743 Lymphatic microvessel density and mast cells in molecular types of breast cancer

P. Gaje[1], A.M. Cimpean[1], M. Raica[1], D. Ribatti[2]. [1]*Victor Babes University of Medicine and Pharmacy, Histology, Timisoara, Romania,* [2]*University of Bari Medical School, Anatomy and Histology, Bari, Italy*

Background: The importance of lymphangiogenesis in the natural evolution of breast cancer is well known, but the mechanisms that stimulate this process are not fully understood. Although the contribution of mast cells to tumour angiogenesis is well documented, their involvement in lymphangiogenesis is virtually unknown. In the present work we show a relationship between lymphatic microvessel density and mast cells in HER2 and luminal types of breast cancer.
Material and Methods: There were investigated 55 patients with ductal invasive breast carcinoma and from these, 26 had lymph node metastases. Patients were stratified according the molecular classification, based on the immunohistochemical expression of estrogen receptor, progesterone receptor, HER2 protein, cytokeratin 5, p53, epidermal growth factor receptor, and Bcl-2. Double staining for D2−40 and mast cell tryptase was performed to evaluate the number of lymphatic vessels (LVs) and mast cells (MCs). Counting of LVs and MCs was performed in the same fields, at magnification $\times$200. Results were statistically analyzed, taking into account the clinico-pathological factors of prognosis and the molecular type of carcinoma.
Results: We found basal-like carcinoma in 8 cases (14.54%), luminal A in 26 (47.27%), luminal B in 7 (12.72%), HER2 in 10 (18.18%), and unclassified tumours in 4 cases (7.27%). LVs were found in the peritumoural stroma in all cases (0.6–15.3/x200) and in the intratumoural area in 39 cases (range 0 to 6.6). MCs were found in both intra-/peritumoural area (3 to 123.33, and 7 to 61.6, respectively). No correlation was found between LVs/MCs, and stage and grade of the tumour. A strong positive correlation was found between LVs/MCs and lymph node metastasis in HER2 and luminal types of breast cancer. A significant negative correlation was found between LVs/MCs and basal-like carcinoma.
Conclusion: Our data suggest that MCs could be involved in breast cancer-associated lymphangiogenesis, and LVs/MCs count is a useful predictor of lymph node metastasis.

744 WWOX tumour suppressor gene is involved in Wilms tumour cancerogenesis in a haploinsufficiency way

W.I. Wujcicka[1], E. Pluciennik[1], E. Zielinska[2], A.K. Bednarek[1]. [1]*Medical University in Lodz, Department of Molecular Cancerogenesis, Lodz, Poland,* [2]*Medical University in Lodz, Paediatric Oncology and Hematology Clinic, Lodz, Poland*

WWOX gene (*locus* 16q23.3–24.1) is known to play crucial role in suppression of tumourigenesis in different cancers. It encompasses common chromosomal fragile site FRA16D characterized with frequent LOH and chromosomal rearrangements observed in many cancers along with Wilms tumour (*nephroblastoma*). Despite of available molecular data on childhood *nephroblastoma* tumourigenesis, along with LOH and methylation level alterations of 16q chromosomal fragment, detailed modifications of *WWOX* gene associated with this tumour type lesions still remain unclear. The main aim of our study was to investigate epigenetic alterations of *WWOX* gene associated with *nephroblastoma* cancerogenesis and to assess this gene function in progression of this tumour.
We analyzed 24 Wilms tumour samples stored at −80ºC till RNA extraction. To evaluate LOH frequency of FRA16D we performed genomic analysis of three highly polymorphic microsatellite markers: D16S518, D16S3096 and D16S504 residing inside *WWOX* gene. Meanwhile, to assess methylation level of two *WWOX* promoter regions we performed MethylScreen assay. By comparative quantitation analysis we assessed expression level of *WWOX* gene and in addition other genes potentially associated with tumourigenesis (*BCL2*, *BAX, CCND, CCNE1, KI67, CDH1*, *TP73, EGFR, ERBB2* and *ERBB4*). To analyze WWOX protein expression we performed Western-blot assay with anti-WWOX rabbit primary polyclonal antibodies.
We identified for the first time in Wilms tumour relatively high LOH frequency of FRA16D markers with the lowest value for D16S518 marker. Hemizygosity was observed at frequency of 16.33%, 49% and 46.83% for D16S518, D16S3096 and D16S504 markers, respectively. In analyzed tumours the level of *WWOX* gene expression was positively correlated with *BCL2* expression ($p < 0.0001$) and *BCL2*/*BAX* ratio ($p = 0.035$), *CDH1* ($p = 0.001$) and *ERBB4* expression ($p = 0.003$). Our results showed also association of *WWOX* gene expression with methylation of its promoter.
Loss of heterozygosity observed in FRA16D region demonstrate involvement of *WWOX* gene in kidney tumourigenesis in a haploinsufficiency fashion. Additionally, positive correlation of *WWOX* gene expression level with lowered apoptosis driven by elevated expression of anti-apoptotic marker − *BCL2* gene, association with expression of other tumour suppressor gene − *CDH1*, coexpression with membranous ERBB4 receptor and association with clinical data confirm tumour suppressor function of investigated gene in Wilms tumour.

