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Yeast-2-Hybrid screen identifies Rab-interacting proteins as interacting prey targets for the OPCML tumour suppressor, highlighting a general theme of membrane trafficking

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To identify proteins which interact with the OPCML ovarian cancer tumour suppressor (1), we undertook a yeast-2-hybrid (Y2H) screen using the Hybrigenics custom screening facility.

A highly representative brain library containing 10 million independent clones were screened to saturation thanks to their Cell-to-Cell mating protocol. This protocol allows for testing an average of 97 million interactions per screen ensuring the exhaustivity of the screen and likelihood of identifying rare interactions (from the rarest transcripts). The depth of the screening procedure substantially impacts on the false-negative rate. Hybrigenics' bioinformatic analysis offers a comprehensive coverage and reproducibility rate of 90% (2). A common theme associated with Y2H screens is the occurrence of false-positive interactions. However, the high reproducibility of the Hybrigenics screening strategy allows the development of a systematic statistical approach in assigning the confidence score to each clone; the Predicted Biological Score (PBS).

The Hybrigenics screen identified 71 clones interacting the extracellular domains of OPCML as bait which included multiple, interacting clones for 3 known proteins given a high PBS confidence score and 9 single cDNA clones for known proteins of moderate PBS score. The two highest scoring candidate interacting proteins (RIM2 and Synaptotagmin1 (SYT1)) were found to be involved in the same functional process namely Ca2+-dependent vesicle fusion. Three further candidates from the lower PBS values involved in this process were also identified and thus a theme appears of Ca2+- dependent membrane trafficking between cellular compartments; a process known to be important for Receptor Tyrosine Kinase (RTK) signal regulation. Thus, from the Hybrigenics bioinformatics we have a supporting evidence that OPCML may interact with at least 2 Rab-interacting proteins involved in membrane trafficking with RIM2 having the potential to cluster other signaling molecules to membrane locations. Emerging evidence implicates alterations of the Rab proteins and their associated regulatory proteins and effectors in multiple human diseases including ovarian and breast cancer.

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Sanguinarine sensitizes tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-resistant gastric cancer cells though downregulation of Akt and activation caspase-3

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Sanguinarine, a benzophenanthridine alkaloid derived from the root of Sanguinaria canadendid, has been shown to possess anti-proliferative, anti-inflammatory, and anti-oxidant properties. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a promising anticancer agent that induces apoptosis in multiple tumor cell types while sparing most normal cells. However, the cytotoxic effect of the TRAIL limited in some cancer cells, including AGS gastric adenocarcinoma cells. In the present study, we suggested that sanguinarine exerted a synergistic effect leading to apoptosis rates unachievable by incubation with TRAIL alone. Combined treatment with sanguinarine and TRAIL significantly enhanced apoptosis and the apoptosis induction was associated with up-regulation of pro-apoptotic Bax and down-regulation of anti-apoptotic Bcl-2, loss of mitochondrial membrane potential (MMP), activation of caspase-3, degradation of poly-(ADP-ribose) polymerase(PARP) and β-catenin. However, combined treatment-induced apoptosis were significantly inhibited by z-DEVD-fmk, a caspase-3 specific inhibitor, indicating the important role of caspase-3 in the observed cytotoxic effect. In addition, pre-treatment of LY294002, a PI3K/Akt inhibitor, significantly increased combined treatment-induced apoptosis in AGS cells. The present results indicate that caspase-3 is a key regulator of apoptosis in response to combined sanguinarine and TRAIL in human gastric adenocarcinoma AGS cells through inactivation of Akt and mitochondrial dysfunction. Furthermore, the use of TRAIL in combination with subtoxic doses of sanguinarine may provide an effective therapeutic strategy for safely treating some resistant gastric cancer cells.

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Prognostic factors in multiple myeloma

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Introduction: Multiple myeloma is a malignant neoplasm originating in plasma cells. It is frequently associated with poor prognosis and characterized by the production of a monoclonal immunoglobulin called the M component. Multiple myeloma (MM) is connected to a numer of chromosomal abnormalities, in most cases IgH translocation with FGFR3, CCND1, CCND2, CCND3, c-MAF in early stages. Further chromosomal changes appear with the progression of the disease the most frequent of which are monoallelic deletion or monosomy of chromosome 13, trisomies of chromosome 8, 9, 15 and many others. It has also been revealed that some proteins controlling cell the cycle and apoptosis (p53, p16, FGFR3, cyclin D 1, 2, 3, Bcl-2, caspase 3, 8, 9) seem to play an important role during MM pathogenesis and progression. However, there are no reliable data on their prognostic significance in the various stages of disease (monoclonal gammapathy of uncertain significance – MGUS, smouldering MM and advanced MM). Therefore the aim of this pilot study was analysis of expression of these proteins in various stages of MM and potentially extend the panel of prognostic markers which allows differentiation of the above-mentioned disease stages.

