ICI treatment. E2 treatment caused down-regulation of ERα after 1h, but no significant change in Jab1 expression. Previous data showed that the subcellular distribution of Jab1 can be regulated. Thus, we also investigated if E2- or anti-E2 treatment could affect the cellular distribution of Jab1, using confocal microscopy and immunofluorescence. Neither E2 nor anti-E2 treatment resulted in a major shift of Jab1 between the nucleus and the cytoplasm. Interaction of ERα and Jab1 under E2- and anti-E2 treatment was investigated using coimmunoprecipitation (Co-IP). A small amount of ERα was Co-IPed with Jab1, which was enhanced by 4-HT treatment. Pre-treatment of MCF7 cells with curcumin increased the portion of Co-IPed ERα. In addition, using siRNA to knock-down Jab1 expression, a significant down-regulation of ERα was observed (P = 0.049). To conclude, a strong correlation between Jab1 and ERα expression occurs in breast tumors in vivo. In ERα+ MCF7 breast cancer cells, there is no short-term regulation of Jab1 expression by E2 and/or anti-E2 treatment. However, Jab1 and ERα may interact directly or within a complex, and this may be influenced by ligand. As well transient knock-down of Jab1 using RNAi resulted in a small but significant decrease in ERα expression suggesting that longer term knockdown of Jab1 may decrease ERα steady-state levels further. Jab1 expression is generally over-expressed in breast cancer compared to normal breast tissue and therefore may have a role in upregulating ERα expression which occurs during breast tumorigenesis.

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The identification of new tumour suppressor micrornas epigenetically silenced in drug resistant cancer cells

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Background: MicroRNAs (miRNAs) are a recently discovered class of non-coding short length RNAs (21-24 nucleotides in length) that play a fundamental role in gene regulation. These molecules down-regulate the expression of their target genes by base pairing to 3' UTR of the target messenger RNAs (mRNAs). These small RNAs are involved in the control of several biological processes, from cell differentiation to cell proliferation, thereby playing an important role in cancer. It was previously demonstrated that miRNA-127 (miR-127), is embedded in a CpG island and is highly induced from its own promoter after treatment with the demethylating agent 5-aza-2'-deoxycytidine (AZA) and the chromatin-modifying drug 4-phenylbutyric acid (PBA). In addition, it is usually expressed as part of a miRNA cluster in normal cells but not in prostate, bladder, and colon cancer cells, suggesting that it is subject to epigenetic silencing.

Materials and methods: Real Time PCR was used in order to evaluate the expression of miRNAs and their corresponding host genes in cancer cell lines. We analysed promoter sequences in cell lines by bisulfite-sequencing and methylation-specific polymerase chain reaction (MSP) to assess methylation status.

Results: Transcriptional silencing in cancer by CpG island methylation of genes that contain miRNAs can down-regulate the expression of the miRNAs as well while up-regulating mRNA expression, classifying both as tumour suppressors. We have mapped several miRNAs inside the introns of putative tumour suppressor genes and have observed down-regulation in cancer cell lines compared to the non invasive counterpart, and in cancer cell lines compared to the drug resistant counterpart, once again classifying both as tumour suppressors. Furthermore, this down-regulation appears to be due at least in part, to CpG island methylation.

Conclusions: This work permitted us to identify new miRNAs and new genes that are silenced in cancer due to an epigenetic event.

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The role of the estrogen-responsive B box protein (EBBP) in cancer cell cycle progression

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We have previously identified EBBP as a transcription factor involved in the retinoid anti-cancer pathway in Neuroblastoma. Currently, we aim to characterise EBBP's mechanism and role as a novel regulator of cancer in cell cycle progression. EBBP is a member of the evolutionarily conserved RBCC/TRIM (RING finger, B-box, coiled-coil/tripartite motif) group of proteins, which have diverse functions including; apoptosis, proliferation, differentiation, and transcriptional regulation.