745 The PPARgamma-independent antiproliferative effects of thiazolidinediones in breast cancer cells are partially mediated by an ER-stress-related induction of EGR1

S. Chbicheb[1], X. Yao[1], I. Grillier-Vuissoz[1], J.L. Rodeau[2], M. Boisbrun[3], Y. Chapleur[3], S. Mazerbourg[1], S. Flament[1]. [1]*Université Henri Poincaré, EA SIGRETO, Vandoeuvre-lès-Nancy, France,* [2]*INCI, UPR CNRS 3212, Strasbourg, France,* [3]*Université Henri Poincaré, UMR CNRS 7565, Vandoeuvre-lès-Nancy, France*

Background: The treatment of human breast cancer cell lines with agonists of peroxisome proliferator-activated receptor gamma (PPARγ) inhibits proliferation. However, these antiproliferative effects seem mainly to be the result of a PPARγ-independent action. In the present study, we aimed at characterizing the PPARγ-independent signaling pathway of PPARγ agonists in the hormone-dependent breast cancer cell line MCF7.
Material and Methods: MCF7 cells were exposed to various PPARγ agonists: Rosiglitazone (RGZ), Ciglitazone (CGZ), Troglitazone (TGZ) and the prostaglandin 15d-PGJ_2. A derivative of TGZ (Δ2-TGZ) devoid of PPARγ agonist activity was also used. Gene expressions were studied by RT-PCR and microarray. Proteins were analyzed by western blotting and immunolocalization. The involvement of signaling pathways was demonstrated using pharmacological agents and RNA interference. Calcium was measured by fluorescent imaging.
Results: EGR1 (Early Growth Response gene 1) mRNA and protein levels peaked after 3 hours of incubation with 25 μM TGZ, CGZ or 15d-PGJ_2 and then gradually decreased. RGZ, the most potent activator of PPARγ, did not show this effect. The PPARγ antagonist GW 9662 did not block EGR1 mRNA induction which also still occurred in case of PPARγ silencing as well as in case of treatment with the PPAR?-inactive compound Δ2-TGZ. Moreover, the MEK/ERK inhibitor U0126 abolished the EGR1 mRNA induction triggered by Δ2-TGZ, TGZ, CGZ and 15d-PGJ2. ERK1/2 phosphorylation and EGR1 mRNA induction were not blocked by EGF Receptor (EGFR) inhibitors (AG1478 and PD153035) whereas these events were prevented by calcium chelation suggesting an increase in cytosolic calcium. Indeed, Δ2-TGZ triggered a rise in intracellular calcium that was associated with endoplasmic reticulum (ER) stress as suggested by ER enlargements and demonstrated by several markers including activated PERK, phosphorylation of eIF2α splicing of XBP-1 and expression of DDIT3. Finally, siRNA targeting EGR1 demonstrated that the early induction of this gene is involved in the antiproliferative effects of Δ2-TGZ.
Conclusions: Taken together, our results show that in MCF7 breast cancer cells, the expression of EGR1 is triggered in a PPARγ-independent manner and is involved in the antiproliferative action of thiazolidinediones. The development of compounds able to disturb calcium homeostasis and to trigger ER stress could be interesting for breast cancer treatment.

