human SNP array 6.0 and we have identified a number of highly recurrent regions. In addition, we have analysed over 27,000 CpG sites for methylation status and used global gene expression to identify a number of genes that undergo methylation and expression silencing in osteosarcomas. By integrating different levels of genome-wide information, i.e. DNA copy number changes, loss of heterozygosity, mRNA gene expression and methylation, we aim to identify important genes and transcriptional networks for osteosarcoma development. The identified genes and networks will be investigated in an osteosarcoma tumour panel at a later stage. The EuroBoNeT osteosarcoma cell line panel will serve as a well-characterised genetic and epigenetic model system for basic and preclinical studies within the EuroBoNeT network and elsewhere.

IN VITRO AGING OF MESENCHYMAL STEM CELLS FROM OSTEOSARCOMA PATIENTS AND HEALTHY CONTROLS IS ASSOCIATED WITH THE INCREASED OCCURRENCE OF BINUCLEATE CELLS

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Background: High grade osteosarcoma (OS) is the most common primary malignant bone sarcoma and is characterized by excessive genomic instability. Benign precursors for OS are lacking and the progenitor cells are elusive, however recent studies favour the mesenchymal stem cell (MSC) as a probable cell of origin for OS. Generation of tetraploidy as an initiating event for malignant transformation and gross aneuploidy of MSCs and other cell types is observed in several studies in mice and man. We hypothesize that an increased susceptibility of MSCs from OS patients to become tetraploid may account for the high genetic instability and the early age of onset of OS.

Methods: We have cultured bone marrow derived MSCs (BM-MSCs) of seven newly diagnosed OS patients and five healthy bone marrow donors. Genome wide expression analysis was performed on RNA isolated from early passages (p3 to p6) of these MSCs using Illumina microarrays. In addition, we are performing long term culture (currently up to 276 days or 27 passages) to investigate the changes associated with aging of MSCs of patients and healthy controls and to investigate if these cells transform *in vitro*. To determine the occurrence of binucleate cells (implicating possible tetraploidy) we have performed fluorescent stainings of the aforementioned MSC cultures (high and low passages) and a new cohort of MSCs obtained from five healthy donors and five patients.

Results: Five genes had significantly lower expression levels in MSCs of OS patients compared to MSCs of healthy donors using Significance Analysis of Microarray (SAM, false discovery rate of 20.5%). One of the differentially expressed genes is eukaryotic elongation factor 1 alpha 1 (EEF1A1). Decreased expression of EEF1A1 has recently been associated with a high proportion of binucleate, tetraploid cells. In aging MSCs we have identified the occurrence of binucleate cells both in MSCs derived from patients as well as in MSCs from healthy controls. In addition, we have determined the occurrence of binucleate cells in low passages of OS patient derived MSCs.

Conclusion: Our study suggests a possible role for tetraploidization as an initiating event in OS pathogenesis. We are currently validating these results and are investigating the proportion of binucleate cells in low and high passages of MSC cultures of patients and controls.

Abstracts Research Line 3 talks

SCARRING AREAS IN GIANT CELL TUMOUR OF BONE: OF BIOLOGICAL IMPORTANCE?

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Background: Giant cell tumour of bone (GCTB) is a locally aggressive, benign tumour displaying unpredictable biological and clinical behaviour. It is morphologically characterized being heterogeneously composed of cellular and fibrotic (scarring) areas. Although no literature is present, these fibrotic foci are believed to be 'low-grade' counterparts of the tumour as is illustrated by the paucity of mitotic figures, mononuclear and osteoclastic –like giant cells. Above all, these areas are hypovascular. Recently, it was shown that hypoxia-induced factor (HIF) is expressed in GCTB being of importance in its osteoclastogenesis and therefore in its pathogenesis. Moreover, it is generally known that hypoxia-induced gene transcription promotes tumour phenotype and behaviour such as proliferation, angiogenesis, invasive and metastatic growth and dedifferentiation. These effects are mediated, at least in part, by targets of HIF.

Methods: In this study we determined the immunohistochemical expression of different HIF-downstream targets and of markers of proliferation, apoptosis and blood vessels in a tissue micro-array set of 133 formalin-fixed and paraffin embedded non-pre-treated GCTB, classified according to accepted guidelines (WHO). These markers were subdivided into different groups according to their known cell biological involvement: neo-angiogenesis (VEGF, VEGFR1, Ang2, Tie2, Endostatin), proliferation and apoptosis (Cyclin D1, Ki67, p53, bcl-2, PML, EiF4E), dedifferentiation (Oct4), invasion and metastatic potential (CXCR4, CXCL12, MMP2, MMP9, PAI1). As blood vessel markers CD31, CD34 and CD105 were used. Special attention was paid to their expression in fibrotic compared to (normo)cellular areas of GCTB.

Results: It was shown that different expression profiles are present in fibrotic compared to cellular areas. For all groups of markers, the expression was higher in the cellular areas. Most differentially expressed where Tie2 and Endostatin (neo-angiogenesis), eIF4E (proliferation and apoptosis), CXCL12, MMP2, MMP9 (invasive growth and metastatic potential). As expected, blood vessel markers were strongly positive in cellular areas.

Conclusion: In this study the findings of stronger expression of proliferation markers together with HIF-downstream targets in cellular areas when compared to fibrotic areas indicate that areas of fibrosis in GCTB display a less aggressive protein profile and that scarring, perhaps linked to hypoxia, may be of potential importance in determining GCTB morphology and biological behaviour.

ESTABLISHMENT OF THE CHICK CHORIO-ALLANTOIC MEMBRANE ASSAY FOR GIANT CELL TUMOUR OF BONE

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Background: Because of the lack of a suitable *in vivo* model for giant cell tumours of bone little is known about their biological behaviour and mechanisms of metastasis. No existing cell line contains all tumour components, so that testing of anti tumour agents is hardly possible. We therefore modified the chick chorio-allantoic membrane (CAM) assay for giant cell tumour of bone (GCTB).

Methods: Out of tumour tissue obtained during surgery of 5 patients a solution was produced. The solute was grafted onto the CAM at day 10 of embryonic development. The growth process was monitored by daily observation and photo documentation using *in vivo* microscopy. After 5 to 6 days of tumour growth the samples were fixed in formalin and further analyzed using standard histology (haematoxylin and eosin stains).

Results: The tissue solute of all 5 patients formed solid tumours when grafted to the CAM. *In vivo* microscopy and standard histology revealed a rich vascularisation of the tumours. The tumours were composed of the typical components of GCTB including multinuclear giant cells.

Conclusion: A reliable protocol for grafting of human giant cell tumours onto the chick chorio-allantoic membrane was established. This model is the first *in vivo* model for giant cell tumours of bone. Further characterization of the growing tissue is necessary in further experiments.

GLOBAL MOLECULAR PROFILE IN GIANT CELL TUMOURS OF BONE

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Supported by EU Project EuroBoNeT Consortium

Background: Our purpose is to identify a specific gene/protein signature associated with aggressiveness and risk of adverse events in patients with giant cell tumor (GCT) of bone.

Methods: First, we performed an overall investigation of the molecular background by using high-throughput screening techniques, such as microarray and proteomics, in a selected number of cases (5 disease-free (DF) and 5 metastatic patients), defining a cluster of differentially expressed molecules according to prognosis.
To date we validated the expression levels of Tenascin C (TNC), Osteocalcin (BGLAP), Chondroadherin (CHAD) integrin (ITAGBL1), fibromodulin (FMOD), by Real Time PCR comparative method and immunohistochemistry (IHC), in a larger series of cases inclusive of 34 disease-free and metastatic primary tumours, plus 12 local recurrences and 5 metastasis specimens.

Results: TNC was significantly more expressed in relapsed compared to non-relapsed tumours both at transcriptional and protein levels. In addition, at a minimum follow-up of 4 years, patients with TNC and BGLAP mRNA levels above the set threshold showed a significantly lower disease-free survival (p=0.002 and *p*=0.026 respectively). When enhanced TNC expression in primary lesions was compared in a multivariate analysis with the most significant parameters (grade, ploidy, BGLAP, ITAGBL1), it emerged that up-regulation of TNC defines a 6-fold increased risk for a post-surgical metastatic event.
On other hand, the global protein profile analysis by MALDI-TOF, identified Glutathione Peroxidase 1 (GPX1), Carbonate Dehydratase 2 (CAII), and the Heat Shock Protein 27 (HSP27) as candidate proteins.
When HSP27 expression was validated by WB and IHC analysis, we found that its amount and positive immunoreactivity was associated with metastatic event, assessing the prognostic value of this protein in giant cell tumours of bone.
Expression of CAII protein was evident in multinucleated cell pattern with a variable staining intensity depending on bone microenvironment conditions.

ACUTE HYPOXIA AND OSTEOCLAST ACTIVITY: A BALANCE BETWEEN ENHANCED RESORPTION AND INCREASED APOPTOSIS

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Background: Hypoxia is a central component of primary bone tumours and cancer metastases to bone. Hypoxia regulates gene expression via the transcription factor HIF (hypoxia-inducible factor), which is emerging as a central regulator of osteoblast function. Given the impact of osteoclasts, the primary mediators of pathological bone resorption, on the pathobiology neoplastic skeletal conditions, we investigated effects of hypoxia on human osteoclasts.

Methods: Mature osteoclasts were obtained by differentiation of PBMC with M-CSF + RANKL or by curettage of Giant Cell Tumour of Bone. Osteoclasts were exposed to a constant hypoxic environment then assessed for parameters including resorption (toluidine blue staining of dentine slices), membrane integrity (trypan blue exclusion), apoptosis (TUNEL, DAPI) and osteolytic enzyme activity (TRAP, Cathepsin K).