In the absence of retinoid, EBBP overexpression induced growth inhibition and apoptosis in both retinoid-sensitive and -resistant cancer cells. Growth arrest correlated with reduced Cyclin D1 expression and phosphorylation of Rb. Furthermore, EBBP induced growth arrest in 7 human cancer cells in the absence of retinoid, but not in 4 normal cell lines. Retinoid treated neuroblastoma cells (retinoid-sensitive) displayed increased EBBP in nuclear aggregates. Contrastingly, retinoid-resistant breast cancer cells treated with retinoid displayed peri-nuclear EBBP aggregations. We have identified E2F transcription factor 1 (E2F-1), and Vimentin, both as EBBP-binding proteins by mass spectrometry and co-immunoprecipitation. EBBP also modulated E2F-1 and Vimentin protein expression in neuroblastoma cells as demonstrated by EBBP transfection and siRNA knock-down experiments.

Like other TRIM family members, EBBP may act as a corepressor in protein-protein complexes, or depending on the cell context it may act as a coactivator. Recently, we demonstrated that down-regulation EBBP expression with specific EBBP siRNA also reduced cell proliferation, induced apoptosis, and blocked phosphorylation of pRb, in retinoid-sensitive cancer cells, but not in retinoid-resistant cancer cells. To determine whether EBBP overexpression influences tumour-forming ability and sensitivity to retinoid treatment in vivo, we established breast cancer (MDA-MB-231) and neuroblastoma (BE(2)-C) stable cell lines overexpressing EBBP. As anticipated, exogenous EBBP induced growth inhibition and increased retinoid sensitivity in these stable clones.

Thus, EBBP has both retinoid-dependent and -independent functions, which may relate to cell cycle regulation and cell structure. These properties make EBBP an exciting new therapeutic target for anticancer compounds that are designed to target cancer cells while having reduced side effects on normal cells.

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Hh-Gli signaling in tumors; Hh-Gli activation and effects on cell cycle progression

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Ovarian dermoid cysts (DC) or benign cystic teratomas are benign tumors descending from germinal cells composed of elements descending from all three of the germinal layers.

We present an investigation of Hh-Gli signaling pathway in ovarian dermoids. Previously, we have shown that methylation of Ptch promoter may contribute to pathway malfunctioning.

We developed several different clone lines derived from primary cell cultures of ovarian dermoid tissue. RNA was isolated and Real-Time PCR analysis was performed. Real-Time PCR demonstrated expression of the Hh-Gli pathway genes. This expression, although present in all clone lines, differs among them, confirming the heterogeneity of this tumor type.

Some of the clone lines were additionally analyzed by immunofluorescent staining. Our results show difference in localization of some of Hh-Gli pathway proteins among the clone lines, and some of them show reactivity to cyclopamine treatment, on both mRNA and protein level. I.e. we have seen difference in localization of Ptch and Smo during cell cycle.

For cell cycle analysis cells were treated with cyclopamine, tomatidine or Shh protein, stained with propidium iodide and analyzed by flow cytometry. In this way we have also demonstrated effect of cyclopamine treatment on cell cycle progression of these clone lines. Taken together, this data suggests Hh-Gli pathway involvement in tumorigenesis and cell cycle progression of ovarian dermoids.

Since similar results were previously shown on ovarian carcinoma, we suggest Hh-Gli pathway aberration is an early event in transformation of ovarian cells in their progression towards malignancy.

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The Na+/H+ exchanger regulation factor (NHERF1) is a component of epidermal growth factor receptor (EGFR) signalling complex and regulates EGFR degradation

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Background: NHERF1 (Na+/H+ exchanger regulating factor 1) is a PDZ domain-containing protein that recruits membrane receptors and transporters and cytoplasmic signalling proteins into functional complexes. Recent evidence obtained from our laboratory and from other groups shows that NHERF1 is an important player in cancer progression. Interestingly, NHERF1 was shown to associate with proteins involved in cancer progression. Some of these are tumor and metastasis suppressors, such as PTEN (phosphatase and tensin homologue deleted on chromosome 10). Other NHERF1 associated proteins are oncogenic, such as EGFR.

Here we report that NHERF1 and PTEN interact with EGFR in a ternary complex, upon EGF stimulation, in metastatic breast cancer cell lines, regulating its degradation.