746 Combination of Smac-mimetics and TNFalpha induce apoptosis in glioma cell lines

C. Battaglia[1], M. Marizzoni[2], P. Seneci[2], C. Drago[2], C. Scolastico[2]. [1]*University of Milan, Scienze e Tecnologie Biomediche, Milan, Italy,* [2]*University of Milan, Center for Intedipartimental Studies and Industrial Application (CISI), Milan, Italy*

Smac/DIABLO, through its AVPI motif, is able to antagonize the anti-apoptotic function of XIAP and to induce cIAP1−2 degradation. XIAP, cIAP1−2 belong to a class of central apoptosis regulators called Inhibitor of Apoptosis Proteins (IAPs) and are upregulated in different tumours. For these reasons, many efforts have been made to design molecules that mimic the activity of the endogenous Smac/DIABLO (Smac-mimetics), because they could represent a new class of anticancer drugs able to overcome the resistance to apoptosis of cancer cells. Two types of Smac-mimetics have been developed. Monovalent molecules mimic the binding of a single AVPI binding motif to one BIR domain of IAP proteins, while bivalent compounds, containing two AVPI binding motifs tethered together through a linker, can bind to two BIR domains. Recently, it has been demonstrated that Smac-mimetics can potentiate TRAIL and TNFα mediated cell death in tumour cell lines [1].
Astrocytoma is the most common glioma and can differentiate in glioblastoma multiforme (GBM), one of the most malignant cancers. GBM is marked by high resistance to chemo- and radiotherapeutics and by a median survival of less then 1 year. The initial therapy produces only palliative effects, the recurrence of the tumour is leading to rapid death [2]. Since novel therapies for malignant gliomas are desperately needed, we examined whether newly synthesized Smac-mimetic compounds [3], could sensitize astrocytoma and glioblastoma cell lines to apoptosis. To this purpose, we assessed the viability of T98G, U87MG and CCF-STTG1 cell lines using MTT assay. The cells have been treated with several Smac-mimetic compounds, alone or in combination with TNFα.
We have identified a dimeric Smac-mimetic compound (Smac-083) that, in combination with low doses of TNFα (0.01 ng/mL), significantly sensitizes T98G cell lines to apoptosis already at nanomolar concentrations. Moreover,

the combination induced a good inhibition of CCF-STTG1's cell growth but was inactive on U87MG cells. Microarray gene profiling and apoptosis pathway proteins' assessments will also be presented to further characterize genes or pathways involved in the synergistic effect of Smac-083 TNFα-induced apoptosis in glioblastoma cell lines.

Reference(s)

[1] Li, L. *et al* (2004) *Science* 305(5689): 1471−4.
[2] DeAngelis L.M. (2001) *N Engl J Med* 344(2):114−23.
[3] Seneci, PF. *et al* (2009) *Bioorg Med Chem* 17(16):5834−56.

747 Human arm protein lost in epithelial cancers, on chromosome X 1 gene is transcriptionally regulated by CREB and Wnt/beta-catenin signaling

A. Takeda[1]. [1]*International University of Health and Welfare Hospital, Surgical Oncology, Tochigi, Japan*

Background: The aberrant activation of the Wnt signaling is a key process in colorectal tumourigenesis. The canonical Wnt signaling controls transcription of target genes via b-catenin and T cell factor/lymphoid enhancer factor family transcription factor complex. *Arm protein lost in epithelial cancers, on chromosome X 1 (ALEX1)* is a novel member of the Armadillo family which has two Armadillo repeats as opposed to more than six repeats in the classical Armadillo family members. The regulatory mechanism of *ALEX1* gene in normal and cancer cells remain largely unknown. Here we examined cis-regulatory elements and trans-acting factors involved in the transcriptional regulation of *ALEX1* gene.