Results: 24 h exposure to 2% O_2 produced a 2.5-fold ($p<0.01$) increase in resorption associated with increased TRAP (40% increase, $p<0.01$) and Cathepsin K (15% increase, $p<0.01$) enzyme activity. HIF-1α protein was induced at 2% O_2 and HIF-1α siRNA completely ablated the hypoxic increase in osteoclast resorption ($p<0.001$) via a mechanism independent of VEGF. At the same time as increasing resorption, 24 h at 2% O_2 reduced the number of VNR-positive osteoclasts (76% reduction, $p<0.01$) and increased the proportion with compromised membrane integrity from 6% to 21% ($p<0.05$), with no change in total osteoclast number or the proportion of late stage apoptotic cells. Transient reoxygenation returned the percentage of trypan blue-positive cells to normoxic levels, suggesting that osteoclasts can recover from the early stages of cell death. Over 14-21 days, constant 2% O_2 dramatically inhibited osteoclast formation ($p<0.05$) and activity ($p<0.001$), whereas hypoxia / reoxygenation enhanced osteoclast differentiation at this pO_2 ($p<0.001$).

Conclusion: We have described time- and O_2 concentration-dependent effects on the activity and viability of human osteoclasts. These results suggest that within the normal bone microenvironment of 7-9% O_2, osteoclast resorptive activity and viability is comparable with that under standard tissue culture conditions. However in diseased bone, where the pO_2 may fall to $\leq$2% O_2, a delicate balance between osteoclast activation and apoptosis, modulated by periods of transient reoxygenation, mediates pathological bone resorption.

INVOLVEMENT OF *TNFRSF11A* POLYMORPHISMS IN THE DEVELOPMENT OF SPORADIC PAGET'S DISEASE OF BONE

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Background: RANK (receptor activator of nuclear factor-κB), encoded by the *TNFRSF11A* gene, is one of the most important proteins in differentiation of osteoclasts and in bone remodelling. Duplications in *TNFRSF11A* have been reported to cause Paget's disease of bone (PDB) –like diseases such as familial expansile osteolysis, expansile skeletal hyperphosphatasia and early onset Paget's disease of bone. Moreover, miss-sense *TNFRSF11A* mutations have been found in families with an osteoclast-poor form of osteopetrosis. Yet, neither *TNFRSF11A* mutations have been identified in the classic form of PDB nor association studies with *TNFRSF11A* polymorphisms have been performed in sporadic PDB.

Methods and Results: We have conducted an association study in our Belgian populations of 196 patients with sporadic PDB (83 females and 112 males) and 212 gender- and age-matched controls (86 females and 126 males). Based on HapMap, 27 tagSNPs and 5 multi-marker tests (MMTs) were selected in and around *TNFRSF11A* (±4kb). In addition, we have included 1 non-synonymous SNP (rs35211496C/T, H141Y) which is not present in HapMap. Genotyping was carried out by TaqMan assay and direct sequencing. Statistical tests (SPSS and WHAP) result in 13 single SNPs and 2 MMTs with a significant *P* value below 0.05 (P=3.17x10^{-4}–0.037). The association turned out to be within the female cohort for eleven out of the 13 single SNPs and the 2 MMTs. 6 single SNPs and 1 MMT withstand the Bonferroni correction (P<0.002) with the most significant result for the non-synonymous rs1805034C/T (A192V) with P=8,25x10^{-4} (OR of *T* allele= 0.619, 95%CI: 0.465-0.823).
In order to confirm our results, genotyping of the 2 non-synonymous SNPs was carried out in a Dutch (78 cases [35 females & 43 males] and 95 controls [46 females & 49 males]) and a British (282 cases [144 females & 138 males] and 325 controls [166 females & 159 males]) sporadic PDB population. Statistics of the 2 populations show significant *P*-values for both SNPs, with rs1805034C/T being the most significant one (P=8.8x10^{-5} in the Dutch and P=0.005 in the British). For rs35211496C/T, significance is only observed in the females of the 2 populations. However, for rs1805034C/T the males of both populations also give a significant *P*-value.
Finally, meta-analysis of the 2 non-synonymous SNPs was performed in all 3 European populations, resulting in a *P*-value of 4.73x10^{-8} for rs1805034C/T (common OR of *T* allele=0.635, 95%CI: 0.540-0.747) and a *P*-value of 0.004 for rs35211496C/T (common OR of *T* allele=0.728, 95%CI: 0.588-0.900).

Conclusion: In conclusion, these results provide a very strong indication that the *TNFRSF11A* polymorphisms 'rs35211496C/T' and 'rs1805034C/T' might be involved in the development of sporadic PDB in the Belgian, Dutch and British population. Further functional investigation will be needed to validate whether these 2 non-synonymous SNPs are truly the causative SNPs in *TNFRSF11A*.

Abstracts Research Line 3 posters

CD33(+) CD14(-) PHENOTYPE IS CHARACTERISTIC OF MULTINUCLEAR OSTEOCLAST-LIKE CELLS IN GIANT CELL TUMOR OF BONE: IMPLICATIONS FOR ITS OSTEOCLASTOGENESIS

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Background: GCTB is composed of uniformly distributed osteoclastic giant cells, thought to originate from the fusion of monocyte-macrophage lineage cells, in a background consisting of mononuclear rounded cells and spindle-shaped cells. Several reports revealed the specific expression of markers, such as CD14 on the mononuclear rounded cell population, however lacking on osteoclastic giant cells. Blood monocytes which were CD14+, CD33+ or CD14+/CD33+ have also been shown to be programmed as pre-osteoclasts. The macrophage marker CD33 is expressed earlier than CD14 in macrophage maturation while CD14 is expressed longer than CD33. The marker SIRPalpha is known to be expressed by cells undergoing phagocytosis. The aim of this study was first to investigate CD14 / CD33 expression profiles in GCTB. Second, to determine which cells could fuse into osteoclastic-giant cells.

Methods: 19 GCTB tumour samples of 19 patients were studied. Immunofluorescent and immunohistochemical analyses were performed with monoclonal antibodies against CD14, CD33, RANK, CD51 and SIRPalpha. In order to unambiguously further prove the expression of these molecules quantitative RT-PCR was employed with subsequent sequencing of its products.

Results: All samples showed similar immunoreactivity profiles. The mononuclear rounded cell population was positive for RANK, CD51, CD14, CD33 and SIRPalpha. The osteoclastic giant cell population expressed RANK and CD51, as well as CD33, but was consistently negative for CD14 and mostly negative for SIRP-alpha expression. Most of the CD14 mononuclear cells were SIRPalpha positive. The CD14 en CD33 profiles were confirmed by quantitative RT-PCR. These RT-PCR products were sequence verified.

Conclusion: Osteoclasts in GCTB are the result of fusion of CD33-expressing pre-osteoclasts that further fuse with CD14+/SIRPalpha+ mononuclear cells. Although these results reflect a static rather than a dynamic spectrum, we strongly believe that osteoclastogenesis seems not to be the exclusive result of fusion of intra-tumoural CD14+ mononuclear cells. Moreover, CD33-modulated osteoclastogenesis opens up the possibility for novel therapeutic directions.

CHROMOSOMAL INSTABILITY IN GCTB AND ITS POSSIBLE ASSOCIATION WITH CENTROSOME ABNORMALITIES

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Background and Methods: We investigated genetic instability in giant cell tumour of bone (GCTB) and correlated to its clinical behaviour, by analyzing 52 frozen tissue samples. Concentrating on the mononuclear cell population, ploidy-value was determined. A relocalization technique was used to perform FISH analysis on CD68 immunostained smears (allowing separation of CD68-positive and negative cells), examining chromosome X, 3, 4, 6, 11 centromeric and 11p sub-telomeric regions. To reveal genome-wide alterations, array-CGH was used on magnetically separated CD68-negative tumour cells.

Results: We found no significant numerical chromosome and telomeric alterations between CD68-positive histiocytes. Array-CGH and FISH showed clonal aberrations almost exclusively in the malignant group. Benign non-recurrent GCTBs displayed low levels of random aneusomy (10.65±3.66%), whereas in the recurrent group it was significantly higher (36.01±11.94%). Clonal alterations were characteristic only for the malignant group. Our results suggest that ploidy determination combined with FISH analysis can predict potential recurrence of GCTB.
The mechanism that generates the genetic instability in GCTB is poorly understood. Numeral and functional abnormalities of centrosomes result in mitotic spindle defects, leading to chromosome segregation errors, and are one of the major causes of chromosome instability in cancer, which accelerates the step-wise tumour progression.

Plans: We have started to investigate the possible centrosome alterations in GCTB using gamma-tubulin and pericentrin immunostaining on cases from Budapest and Oxford (smears from frozen material as well as TMA sections). Performing FISH combined with gamma-tubulin immunostaining we are able to examine the possible relationship between centrosome alteration and the aneusomy found in GCTB.

NON-CANONICAL PATHWAYS OF OSTEOCLAST FORMATION

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Background: Two growth factors essential for osteoclast differentiation are M-CSF, a macrophage survival and proliferation factor, and RANKL, required for osteoclast formation. A number of other cytokines and growth factors are able to substitute to some extent for either M-CSF or RANKL. We have identified three novel RANKL substitutes: APRIL, BAFF, and NGF. We studied the effect of these, and other previously identified MCSF substitutes, on osteoclast differentiation, formation and activity.