Materials and methods: Human mammary cell lines were transiently transfected with wild-type mouse NHERF1 cDNA inserted into the pcDNA vector and the experiments were conducted 48h later. After treatment monolayers were lysed in fractionation lysis buffer (HEPES 10mM pH 7.9, KCl 10mM, EDTA 0.1mM, EGTA 0.1mM). The nuclear fraction was obtained by centrifuging the homogenate at 600 X g for 10 min. The resulting supernatant was centrifuged at 3,500 X g for 10 min to obtain a pellet containing the endosomal fraction; the supernatant was centrifuged again at 100000 X g for 1.5 hr resulting in a plasma membrane pellet, the soluble cytoplasmic fraction in the supernatant. 50 µg of each cellular fractions were resuspended in ice-cold coimmunoprecipitation lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate,100 mM Na3VO4, and 1 mM NaF, protease inhibitors). 150 µg of total cellular protein was incubated at 4°C with 1 µg of primary antibody and protein A/G Plus-Agarose overnight. Immunoprecipitates were loaded in SDS sample buffer, run on 10% SDS-PAGE, analyzed by Western blotting.

Results: NHERF1 and EGFR signaling molecules interact after EGF stimulation. Interaction of endogenous NHERF1 with EGFR requires a NHERF1 functional PDZ1domain. The over expression of NHERF1 inhibits EGFR degradation, permits the interaction of the two proteins in not stimulated conditions. Moreover PTEN could be an important molecular integrator of the downstream events of EGFR.

Conclusions: Taken together, our studies suggest that NHERF1 senses signal of EGF and with PTEN regulates ligand induced degradation of EGFR.

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Validation of phosphoprotein array assay for determination of human epidermal growth factor receptors downstream signalling phosphoproteins in breast carcinoma

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Background: human epidermal growth factor receptors (HER) downstream signalling kinases have major consequences on tumor response to anti-HER monoclonal antibodies and tyrosine kinase inhibitors targeted therapy. The present study was designed to validate the use of phosphoprotein array assay to investigate HER downstream signalling functionality.

Materials and methods: the expression of phosphorylated EGFR and HER downstream signalling proteins (AKT, P70S6 kinase, ERK1/2, GSK3b) was measured by multiplex analysis using phosphoprotein array in 49 ductal infiltrating breast carcinoma frozen specimens taken at diagnosis and compared with western blot analyses. Based on routine immunohistochemistry, HER2 expression was overexpressed (3+) in 19 specimens. Ten tumors were triple-negative (HER2, estrogen and progesterone negative). Standard operating procedures were optimized regarding sample size, homogeneity, tumor content, freezing and protein extraction. The validation of the assay was based on the FDA guidelines for Bioanalytical Method Validation.

Results: linear regression and Bland-Altman analyses showed highly significant quantitative correlations between the phosphoprotein array assay and western blot analysis with regression coefficient values ranged from 0.790 to 0.852 ($P<0.001$). With limits of detection established at a signal-to-noise ratio of 3, great variations of phosphoprotein expression, up to several thousand-fold, were observed among the 49 tumor specimens. No significant variation in phospho-ERK1/2 as well as in phospho-AKT and related downstream phosphoproteins was observed between HER2 overexpressing and HER2 negative tumors. Lower expression of all HER downstream signalling phosphoproteins was observed in triple-negative tumors.

Conclusion: these results showed that before any treatment initiation, MAP kinase and PI3 kinase/AKT signalling pathway functionality can dramatically differ from patient to patient and could probably explain discrepancies in clinical response to signalling kinase targeted therapy even when based on individual immunohistochemistry target determination.

These results validate the use of multiplex phosphoprotein array assay in size-limited tumor specimens and warrant further prospective evaluation of HER downstream signalling phosphoproteins as predictive and/or surrogate marker for clinical response to anti-HER drugs.