Material and Methods: Human colon cancer cell lines (HCT116 and SW480) and pancreatic cancer cell line (PANC-1) were used in this project. The putative promoter region of the human *ALEX1* gene from −1933 to +487 was amplified by PCR and subcloned into the pCR-Blunt II-TOPO plasmid. The luciferase reporter plasmids driven by deleted mutant types of *ALEX1* promoter were generated by the same PCR-based method. The luciferase reporter assay was performed using the Dual-Glo Luciferase Assay System. PANC-1 cells were transfected with the ON-TARGETplus Non-Targeting Control siRNA or siRNAs targeting CREB using DharmaFECT1 siRNA. Chromatin immunoprecipitation was carried out with Dynabeads Protein G and polyclonal anti-CREB antibody. Indirect immunofluorescence for b-catenin was performed with Rhodamine-conjugated anti-mouse IgG antibody, whose images were obtained using a Axiovert 200M fluorescent microscope.

Results: Site-directed mutations of a cyclic AMP response element (CRE) and an E-box impaired the basal activity of human *ALEX1* promoter in colorectal and pancreatic cancer cell lines. Moreover, overexpression of CRE-binding protein (CREB) increased the *ALEX1* promoter activity in these cell lines, whereas knockdown of *CREB* expression decreased the expression level of *ALEX1* mRNA. Interestingly, luciferase reporter analysis and quantitative real-time RT-PCR demonstrated that the *ALEX1* promoter was upregulated in a CRE-dependent manner by continuous activation of Wnt/b-catenin signaling induced by a glycogen synthase kinase-3 inhibitor and overexpression of b-catenin.

Conclusions: These results indicate that the CRE and E-box sites are essential cis-regulatory elements for the *ALEX1* promoter activity, and the *ALEX1* expression is regulated by CREB and Wnt/b-catenin signaling.

748 Regulation of expression of the VE-statin/egfl7 gene in endothelial cells: a critical role for ETS and GATA factors

F. Soncin[1], A. Le Bras[1], C. Samson[1], M. Trentini[1], B. Caetano[1], E. Lelièvre[1], V. Mattot[1], S. Guichard[2], F. Beermann[2]. [1]*Institut de Biologie de Lille, CNRS UMR8161, Lille, France,* [2]*Swiss Institute for Experimental Cancer Research, Centre de Phénotypage Génomique, Lausanne, Switzerland*

The *VE-statin/egfl7* gene is specifically expressed in endothelial cells during development and in the adult [1]. We studied here the regulatory mechanisms that control this specific expression *in vitro* and *in vivo*.

A specific expression in endothelial cells is not observed with *VE-statin/egfl7*'s closest neighbor genes *notch1* and *agpat2* on chromosome 2. Further, the acetylation state of histones around the two *VE-statin/egfl7* transcription start sites shows that the chromatin is opened in endothelial cells, not in fibroblasts at the *VE-statin/egfl7* locus. Two regions are important for the endothelial-specific expression of the gene; a −8409/−7688 enhancer and the −252/+38 region located ahead of the exon-1b transcription start site. The latter contains important ETS- and GATA-binding sites which are crucial for expression of the gene in endothelial cells. Analysis of expression, chromatin immunoprecipitation and RNA interference of endogenous transcription factors showed that Erg and GATA-2 are, by far, the most highly expressed in endothelial cells and that these two factors directly control expression of *VE-statin/egfl7*.

This first detailed analysis of the mechanisms that govern the expression of the *VE-statin/egfl7* gene in endothelial cells pinpoints the specific importance of Erg and GATA-2 factors in the regulation of genes in these cells.

Reference(s)

[1] Soncin F et al EMBO J. 2003 Nov 3;22(21):5700−11.

749 C6orf69, a PKCe interacting BTB-containing protein, is a novel Cullin3 binding partner

D. Garczarczyk[1], J. Hofmann[1]. [1]*Innsbruck Medical University, Biocenter Division of Medical Biochemistry, Innsbruck, Austria*

Protein kinase C (PKC) family members are key signaling molecules involved in many diverse cellular functions such as proliferation, differentiation, survival and death.

Among PKC isoenzymes, novel PKCε has been reported to act as an oncogene, contributing to malignancy by enhancing cell proliferation or by inhibiting cell death. PKCε seems to be involved also in mechanisms related to tumour cell invasion and metastasis, however its exact function is not known yet. By gene array approach, we identified few novel genes regulated by overexpression of constitutively active PKCε (PKCεA/E). One of the down-modulated genes, termed C6orf69, encodes a 47 kDa protein containing a single BTB/POZ domain.