Methods and results: APRIL and BAFF are members of the TNF superfamily and NGF is a member of the neurotrophin family. We cultured peripheral blood mononuclear cells (PBMCs) with M-CSF (25ng/ml) and 5-200ng/ml of either APRIL, BAFF, or NGF. We found that at 25-200ng/ml each of these growth factors induced the formation of TRAP^{+} and VNR^{+} multinucleated cells capable of resorbing bone. On average each of these growth factors were able to resorb 2% of a dentine slice as compared to the positive control, M-CSF plus RANKL. By using osteoprotegrin, the endogenous decoy receptor of RANKL, we determined that the resorption induced by APRIL and NGF was independent of RANKL.
In the presence of RANKL, the M-CSF substitutes HGF (25ng/ml), VEGF (20ng/ml), PlGF (50ng/ml) and FLT3 Ligand (50ng/ml) induced osteoclast differentiation from PBMCs. Each substitute induced the formation of multinucleated TRAP^{+} and VNR^{+} positive cells capable of forming F-actin rings and lacunar resorption pits. The resorption observed was between 2-5% relative to the positive control, MCSF plus RANKL. Interestingly, we found that in combination MCSF, RANKL, HGF and VEGF synergistically increased resorption relative to MCSF and RANKL by 72%.
GCTB contains three cell populations: osteoclastic giant cells, mononuclear cells, and stromal cells. When APRIL (25ng/ml), BAFF (25ng/ml), and NGF (25ng/ml) were cultured for 24 hours with the osteoclastic giant cells, the resorptive activity of these cells was increased 2-3 fold above the negative control. When MCSF, RANKL or the MCSF substitutes were added to these cells resorption increased in 30% of GCTB cases by 2 fold, relative to the negative control.
Immunohistochemical staining of GCTB tissue micro-arrays demonstrated the presence of MCSF, RANKL, HGF, VEGF, FLT3 Ligand, PlGF, and APRIL, expressed by both the giant cell and stromal cell populations.

Conclusion: We have identified a range of MCSF and RANKL substitutes capable of inducing osteoclastogenesis. Further, these substitutes have been shown to interact and increase the resorption activity of GCTB osteoclastic giant cells. Consequently the presence of these osteoclastogenic factors in the GCTB microenvironment may partially account for the rich giant cell environment and the extensive bone destruction associated with this tumour.

GENE EXPRESSION ANALYSIS IN GIANT CELL TUMOUR OF BONE: PREDICTOR OF CLINICAL OUTCOME? A FIRST APPROACH

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Background: Predicting GCTB's biological behaviour has been a subject of increasing clinical interest. Until now, no specific or reliable predictive histological or biological parameters have been found, especially taking worst case scenarios into account such as metastases and recurrences.
As a first approach, a search for differential gene expression profiles was performed in the heterogeneous group of GCTBs with regard to specific clinical orientated questions. At a later stage (not part of this abstract) the molecular identities of the different groups will be specifically characterized and validated.

Methods: Following quality control of each preselected case, eight patient groups were selected (N=36).

- g0: pure GCTB without degenerative histological changes (n=4),
- g1: pure GCTB with degenerative histological changes (blood lakes, fibrosis, islands of foam cells) (n=5),
- g2: recurrent GCTB (at least third recidive) (n=2),
- g3: distal radius GCTB (n=5),
- g4: GCTB + ABC (n=5),
- g5: sacral GCTB (n=5),
- g6: ABC (n=5),
- g7: lung metastases (n=5).

Gene expression analysis was performed using Illumina Human-6 v2 Expression BeadChip microarrays on RNA lysates from the different GTCB-samples (TP3.1). Unsupervised and supervised analyses were performed using univariate and multivariate statistical procedures based on a framework established in the WWU and used by TP6.1.

In view of the supervised analysis, following questions were formulated:
1) Do specific locations (i.e. Distal Radius, Sacrum) display another mRNA profile?
a1) g3≠g0?, a2) g5≠g0?, b) g3+g5 ≠ g1+g0?, c) g3+g5 ≠ g0?
2) Is the presence of secondary ABC an separate phenomenon?
a) g4 = g1?, b) g6 = g4 ?
3) Do recurrences display another mRNA profile among the GCTB tumour group?
a) g2 ≠ g0 + g1?,b) g2 ≠ g3 + g4 + g5 + g0 + g1?, c) g2 ≠ g7 ?
4) Do regressive changes (pseudocyst, bleedings, fibrosis) display another mRNA profile?
a) g1 ≠ g0?, b) g1 ≠ g0 + g2 + g3 + g4 +g5 ?
5) Can we predict malignant / metastatic behaviour?
g7 ≠ g1 + g2 + g3 + g4 + g5 + g0 ?

Results: Unsupervised Analysis: Three subgroups can clearly be demonstrated: metastatic GCTB, primary ABC and a heterogeneous group of non-metastatic GCTBs.

Supervised Analysis

Question	Result	Number of differentially expressed genes	
		No MTC	**MTC (BH)**
1a1	No difference	10	0
1a2	No difference	3	0
1b	No difference	27	0
1c	No difference	13	0
2a	No difference	17	0
2b	Different (NS)	267	0
3a	Slight difference (NS)	27	1
3b	No difference	11	0
3c	Different	98	0
4a	No difference	4	0
4b	No difference	17	0
5	Different	N/A	685

NS: Not Significant / MTC: Multiple Testing Correction / BH: Benjamini-Hochberg

Discussion and Conclusion

1. Metastatic GCTB, primary ABC's and Non-metastatic GCTB form different subgroups
2. Out of the group of non-metastatic GCTB, RN7SK, a small nuclear RNA, could be identified as differentially expressed in the high recurrence group. This protein is involved in transcription of genes, serving as an important feed-back loop modulating the activity of RNA Pol II.
3. Metastatic GCTB significantly demonstrated differential gene expression.
4. Associated degenerative lesions and secondary ABCs seems not to be of importance

Conclusion: GCTB's worst case scenario's - recurrences and metastases – show a differential expression profile in a first approach by using gene expression analyses. Further analysis with subsequent validation is ongoing.

TELOMERE LENGTH ASSAY IN CLINICAL SAMPLES: IS TELOMERE LENGTH ARTIFICIALLY REDUCED BY STRESSFUL SURGERY?

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Introduction: Telomere length measurement is a popular and important technique in studying telomere biology of all sorts of tumours. Since it is well documented that in a short time interval of stress (30 minutes – 2 hours) the mean telomere length of peripheral blood can be reduced, it is of most interest in a “tumour biology - point of view” to determine its effect under surgical stress.

Materials & Methods: Twenty patients were selected undergoing a posterior lumbal intervertebrate fusion (PLIF). This surgical procedure takes about 2-3 hours and is known for its surgical stress. Peripheral blood samples were taken on different time points: before induction (t=0), one and a half hour after incision (t=1½) and twenty four hours after incision (t=24). In our experience t= 1½ reflects the time point of highest surgical stress. The peripheral blood samples (N=60) were collected in EDTA-tubes and after a maximum of 24 hours these samples were centrifuged. Directly after this procedure DNA was isolated and digested according to the manufacture’s protocol (Roche Telomere Length Assay Kit). To determine and compare the telomere lengths of each sample, gel-electrophoresis followed by southern-blotting was performed.

Results: After comparing the telomere lengths in each patient at different time points, no significant reduction in the mean telomere length could demonstrated.

Discussion & Conclusion: Under the influence of different sorts of stress, such as sporting or surgical stress, the mean telomere length in samples taken from the peripheral blood is reduced. Since telomere length measurements are in use in investigating telomere biology of tumours such as sarcomas, it is of most interest to determine any potential effect of surgery on this matter. In this study, according to our experience, PLIF was used as an example of stressful surgery (for the patient). Using different time points, we were not able to demonstrate any significant reduction in telomere lengths in the peripheral blood of these patients. In summary, surgical stress does not have any significant impact on telomere reduction. Therefore post-surgery measurements of telomere lengths in clinical samples, such as tumours, reflect true intrinsic mean telomere lengths.

Abstracts Research Line 4 talks

TRIGGERING OF CD99 INDUCES APOPTOSIS OF EWING'S SARCOMA CELLS THROUGH RE-ACTIVATION OF P53 FUNCTIONS

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Background: CD99 is a transmembrane 32 KDa protein, whose expression is consistently associated to Ewing's Sarcomas Family of Tumours (EWSFT) a class of child bone tumours with particular poor prognosis. Despite CD99 role in physiological and cancer scenery has not been yet fully elucidated, the employment of specific antibodies has brought insight into the molecular functions and specific pathways triggered by this protein, thus showing promising efficacy for the treatment of Ewing Sarcomas.

Methods and results: Particularly we evaluated the effects of the agonist monoclonal antibody, 0662, through Microarray and Phosphoarrays studies, showing that CD99 triggering is sufficient to modulate many important pathways involved in: I) adhesion; II) migration and metastasis; III) cell-cycle regulation and cell signalling; IV) apoptosis and death receptor signalling. These data are consistent with our previous analysis of 0662 effects in vivo and in vitro (Scotlandi et al. Cancer Research 2000): anti-CD99 monoclonal antibody triggers indeed homotypic aggregation and a rapid death response of ES cells, thus resulting in growth inhibition and reduced metastatic/colony-forming potential. We therefore validated micro/phosphor-array data by western blotting upon 0662 treatment in various ES cell lines and focused on its effects on signalling, cell cycle regulation and induction of apoptosis (points III and IV) to elucidate the molecular mechanism/s underlying these processes. Our studies are pointing out to the involvement of p53 and of its stability regulator, MDM2, in 0662 mediated apoptosis rather than a direct activation of caspases. Plus degradation of IKBa with subsequent activation of NfKB, might be either dependent on p53 activation and contributing to cell death as recently demonstrated by Kevin et al. (Nature 2000). These results are consistent with a higher sensitivity of p53 WT cell lines to 0662 treatment: whereas 6647, a $p53^{+/+}$ cell line, shows rapid induction of p53 canonical targets (p21, BAX), no induction of p21 is revealed in TC71, a $p53^{-/-}$ cell line. Moreover p53 involvement might give reason of the higher striking effectiveness we reported against local tumours and metastases in the combined employment of CD99 monoclonal antibodies and Doxorubicin (Scotlandi et al. Eur. Jour. Canc. 2006).