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The alpha5beta1 integrin predicts glioblastoma chemotherapy outcome through modulation of p53 pathways

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Background - Of solid tumors, glioblastoma remains the most resistant to therapy and new therapeutic approaches are needed. The potential role of $\alpha5\beta1$ integrins in cancer has recently attracted much interest. Its overexpression in tumoral neovessels and in glioblastoma makes it a potential interesting therapeutic target. We investigated its role in glioblastoma carcinogenesis and chemoresistance.

Methods - The $\alpha5$ integrin subunit was overexpressed or knocked down in U87MG cells and proliferation and clonogenicity were examined. Ellipticine, a DNA intercalating agent and topoisomerase II inhibitor, which demonstrated specificity towards brain tumor cell lines, was investigated as a chemotherapeutic agent. Ellipticine effects on proliferation, cell cycling, apoptosis and senescence were investigated in the presence or absence of SJ749, a $\alpha5\beta1$ integrin specific antagonist.

Results - $\alpha5$-overexpressing U87MG cells exherted increased aggressivity as low expressing cells confirming a specific role of the $\alpha5\beta1$ integrin in glioblastoma tumorigenicity. Accordingly, blocking the integrin with SJ749 inhibited glioma cell adhesion to fibronectin and reduced cell proliferation / clonogenicity via a G0-G1 cell cycle arrest without induction of apoptosis. This effect was dependent on the $\alpha5$ subunit expression level. The main effect of ellipticine in U87MG cells was to induce premature senescence through the activation of wild type p53. By contrast, ellipticine mostly induced apoptosis in U373 cells expressing mutant p53. Association of chemotherapy (using ellipticine) and targeted therapy against $\alpha5\beta1$ integrin (using SJ749) led to an additive reduction of U87MG cell proliferation. Interestingly, combined therapy decreased senescence and increased apoptosis in U87MG but not in U373 cells. We also showed that knocking down the $\alpha5\beta1$ integrin in U87MG impaired the ellipticine-induced p53 activation (as observed with SJ749) but surprisingly drastically enhanced the senescent cell population.

Conclusions – The $\alpha5\beta1$ integrin is a marker of glioblastoma aggressiveness and modulates glioblastoma chemosensitivity through p53 pathways. p53 status and $\alpha5\beta1$ integrin expression level may predict outcome of combined therapies (chemo- and targeted therapies) for glioblastoma.

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Identification of therapeutic targets in uterine and soft tissue leiomyosarcomas

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Background: Leiomyosarcoma is a rare mesenchymal tumor, with poor prognosis and response to cytotoxic chemotherapy. It shares some similar phenotypic and histological features with gastrointestinal stromal tumors (GISTs) which the prognosis was dramatically changed with the selective tyrosine kinase inhibitor Imatinib mesylate. However, in contrast to GISTs, patients with advanced LMS do not benefit from Imatnib therapy. So, an effective treatment lack for this neoplasm. Thus, a better understanding of the LMS' biology needs to be elucidated in order to develop new therapeutic agents, notably tyrosine kinases inhibitors.

Objectives: We aim to clarify the molecular mechanisms of signal transduction of LMS by a large screening of phosphorylated protein kinases.

Methods: A screen of 630 phosphorylated protein kinases was performed on one Leiomyosarcoma tumor using the Kinex antibody micro array. Also, by Western Blot analysis, a more specific approach, we investigated the expression/activation of specific tyrosine kinases receptors like c-Kit, CSF1-R, and c-Met receptor tyrosine kinases in 13 primary LMSs.

Results: In our screen, we observed higher levels of phosphorylated Her2/Neu, FAK, Src, IRS1, MEK1 (MAP2K1), MEK3 (MAP2K3), ERK5, and MAPKAPK2a/b in the leiomyosarcoma tumor analyzed, than in the healthy muscle used as a control. Furthermore, western blot analysis revealed the expression of c-Kit, CSF1-R, c-Met in all cases but only Kit was phosphorylated, thus activated in these tumors.

Conclusion: To date, no causal event in the development of leiomyosarcoma was uncovered yet. A better understanding of the leiomyosarcoma biology will lead us to explain its non-response to Imatinib mesylate despite Kit activation, and especially to identify potential therapeutic targets as tyrosine kinases receptors.