Materials and methods: Bone marrow from 35 patients treated by the same chemotherapy protocol (VAD) and autologous transplantation were used. Standard indirect immunohistochemistry on formalin fixed, paraffin-embedded sections was used for the detection of p53, p16, FGFR3, cyclin D 1, 2, 3, Bcl-2, caspase 3, 8, 9 using a high temperature epitope retrieval technique. Immunohistochemical staining was evaluated by a semi-quantitative method using a histoscore which is the multiplication of positivity by intensity of staining. Intensity of staining was scored as weak (1), moderate (2) or strong (3) while positivity of staining was assessed as percentage of tumour cells.

Results: Bone marrow samples of patients in advanced stages of MM showed high expression of Bcl-2 in tumor cells, in contrast to those in remission which showed weak or no positivity for Bcl-2. p53 and p16 were completely negative. Caspase 8 was negative in most cases. However, we detected a few caspase 8 positive cells in patients in remission. Caspase 9 was negative in biopsies taken before treatment but we detected several caspase 9 positive cells in patients after treatment.

Conclusions: Based on decreased BCl-2 expression in patients in remission, these preliminary data suggest that detection of low BCl-2 expression in bioptic samples of MM might be used as positive prognostic factor. Negativity of p16 can be explained by hypermethylation which leads to p16 deactivation. Mutation of p53 is probably infrequent in multiple myeloma but the mechanism of its impaired function needs further study.

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Up-regulation of thymosin β4 is a determinant of the transformed phenotype and invasiveness of mouse fibrosarcoma cells

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Background: Understanding the mechanisms of tumor cell invasion is essential to prevent cancer deaths. S-adenosylmethionine decarboxylase (AdoMetDC), a key enzyme in the synthesis of polyamines, induces transformation of murine fibroblasts upon over-expression. As these transformed cells form highly invasive fibrosarcomas in nude mice, they provide a valuable model to study cell invasion. Materials and methods: We analyzed by DNA microarrays the transformation-related gene expression changes in AdoMetDC-transformed cell lines in comparison to normal NIH3T3 cells, and further studied the roles of the identified genes, in two- and three-dimensional cell cultures (collagen I and Matrigel), using antisense RNA expression, siRNAs, and a sponge toxin latrunculin A. Results: We found marked up-regulation of thymosin β4 to be the most prominent change in these invasive fibrosarcoma cells, and it was further confirmed at the protein level. Thymosin β4 is a major actin sequestering protein, forming 1:1 complex with monomeric actin. Interestingly, we found a sponge toxin latrunculin A, which inhibits the binding of thymosin β4 to actin, to profoundly affect the morphology and proliferation of the AdoMetDC-transformants and to block their invasion/migration in three-dimensional Matrigel. In addition, we detected the up-regulation of thymosin β4 also in ras-transformed mouse fibroblasts and metastatic human melanoma cells. Conclusions: Elevated thymosin β4-expression appears to be related to increased tumorigenicity and metastatic potential, consistent with other studies on fibrosarcoma, melanoma, and colon carcinoma cells. Our results encourage testing latrunculin A-like and other

agents interfering with thymosin β4 (either its up-regulation or actin sequestering function) for the treatment of thymosin β4-overexpressing tumors with high invasive and metastatic potential.

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Erythropoietin and steroid membrane initiated actions interact in breast cancer cells leading to enhanced cell survival

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Background: Erythropoietin (EPO) is a hormone primarily involved in erythropoiesis but dotted equally with an array of autocrine/paracrine effects. EPO can regulate major cell functions of normal and cancer cell types including breast cancer, a steroid hormone dependent neoplasm. Steroid effects can be nuclear- and/or membrane-initiated, with the latter depending, among others, to a cross-linking with various growth factor receptors. Previously, in breast cancer specimens we have reported a correlation of erythropoietin, its receptor (EPOR) and membrane androgen sites. In the present work we further explore this interaction and the possible mechanism involved. Material and Methods: We assayed the effect of serum deprivation- and testosterone-BSA-induced apoptosis and cell migration in the presence of erythropoietin and explored the signaling pathways involved. Results: Testosterone-BSA-induced apoptosis and decreased cell migration was reversed by erythropoietin in a dose- and time- related manner. Moreover, the anti-apoptotic effect of EPO was potentiated by the addition of testosterone-BSA indicating an interaction between the two systems. This interaction is not at the membrane-receptor level but is the result of the modulation of specific signaling pathways (switching of p38 and Jnk from pro- to anti-apoptosis and from STAT to Akt and β-catenin signaling), and the enhanced transcription of EPOR by testosterone-BSA. Conclusions: Erythropoietin could be integrated to the ensemble of growth factors that cross-link with membrane steroid receptors, amending tumor cell survival. Their importance to patients' prognosis and selection of the appropriate therapeutic regimen should be considered.