Aim: The main aim of our study is to achieve a greater insight into the molecular bases of ES disease and of CD99 expression-function, eventually establish whether any association of 0662 with other molecular targeted drugs, targeting e.g. the p53 regulators MDM2-MDM4, might enhance the apoptotic response and be therefore of therapeutic interest in the treatment of Ewing's Sarcomas.

HIGH EXPRESSION OF CHEMOKINE GENES BY EWING SARCOMA TUMOURS PREDICTS SURVIVAL

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Background: Chemokines are small, often inducible, cytokines that play key roles in directed migration of cells and angiogenesis, both in homeostatic and pathological processes. In cancer, the chemokine network may influence the extent and composition of the leukocyte infiltrate, angiogenesis and tumour cell growth, survival and metastases. Whether the chemokine system is involved in recruitment of immune cells to Ewing sarcoma (EWS) or affects cancer progression and prognosis is currently unknown. Characterization of the chemokine network and its roles in EWS may provide insight into EWS tumour biology as well as prognostic markers or molecular targets for additional (immuno-) therapeutic strategies.

Design: Twelve primary EWS tumours, eight EWS cell lines and mesenchymal stem cell (MSC) cultures obtained from three healthy donors were analyzed for mRNA expression of all known human chemokines using quantitative RT-PCR analysis. Leukocyte infiltration was detected immunohistochemically, by staining for $CD3^+$, $CD4^+$, $CD8^+$ and $CD14^+$ cells. All patients were treated according to the EuroEwing-99 protocol and follow-up information was available. Transwell migration assays were used to evaluate the chemotactic responses of immune cell subsets to EWS cell culture supernatants.

Results: Although hierarchical cluster analysis perfectly separated primary tumours from cell lines and MSC, extensive inter-tumour heterogeneity in chemokine gene expression levels was observed. A clear positive correlation was observed between expression of the anti-angiogenic, IFNγ-inducible and pro-inflammatory chemokines CXCL9 and CXCL10 and histological response to pre-operative chemotherapy (Student's t-test, $p<0.05$). In addition, expression levels of these chemokines and CXCL12 showed positive correlation with *in vivo* T-lymphocyte infiltration. CXCL11/CXCL12 and CCL5 expression by cell lines correlated with *in vitro* lymphocyte respectively monocyte migration as well. The most striking observation, however, was the significant positive correlation between 'total chemokine expression' in tumours, as determined by adding the expression levels of all individual chemokines, and overall survival (Kaplan-Meier method, logrank test $p<0.05$).

Conclusion: The significant correlation between high chemokine expression by EWS tumours and patient survival suggests that chemokine secretion has an important role in EWS tumour progression and patient outcome. Current research focuses on identification of the sources of individual chemokines within EWS tumours, their regulation and contribution to anti-tumour immunity and/or angiogenesis.

INFLAMMATORY GENE PROFILING OF EWING SARCOMA FAMILY OF TUMOURS REVEALS POTENTIAL TARGET MOLECULES FOR DRUG THERAPY

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Background: Ewing sarcoma family of tumours (ESFT), like other childhood cancers differ in their cell biology and tissue environment from adult-onset cancers that are predominantly carcinomas often preceded by a prolonged period of chronic inflammation. Vast majority of ESFT develop in close contact with muscular tissue. We characterized by bioinformatic tools the inflammatory signals that distinguish ESFT tumours from surrounding muscular tissue and whether these signals originate from ESFT cells or from tumour associated stromal component including infiltrating inflammatory cells.

Methods: Data-analysis was performed on gene expression profiles of 44 ESFT patient samples, normal muscle tissue (n=18) (GEO: accession numbers GSE6798 and GSE3526) and ESFT cell lines (n=11). List of 238 inflammatory genes was selected based on literature. Differential expression of these genes was compared by t-test between ESFT patients, cell lines and normal muscle tissue.

Results: 32 of 238 (13%) inflammatory genes were upregulated in ESFT patient samples vs. muscle cells. Similarly, 38 of 238 (16%) inflammatory genes were upregulated in ESFT patient samples vs. cell lines. We postulated that upregulated genes in both sets represent genes that are potentially stromal/immune cell derived. These genes included *CXCR4, STAT2, SPP1, IL8, IL23, HSPA6, SOCS3, TNFAIP3, FOS* and *CD14*. Genes that were upregulated in primary tumours in comparison to muscle tissue, but not if compared to ESFT cell lines, were regarded as putative ESFT derived genes. This list included e.g. *HMGB1, C5* and *MIF*.

Conclusions: Our results are in line with the previous IHC analyses showing that ESFT have a scarce immune cell infiltration. These results, however, point out several potential drug targets that could play a role in leukocyte migration into ESFT tissues (*IL8, SPP1, MIF, C5*) or be advantageous for tumour growth (*HMGB1, C5*). In near future, these biomarkers will be analysed in ESFT primary tumours at protein level and further validated in functional assays.

ASSESSMENT OF HUMAN MESENCHYMAL STEM CELLS AS A VALID CELLULAR MODEL FOR EWING SARCOMA PATHOGENESIS

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Background: The first relevant aim of this cooperative work inside EuroBoNeT is to elucidate the early molecular events previous to translocation in Ewing sarcoma. Characterization of human mesenchymal stem cells obtained from bone marrow iliac crest (hMSCs) of Ewing sarcoma patients revealed that EWS-ETS fusions are not present in these cells, as determined by RT-PCR, qRT-PCR and FISH (fusion probe). Analysis by flow cytometry showed the absence of Ewing sarcoma specific markers, such as CD13, CD54, CD99, CD117, CD166, and CD271.

Material and result: We have performed the comparison of hMSCs from 3 healthy donors versus 3 Ewing sarcoma patients at both, transcriptomic and proteomic levels. Affymetrix Human Gene 1.0 ST Arrays® and posterior bioinformatic analysis by SAM (significance analysis of microarrays) pointed out a Chitinase-3-like protein 1 precursor (Cartilage glycoprotein 39) as the most significant gene upregulated in all three hMSCs from Ewing sarcoma patients (9.5-fold induction) versus hMSCs from healthy donors. This gene has been associated with RAS signalling pathway in glioblastoma (Pelloski *et al.*, 2006), and high serum concentration is associated with poor prognosis. Further characterization of this gene will disclose its role in Ewing sarcoma initiation or propagation. Proteomic analysis of hMSCs from 3 healthy donors versus 3 Ewing sarcoma patients revealed two sets of proteins being overexpressed in patients with respect to normal donors. One set corresponding to cytoskeleton-related proteins such as actin-interacting protein 1, and a similar to cytoskeleton-associated protein. Another set of proteins comprised those mediating protein folding such as a rotamase and a T-complex protein 1 subunit beta (TCP-1-beta). Characterization of these proteins is in progress. Further experiments for the full characterization of patients and donors hMSCs involve an analysis of full genome promoter methylation and a telomere length assay.

We are also characterizing hMSCs coming from a patient in which Ewing sarcoma was localized in the left iliac bone and punctures were performed at the right iliac crest. In case the translocation event is present, isolation and characterization of sarcoma propagating cells will be carried out.

The second aim is to elucidate the early signalling pathways subsequent to translocation in Ewing sarcoma. For this purpose, we have transfected hMSCs obtained from 3 Ewing sarcoma patients with EWS-FLI1-pTet-Off combi and identified 2 inducible lines (patients 2 and 3, 12 hours induction). RNA was isolated from induction and non induction conditions (mock as control) and hybridization with Affymetrix Human Gene 1.0 ST Arrays® was performed. Bioinformatic did not reveal any significant deregulated gene present in both replicates after the induction time selected. Proteomic analysis of hMSCs from patient 2 transfected with the inducible vector (2 days induction vs. non induction, mock as control) allowed us to identify some proteins involved in biogenesis, folding and dynamics of cytoeskeletal proteins, such as TCP1, cofilin and profilin. Proteomic analysis for hMScs from patient 3 transfected with the inducible vector (2 days induction vs. non induction, mock as control) is in progress.

Reference: Pelloski CE, et al (2006) Clin Cancer Res 12:3935-41

Abstracts Research Line 4 posters

MULTIGENE-DOSAGE ANALYSES FOR THE DETECTION OF PROGNOSTIC RELEVANT DELETIONS IN EWING'S SARCOMA FAMILY OF TUMOURS (ESFT) – A TECHNICAL COMPARISON OF ACGH AND MLPA

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Aims: Various recurrent chromosomal losses in ESFT's have been discovered so far, however their prognostic significance needs to be validated. Therefore, in this study we compared different multigene-dosage analysis techniques to improve the identification and validation of prognostic significant gene deletions in ESFT's.

Methods: 14 Ewing cell lines were tested for deletions of 38 different tumour suppressor genes by multiplex ligation-dependent probe amplification and array comparative genomic hybridization. MLPA analysis was performed at least in duplicate.

Results: Data obtained from independent MLPA runs showed high degree of reproducibility (consistency of deletion call: 94%) .In addition, redundant MLPA probes always show identical results. Comparison of MLPA and aCGH data reveals 90% consistency (mean value). Frequent deletions confirmed by both techniques could be found, amongst others for CDKN2A and CDKN2B (both 9p21) in 8 cell lines (57%), CDH13 (16q24) in 5 cell lines (36%), FHIT (3p14) and CTNNB1 (3p22) in 4 cell lines (29%).