C6orf69 gene is ubiquitously expressed. C6orf69 proteins were up regulated in some cancers (breast) compared to normal counterparts, suggesting a participation of C6orf69 in pathogenesis of certain tumours.

C6orf69 contains BTB domain usually involved in protein-protein interactions and implicated in the stability and dynamics of actin filaments. C6orf69 has been shown to co-immunoprecipitate with PKCε and bind to actin. Recently, BTB-domain containing proteins have been reported to regulate protein stability by recruiting proteins to the Cullin3 (Cul3) ubiquitin ligase leading to their degradation *via* ubiquitin/proteasome. Using co-immunoprecipitation method we could demonstrate that C6orf69 is interacting specifically with Cul3. We also addressed whether C6orf69 could be ubiquitylated, based on the fact that other BTB/POZ adaptor proteins have been shown to be substrates of E3 ligases. Our *in vivo* ubiquitylation assay demonstrated that C6orf69-CFP protein is efficiently polyubiquitylated and that inhibition of 26S proteasome by MG132 accumulated both unmodified C6orf69 as well polyubiquitylated C6orf69. Considering that C6orf69 binds to Cul3 and to PKCε, we are currently investigating if PKCε is ubiquitylated through the C6orf69/Cul3-based ubiquitylation system.

Analysis of the C6orf69 polypeptide showed that there are putative PKC phosphorylation sites. However, C6orf69 seems not to be a direct substrate of PKCε or other PKCs as demonstrated by *in vitro* kinase assay. Part of our current work is focused on determining which kinase (s) is responsible for C6orf69 phosphorylation, fuctional consequences of this PTM as well as determination of the precise position of the phosphorylated residues.

This work was supported by grant from the Austrian Science Fund (No. P19878).

750 Normal breast tissue from cancer patients is different in expression of Bmi-1 and Mel-18 compared to breast tissue from non cancer patients

M. Riis[1], T. Luders[2], V. Kristensen[2], I. Bukholm[1]. [1]*Akershus University Hospital, Surgery, Nordbyhagen, Norway*, [2]*University of Oslo, Institute of Clinical Epidemiology and Molecular Biology (Epi-Gen), Nordbyhagen, Norway*

Background: Polycomb Group (PcG) proteins are epigenetic silencers involved in maintaining cellular identity, and their deregulation can result in cancer. Mel-18 and Bmi-1 are both members of PcG. Bmi-1 was initially considered an oncogene, although recent studies have suggested that Bmi-1 overexpression is associated with good outcome in breast cancer. Mel-18 is considered a tumour suppressor gene. There are different mechanisms to this tumour suppressiveness. Both Mel-18 and Bmi-1 have been studied in tumour tissue, but to our knowledge it has not been studied in normal breast epithelium. Our study compares the expression of the two genes in normal breast epithelium of cancer patients and compares this to the level of expression in breast epithelium of healthy women.

Material and Method: We studied a total of 71, 23 of which we have normal tissue from viscinity of the tumour. In addition we had 6 fibroadenomas, 2 DCIC, and 12 reduction mammoplasties. The tissue samples were stored in RNAlater, RNA was isolated and microarray performed to achieve a molecular profile. These two genes were then studied more closely first on mRNA expression level and later on protein expression level. using immunohistochemistry.

Results: Bmi-1 mRNA is significantly up regulated in normal breast tissue in breast cancer patients compared to normal breast tissue from noncancerous patients, while the mRNA expression of Mel-18 was found to be lower in normal breast from patients operated for breast cancer compared to breast tissue from mammoplasty. mRNA expression of these two genes was inversely correlated. When protein expression of these two genes was evaluated, we observed that most of the epithelial cells were positive for Bmi-1 both groups of tissue samples, although the expression intensity was stronger in normal tissue from cancer patients compared to mammoplasty tissue samples. Protein expression of Mel-18 showed stronger intensity in tissue samples from mammoplasty compared to normal breast tissue from patients operated for breast cancer.