Conclusions: Deletion of CDKN2A/B represents the most prevalent loss of tumour suppressor gene in ESFT's. We could discover a new set of deleted tumour suppressor genes which may be of prognostic impact in ESFT's. MLPA has been proven a reliable method for identification and validation of candidate genes in ESFT's, and should be considered to be included in the analysis of prognostic relevant genes in routine diagnostics.

MUTATION ANALYSES OF TP53 AND CDKN2A IN BONE SARCOMAS

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Aims: While in Ewing's sarcoma (ES) loss of function of p53 and CDKN2A represent predictive markers for chemo-resistance and reduced survival, in osteosarcoma (OS) no such stringent clinical correlation of these genetic markers could be confirmed so far. To describe principle genetic differences in distinct bone tumour entities we correlate TP53 mutation and CDKN2A deletion status with expression of p53 and p16 protein in bone sarcomas.

Methods: TP53 gene mutation status was determined by DNA and RNA sequencing, deletion of CDKN2A/p16 by MLPA and aCGH. Analyses were performed on the complete EuroBoNeT panel of cell lines covering 14 ES, two chondrosarcomas and 18 OS. Tissue arrays of FFPE cell lines were used for protein staining (IHC).

Results: All cell lines showing homozygous deletion of CDKN2A were negative for p16 while 14/17 samples without any CDKN2A deletion were positive for p16. In 16 cell lines mutations for TP53 were found, 11 point mutations (4 non-sense, 7 miss-sense) and 5 insertions /deletions. 6/7 cases harbouring miss-sense mutations show elevated level of p53 protein expression (> 60% of the cells), while 6 cell lines with non-sense mutation or insertion / deletion were negative for p53 protein. 10 of the TP53 wild type cell lines show expression of p53 protein in 2-10% of the cells. 6 TP53 wild type OS were negative for both, p53 mRNA and protein.

Conclusions: While p16 protein expression is strongly correlated with gene dosage in our set of bone tumours, the induction of p53 protein expression by gene mutation was affected by high proportion of genetic changes leading to complete loss of protein expression. In contrast to ES, in OS downregulation of p53 mRNA may be a common mechanism of impairment of p53 signalling.

OSTEOCLASTOGENIC AND TUMOURIGENIC CAPACITY OF EWINGS' TUMOUR CELLS

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Background: Using an in vitro tumour bioassay system, we have previously demonstrated lacunar bone resorption mediated by osteoclasts formed from Ewings' tumour-associated macrophages. We now report a role for Ewings' tumour cells themselves in facilitating bone resorption, as well as modulation of the Ewings' tumour phenotype by local micro-environmental conditions.

Methods and results: Ewings' cell lines (A673, RD-ES, SK-ES-1, SK-N-MC, TC-71) were cultured for 5 days on dentine slices and release of collagen type I C-terminal peptide (CTX-1) was assayed by ELISA. All cell lines stimulated CTX-1 release in the presence of the osteoclastogenic agent RANKL. RANKL mRNA was present in Ewings' cell lines and RANKL protein was detected in Ewings' cells in formalin-fixed tumour sections. This suggests that bone destruction in Ewings' sarcoma is mediated by a combination of lacunar bone resorption by osteoclasts and background release of collagen from bone by tumour cells. Both mechanisms are aided by the presence of RANKL in the tumour, which may additionally influence osteoclastogenesis.
Ewings' tumours also express other osteoclastogenic cytokines, including M-CSF and VEGF. Hypoxia is a major regulator of VEGF, mediated via activation of the hypoxia-inducible transcription factor HIF. Ewings' tumours were positive for both HIF-1α and HIF-2α. All Ewings' cell lines induced HIF-1α and HIF-2α protein under hypoxia (24 h, 0.1-2% O_2), as well as the downstream genes VEGF and Glut-1. HIF-2α and VEGF were also induced by 24 h glucose deprivation, although to a lesser extent than under hypoxia. 24 h hypoxia produced a modest reduction in cell number (cell counts, CellBlue) associated with a 2 to 5-fold increase in apoptosis (caspase 3/7 activation). Conversely, glucose-deprivation dramatically reduced cell number despite no apparent change in apoptotic index. Hypoxia also demonstrates inhibitory effects on cell migration (scratch assay) and tube formation / vasculogenic mimicry. Experiments are underway to investigate effects of HIF-1α and HIF-2α RNAi on these phenotypes.

This data describes direct effects of Ewings' tumour cells on bone resorption. It also suggests that the local tumour microenvironment (hypoxia, glucose deprivation) could play a major role in modulating the tumourigenicity of these cells.

FUNCTIONAL VALIDATIONS OF WWOX, A CANDIDATE GENE IN 16q AND ONGOING WORK IN CDH13

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Background: As a part of the aims delivered by EuroBoNeT 4.1.3, secondary genomic alteration profiling of 52 cases and 16 cell lines from Ewing's Sarcoma (EWS) by BAC-array CGH studies (data previously presented), showed several recurrent copy-number alterations from which 16q loss was the most frequent deletion. In cell lines the minimum region of loss was a 5Mb region in 16q23.1-24.1. WWOX is the first candidate of a series of genes with potential tumour suppressor function placed in this region that our laboratory is functionally validating. The functional/clinical validation of another candidate (cdh13) is ongoing and will be likely presented in the meeting too.

Material and Methods: Real-Time RT-qPCR (Sybr Green): SybrMix (Biorad), 40 cycles, primer Fw TCCTGCCCGTGTCATTGTGG, primer Rv TGTTGGAGAGA GGCGACTGAAG, IQ5 device (BioRad). Western Blot: 30 µg of protein loaded on 12% polyacrylamide gels. antiWWOX serum kind gift of Dr. Kay Huebner (The Ohio State University) 1:20000 Overnight 4ºC. Retroviral Transduction: pMIG-WWOX or pMIG alone were Phosphate Calcium tranfected in HEK-293T along with pGNLV (gag-pol) and pMI-D (vsv-g evelope) vectors. 72 hours after transfection the supernatant was 0.44 filtered, mixed with 0.8 µg/ml polybrene and added to target EWS cell lines. MTT assay: MTT (SIGMA) was applied to the cell culture and incubated 1Hour at 37ºC. Cells were lysed with DMSO and colour was evaluated in a Tecan plate reader. Cell Cycle study: PI and RNAse treated cells were analysed in a *FACSort* (Becton Dickinson) flow cytometer using *CellQuest* (Becton Dickinson) software. Apoptosis study: AnV-DY634 and PI kit (Immunostep) was used to label the transduced population. Cells were acquired in *FACSort* (Becton Dickinson) flow cytometer using *CellQuest* (Becton Dickinson) software. Immunohistochemistry: done using a Discovery (Roche-Ventana Medical Systems) automated station with Ventana reagents. Clinical Correlation: using SPSS software.

Results: **WWOX functional/clinical validation results.** Levels of expression of the cDNA and protein from WWOX were confirmed to correlate with the underlying gene dose in the EWS cell lines by means of Real Time RT-qPCR and Western-Blot. WWOX cDNA was cloned in pMIG retroviral vector, which harbours an IRES-GFP sequence downstream the multiple cloning site. Retroviral supernatant from HEK-293T were used to transduce A4573 and STA-ET1 EWS cell lines. At time points of 48, 72, 96 and 120 hours after transduction the cell population was studied for proliferation (MTT assay), cell cycle state (Propidium Iodide and Flow Cytometry), apoptosis (AnV-PI assay by Flow Cytometry) and WWOX levels were checked by Western blot. No changes were detected among the pMIG-WWOX transduced population and the pMIG (vector alone) one.
Immunohistochemistry was done onto Düsseldorf's TMAs comprising 50 EWS cases. 6 cases were discarded due to the quality of the result. 11 out of 44 cases showed low expression levels. Studies of correlation with clinical parameters are ongoing and are planned to be presented in the meeting.
cdh13 ongoing results. The same methodology described above is being applied to the cdh13 gene (T-cadherin). At this time we have confirmed the correlations among expression and dosage (Real Time RT-qPCR and Western Blot), and cloned the cDNA in pMIG vector.

Conclusions: WWOX seems not to have a tumour suppressor effect on Ewing Sarcoma, at least in functional assays done in EWS cell lines. We expect to have conclusions on cdh13 in the next 2 months.

IS ES/PNET TUMOUR PROGRESSION IN NUDE MICE XENOGRAFT ASSOCIATED WITH PHENOTYPIC AND GENETIC ALTERATION?

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Introduction: Es/PNET is the third most common primary sarcoma of bone. Three main types are recognized: conventional Es, characterized by the classical Es configuration), PNET (with neuro-ectodermal traces and pseudo-rosettes), and atypical Es (large cells, pseudo-endothelial-like, spindle cells, and adamantinoma-like). Almost all Es/PNET display the t(11;22) q(q24;q12) or similar translocation, involving the EWS gene with an ETS member of the transcription factor family.

Material and Methods: A series of 14 Es/PNET cases were transferred to nude mice producing xenografts of the tumours that were maintained by subcutaneous implant for several generations in successive xenotransplants. A histological characterization of the original and transplanted tumours was performed using tissue microarray technology (3 tissue arrays were constructed). Immunostaining tested for HBA 71, HNK 1, Fli 1, CAV1, Ki67, and p53. Genetic studies including FISH for EWS break apart, 9p21 (p16) and p53 were performed in TMA.

Results: Tumour growth in the subcutaneous tissue, located in the back of the animal occurred in 100% of cases after a primary tumour transfer. A tumour size of 2-3 cm was reached within two to three months. No metastases were observed in any case. The histology of the 14 tumours was sub-classified based upon the previously indicated criteria, and their morphology followed in the xenografts. Histology was preserved in all cases and the tumour acquired more undifferentiated morphology during subsequent tumour passages. Immunohistochemistry of the original neoplasms was maintained, but CD99 expression decreased progressively in advanced xenografts. p53 remained stable, while ki67 increased successively in conjunction with a faster tumour growth over successive transfers. The molecular biology analysis revealed EWS/FLI1 gene rearrangement in all the cases: both in the original neoplasms as well as in the subsequent tumour passages. EWS break apart was positive in all cases while the percentage of cells with translocation increased during the subsequent passages. p16 deletion was observed in 4 cases (heterozygous/homozygous deletion), but without correlation with immuno-staining. The p16 methylation was seen as an epigenetic phenomenon in one case. Rare p53 alterations were found by FISH: only two cases displayed chromosome 17 polysomy and in one of these a p53 mutation was detected by molecular biology.

Conclusion: Xenografts of Es/PNET make an excellent model for analysis of the biology and genetics in this family of tumours, preserving their genotype and phenotype after several years of transfers into nude mice, however, a number of new genetic alterations appear associated with the progression of the tumour.

THE EXPERIENCE OF THE Es/PNET DATA BASE OF THE PROTHETS CONSORTIUM. A POSSIBLE BENEFIT TOOL FOR EUROBONET?

Llombart-Bosch A[1], Machado I[1], Lopez-Guerrero JA[2], Picci P[3], Scotlandi K[3], Karseladze A[4], Petrov S[5], Alvarado I[6], Mihaila D[7].

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Introduction: PROTHETS constituted a European Project (Contract Nº: 503036) with the objective of developing a collaborative study to define prognostic markers and new therapeutic targets in the Ewing's sarcoma family of tumours (ESFT). The aim of the project was to evaluate the prognostic relevance of selected markers and the effectiveness of therapeutic approaches targeting some of these molecules. The objective of this presentation is to present the value of the Data Base and the possibility for future applications

Material and Methods: A total of 856 formalin fixed, paraffin embedded tumours were collected and after histological and immunohistochemical evaluation 24 Tissue microarrays were constructed. Additionally a study was performed for the detection of FISH break-apart and molecular biology for EWS/FLI1 translocation. All clinical information available was introduced into the data bank.

Results: The Data Base included: The clinical data from each patient (case number, age, sex, anatomical location, tissue sample origin, clinical history number, biopsy number, diagnosis of the original center, metastasis and recurrence date, last control date, progression, overall survival, relapse free interval, type of treatment and the current status of the patient). Additionally the histological features of the tumour (histological pattern, vascular neogenesis, lobular pattern, filigree pattern, necrosis, apoptosis and mitosis) and EM of the available cases provided the final diagnosis and the differential diagnosis. The immunohistochemical studies comprised: CD99, Fli1, HNK, CCN3, epithelial, epithelial mesenchymal transition, cell cycle regulation markers, and adhesion molecular markers. The results of the molecular biology study indicated the gene fusion type, the presence of p53 mutation or p16 deletion. The FISH study of EWS break apart (translocation or not, and percentage of cell with translocation), 9p21 status (p16 deletion or no), p53 study (normal or polysomy). Additionally, the cultures performed, type of culture (from primary tumour, metastasis or from tumour xenograft) and the results of immunohistochemical, FISH, electron microscopy and molecular studies in Ewing/PNET cell cultures). Morphometric data was also incorporated.

Conclusion: At present, following this study, a new review of the remaining tissue has been made, comprising a set of well-characterized Es/PNET, including clinical follow up, material from 387 cases is available of which 12 new TMA have been constructed. These cases have been incorporated into a new data base which includes the previously indicated information regarding the morphology, phenotype and genotype of all tumours.

CHEMO-RESISTANT EWING SARCOMA ARE SUSCEPTIBLE TO IL15-ACTIVATED NK CELLS WHICH CAN BE MODULATED BY HISTONE DEACETYLASE INHIBITORS

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Depts. of Pathology[1] and Pediatrics[2], Leiden University Medical Center, Leiden, The Netherlands; Institute of Pathology[3], Heinrich-Heine University, Düsseldorf, Germany; Laboratory of Oncology Research[4], Rizzoli Orthopaedic Institute, Bologna, Italy

Background: Despite multimodal therapy, patients with refractory or relapsed Ewing sarcoma (EWS) have poor prognoses. To explore the feasibility of natural killer (NK) cell-mediated immunotherapy for patients with advanced-stage EWS, we investigated susceptibility of both chemo-sensitive and chemo-resistant EWS to NK cell-mediated cytolysis and identified pivotal molecular mechanisms involved in cytolysis. In addition, we studied the impact of histone de-actetylase inhibitors (HDACi) on susceptibility to NK cells

Methods: Expression of ligands for inhibitory and activating NK cell receptors was evaluated in chemo-resistant (n=6) and -sensitive (n=6) EWS cell lines by flow cytometry. Cytotoxicity was determined in chromium release assays, using freshly isolated (resting) and interleukin (IL)-15 activated NK cells obtained from healthy donors. Blocking antibodies against specific ligands/receptors were used to study contribution of these molecules.

Results: All cell lines were lysed by resting NK cells, except for the chemo-resistant CADO-ES. Ligands for activating NK cell receptors DNAM-1 and NKG2D were expressed by all cell lines. Cytotoxicity depended on these receptors, since blocking either DNAM-1 or NKG2D abrogated lysis by resting NK cells. IL-15 activation of NK cells increased efficacy of lysis in all cell lines, including CADO-ES, and resulted in more efficient recognition, since only combined DNAM-1/NKG2D-blockade inhibited lysis. Compared to other cell lines, CADO-ES expressed high levels of HLA class I; in addition, the alleles expressed by these cells are ligands for all inhibitory NK cell receptors (KIR). In CADO-ES, cytolysis by resting NK cells depended on loss of inhibition, since HLA class I-blockade reversed resistance. Pre-treatment with HDACi resulted in increased expression of NKG2D ligands, and preliminary data indicate increased lysis of chemo-resistant EWS cells by NK cells.

Conclusion: The observed susceptibility of chemo-resistant Ewing sarcoma to cytolysis by cytokine-activated NK cells and the possible role of HDACi on this process may provide patients with advanced-stage Ewing sarcoma with an additional treatment modality.

ASSOCIATIONS BETWEEN WNT-BETA CATENIN SIGNALING AND PROGNOSIS IN EWING'S SARCOMA

Maria Cristina Manara[1], Diana Zambelli[1], Marika Sciandra[1], Sakari Knuutila[2], Antonio Llombart-Bosch[3], Piero Picci[1] and Katia Scotlandi[1]

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Material and results: Real Time PCR analysis of Ewing's sarcoma (EWS) cells silenced for CD99 suggested a down-modulation of Wnt signalling. In fact, TC-71 cells deprived of CD99 (TC-siCD99) showed increased expression of some inhibitors of the Wnt pathway (DKK1, sFRP1) and reductions in some ligands (Wnt 2 and 11). Since TCsiCD99 are less malignant than parental cells, these preliminary data indicated a possible relationship between Wnt pathway and EWS aggressiveness. Gene expression profiling of 30 primary EWS tumours confirmed these indications: low expression of Frizzled (Fzd) 2,3 and LRP5/6 as well as low expression of some Wnt ligands (Wnt3, 11) are significantly associated with better prognosis. Thus, we decided to validate these associations at protein level by evaluation of Fzd3 and beta-catenin in EWS tissue arrays. Though we confirmed that lower levels of Fzd3 are associated with better prognosis, we surprisingly did not found beta-catenin nuclear localization in any of the 103 cases examined. In contrast, around 40% of patients showed high expression of beta-catenin at the membrane level and these patients showed better prognosis. It is well known that beta-catenin is an essential co-activator of the canonical Wnt pathway but it also plays a critical structural role in cadherin-based adhesions. Beta catenin contribution to these two functions is dictated from various cell-signalling events. In EWS, beta-catenin membrane associated localization suggested its major role in cell-cell adhesion. Indeed, direct and significant associations were observed with other adhesion proteins, such as ZO-1, desmoplakin and desmoglein ($p<0.05$, Fisher t test). Higher expression of these proteins is also associated with better prognosis, indicating the positive value of adherence and tight junctions in limiting EWS aggressiveness. As far as Wnt pathway is concerned, lack of nuclear beta-catenin in EWS samples suggested that the Wnt canonical pathway is not activated. However, Frzs and Wnts are also involved in the delivery of a beta-catenin independent signalling, which is dependent on cytoskeleton organization and intracellular Ca+ release. Genetic expression of mediators of these non-canonical signalling pathways (CAMK, RAC1) are also associated with EWS prognosis.

Conclusion: In conclusion, our study indicates that: 1. canonical Wnt pathway is not activated in EWS, whereas non-canonical Wnt pathways are and they show prognostic relevance. 2. beta-catenin functions in EWS are more related to regulation of cell-cell adhesion than activating Wnt target genes transcription.

MOLECULAR EVENTS IN THE ANGIOGENESIS INITIATION OF HUMAN SARCOMAS

López-Guerrero JA[1], Machado I[2], Mayordomo E[2], Carda C[2], F. Giner[2], S. Clabauig-Fariñas[2], Pellín A[2], Peydró-Olaya A[2], Antonio Llombart-Bosch[2].

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Background: Tumour growth is mainly supported by vascular neogenesis, provided by the host but induced by the tumour cells. When a growing tumour exceeds the size of 1 mm^3, diffusion fails to provide the essential nutrients for continuous growth. At this point the hypoxia phenomenon begins and the angiogenic process is activated through the well-known hypoxia inducible transcription factors (HIF) which induce the expression of several tumour-derived cytokines, such as vascular endothelial growth factors (VEGF) or fibroblast growth factors. Microvessels lined by endothelium grow into the tumour, providing a blood supply and allowing the tumour to grow beyond the limits imposed by substrate diffusion. We have used a *nude mice* model to study the initiation of this process in xenotransplanted human sarcomas.

Material and methods: Fifteen human sarcomas were evaluated: three Ewing's sarcomas (ES), three osteosarcomas (OS), four chondrosarcomas (Chs), two synovial sarcoma (SS), two gastrointestinal stromal tumours (GIST) and a fibrosarcoma (FS). Tumour pieces of 0.3-0.4 cm in size were implanted into the backs of nude mice (athymic Balb-c nude mice) which were sacrificed at 24, 48, 72 hours, 7, 14, 21 and 28 days from implantation. The tumour was cut and divided into three pieces for EM, histology and molecular biology. RNA was obtained from fresh tissue and the expression of 96 angiogenesis related genes was evaluated by means of quantitative RT-PCR using the micro-fluidic cards technology. Among these genes are: 5 promoters of angiogenesis; 41 growth factors and their receptors; 15 chemokines and cytokines; 5 adhesion molecules; 17 matrix proteins, proteases and inhibitors; 6 transcription factors and other 7 genes. Blood samples from mice were also evaluated for serum levels of human and mice VEGF.

Results: The complete series of experiences is currently being analyzed and the results herein reported should be considered as provisional. We have observed that after 24-48 hours from implantation all tumours expressed high levels of several angiogenic factors, especially VEGF and their receptors, and FGF2. At this point the stroma surrounding the tumour initiates an angiogenic induction producing capillary congestion. Co-option between endothelial cells and tumour cells together with pseudo- vessels constituted only by tumour cells (vascular mimicry) is also observed. Cluster analysis showed that the expression profiles at 48h and 1 week had the same behaviour and in turn these correlated with the angiogenesis induction and the angiogenic remodelling within the tumour respectively.

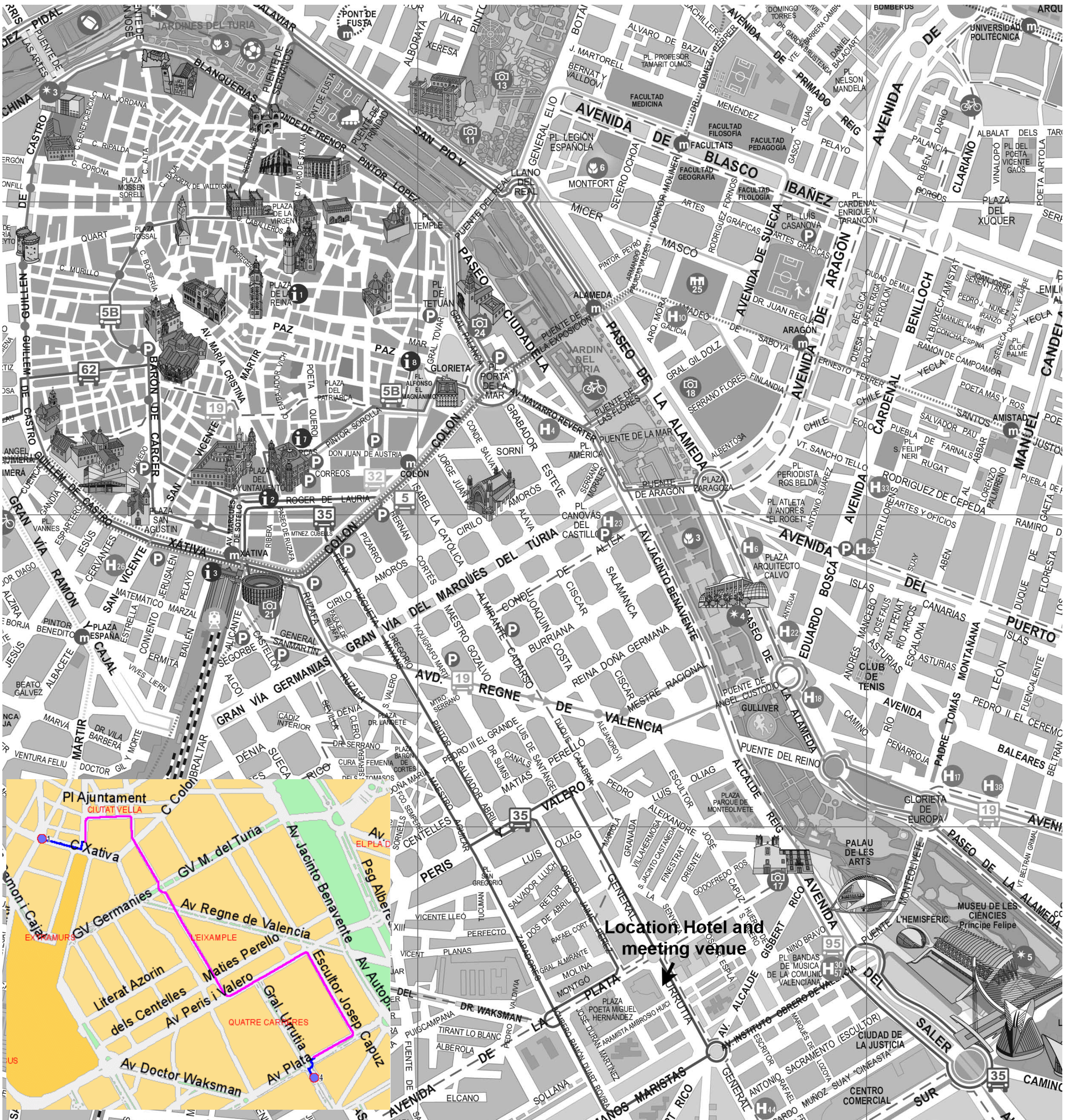
Conclusions: The model herein presented offers an excellent way in which to study the initiation process of tumour angiogenesis and provides new ways to assess the activity of potential inhibitory agents associated with tumour growth.

Map of Valencia

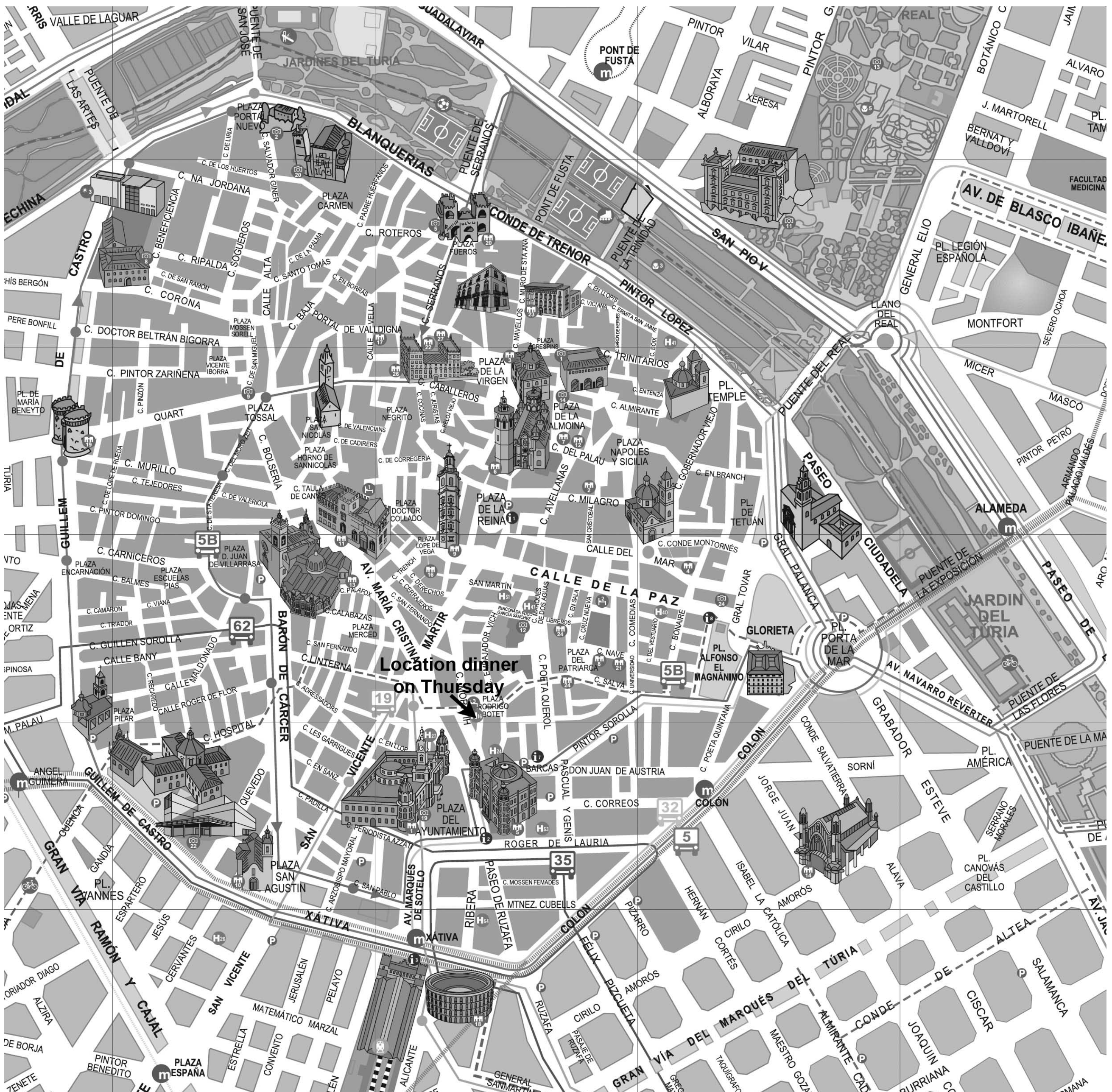
Route summary from the train station (*Renfe, C. XATIVA)* **to the hotel**

See inset:

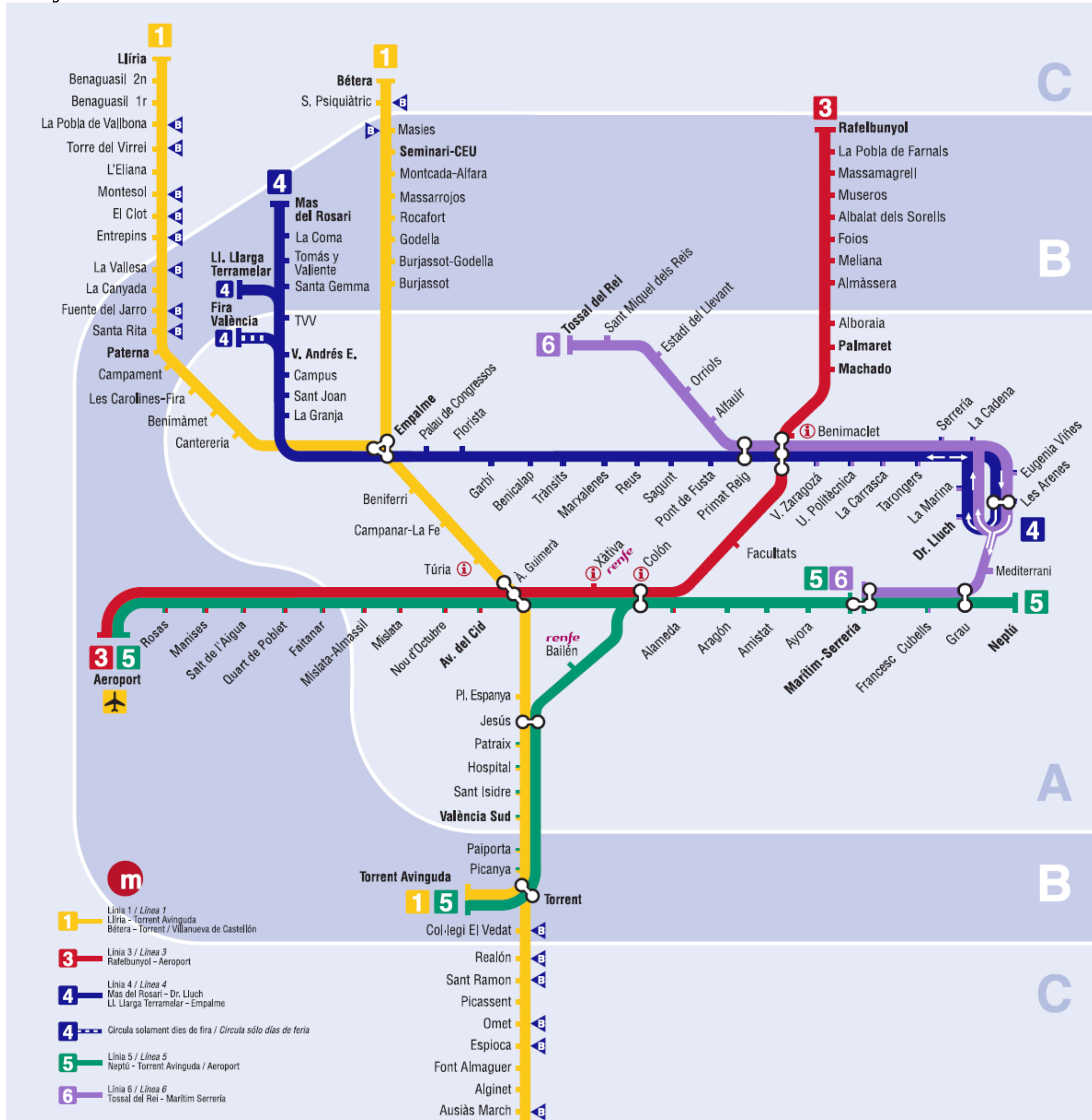
- Walk from *C. XATIVA*, 269 meters to the bus stop *“1632-Marqués de Sotelo – Xátiva”*
- Catch bus line 14
- Get off after 11 bus stops in *“685-la Plata - General Urrutia”*
- Walk from bus stop *“685-la Plata - General Urrutia”* 144 meters to the *C. GENERAL URRUTIA, 48 Hotel Medium*



Old City Centre



Metro system



RESTAURANTS SEE SIDE (BEACH AREA)

Casa Zaragoza
Comida Mediterránea
Paseo Marítimo Malvarrosa 12 Valencia, 46001

La Murciana
Comida Mediterránea
Paseo Marítimo Malvarrosa 10 Valencia, 46001

La Murciana
Comida Mediterránea
Paseo Marítimo Malvarrosa 10 Valencia, 46001

El Trompo
Arrocerías
Paseo Marítimo Malvarrosa 10 Valencia, 46001

RESTAURANTS DOWN TOWN

El Forcat
Comida Mediterránea
Calle Roteros 12 Valencia, 46003

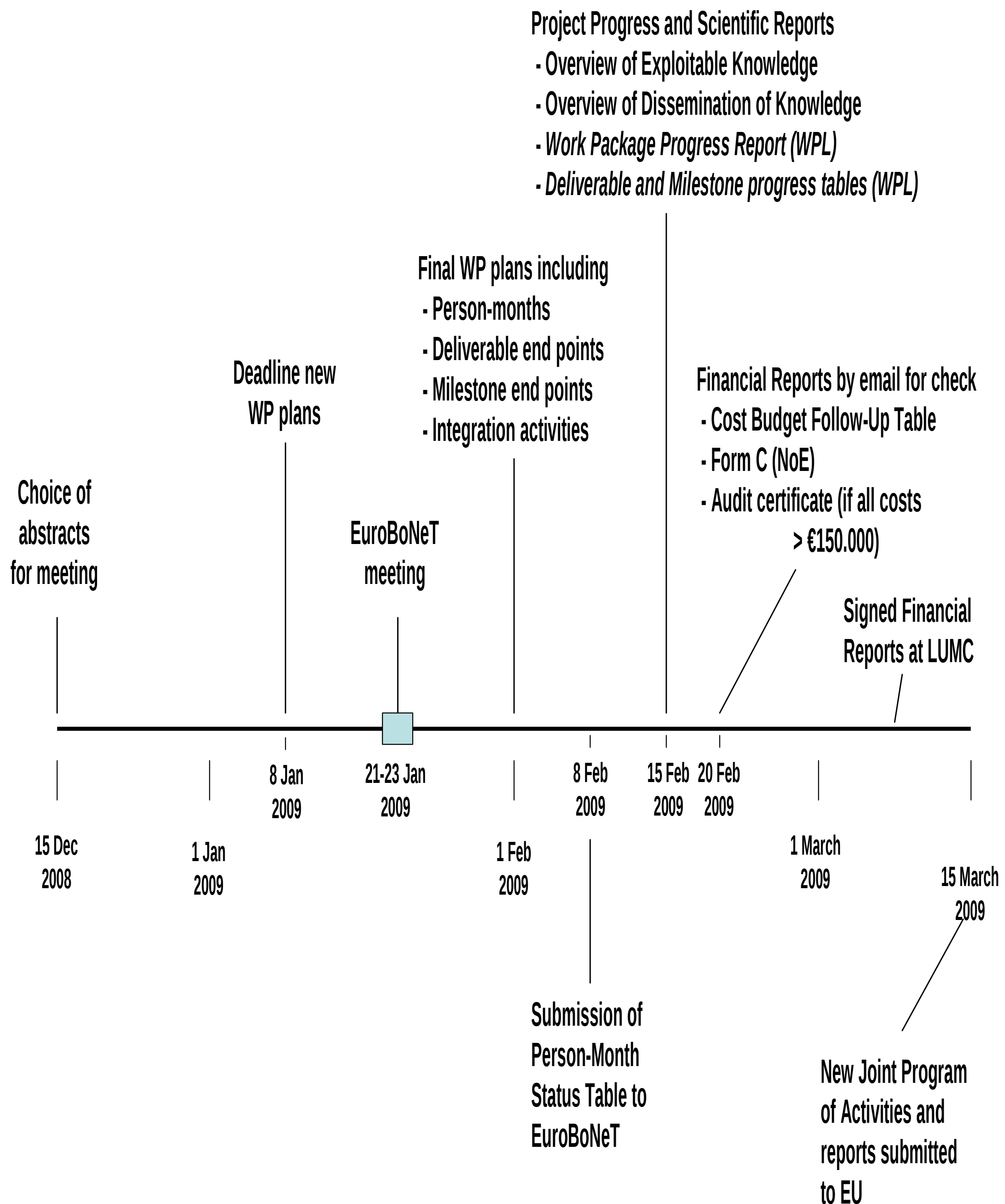
El Generalife
Comida Mediterránea
Caballeros, 5 Valencia, 46001

El Tossal
Arrocerías
Calle Quart 6 Valencia, 46001

Asador del Carme
Asador
Plaza Carmen 6 Valencia, 46003

La Pitanza
Comida Mediterránea
Calle Quart 5 Valencia, 46001

Time Line for reporting



The work package leaders are expected to collect the progress form the partners involved and combine this to one work package progress report. Please inform the partners on time to achieve the deadline of the 15th of February

The submission of the **"Person-Month Status Table"** should be submitted to EuroBoNeT. EuroBoNeT will communicate the person months of each partner to the WP leaders, who will divide this over the deliverables. This is because last year we found discrepancies between the person months reported by the WP leaders and the ones reported by the partners. In this way we hope to avoid these problems this year.