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Src activity is increased in liposarcomas and in gastrointestinal stromal tumors—analysis of associations with clinical and molecular tumor characteristics

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Increased activity of the non-receptor protein tyrosine kinase Src can be found in a variety of human cancers in vitro and in vivo. Several studies have shown that elevated Src activity is associated with an increase in tumor malignancy, as well as poor clinical prognosis. The present study was done to determine whether Src activity is also increased in soft tissue sarcomas such as gastrointestinal stromal tumors (GISTs), and liposarcomas, and if Src activity in these tumors correlates with established tumor characteristics, other molecular determinants, or clinical prognosis. Tumors and normal tissues from 29 patients with GIST and from 17 patients with liposarcoma were analyzed for Src activity by immune complex kinase assays. There was a positive correlation for Src autophosphorylation and phosphorylation of MBP, reflecting the ability of Src to activate an external substrate in GISTs (r=0,751; p<0,001) and liposarcomas (r=0,912; p<0,001). Src activity was significantly higher in tumors than in normal tissues within the 16 GIST patients excluding imatinib responders (p=0,017), and in liposarcoma patients (p=0,033). There was a trend for increased Src activity in GISTs to correlate with positive PDGF-R (p=0,066). Elevated specific Src activity was observed as a trend in tumors with high risk of malignant behaviour according to Fletcher (p=0,07), and in those with positive CD117 (p=0,099). Five GIST patients with recurrence and recent surgery were also analyzed for Src activity and, as a statistical trend, Src activity was now lower than in the primary (p=0,08). Furthermore, specific Src activity was significantly lower in GISTs containing spindle cells (p=0,01) than in epitheloid tumors, or tumors containing both cell types. No significant association with clinical prognosis was observed in this series so far, however, this may be due to the duration of follow-up and will be re-analyzed in the future. This study demonstrates that Src activity is significantly increased in GISTs and liposarcomas as compared to normal tissues, and in trend is associated with CD117, PDGF-R, and the score for malignant risk of GIST.

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Integrin-Rab21-Rasa1 complex regulates integrin traffic in migrating cells

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During malignancy progression, tumour cells acquire the ability to break the basement membrane and invade underlying tissue, a process called metastasis. The seeding of tumour colonies to different sites in the body requires the activity of integrin cell surface receptors that anchor cells to the surrounding extracellular matrix (ECM). The regulation of cellular migration and adhesion is thereby dependent on the continuous turn-over of integrins that need to be internalized at retracting edges and transported to new adhesion sites of the cell.

We discovered that the small GTPase Rab21 critically regulates the endocytic traffic of integrins (Pellinen et al., J. Cell Biol., 2006). Our aim is now to further elucidate the GAPs (GTPase-activating proteins) and GEFs (GDP/GTP-exchange factors) that are decisive for the control of Rab21-activity. Our recent findings that Rasa1 (GAP) regulates integrin-internalisation and migration of breast cancer cells suggests that Rasa1 is a crucial regulator for Rab21-controlled integrin traffic and has therewith an impact on the motility of transformed breast epithelial cells during metastasis.

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Knockdown of oncogenic microRNA-21 displays cytotoxicity in p53 wild-type colon cancer cells

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Although the number of verified human microRNAs (miRNAs) is still expanding, only few have been functionally described. However, emerging evidences suggest the involvement of altered regulation of miRNA in pathogenesis of cancers and these genes are thought to function as both tumours suppressor and oncogenes. Previous data suggest altered regulation of microRNA-21 (miR-21) expression in CRC. In our study, we examined by Real-Time PCR expression levels of microRNA-21 (miR-21) in 60 colorectal tumors and 40 paired adjacent non-tumor tissues and correlated them to selected clinicopathologic features and survival parameters. We used expression of U6 small nuclear RNA (RNU6B) for data normalization and standard ddCt method for relative quantification of miRNA expression. Levels of miR-21 were significantly higher in tumors comparing to normal mucosa ($p < 0,0001$, Wilcoxon matched-pairs test). High expression levels of miR-21 in tumors (based on high tertile) were associated also with a poor survival (long-rank p=0,043). Up-regulation of miR-21 was previously associated with high potential of invasion, intravasation and metastasis in pre-clinical colorectal cancer models. Till now no data exist focused on miR-21 effects on CRC cells proliferation. To elucidate potential involvement of miR-21 in regulation of colon cancer cells (DLD1, SW837, HCT116 wt-p53, HCT116 null-p53) proliferation we tested effects of synthetic 2´OMe-antisense-miR-21 (anti-miR-21) transfection (2´OMe-EGFP as control) on their growth by use of MTT test. Proliferation was not affected in a null-p53 cell line or cell lines expressing mutated p53 (DLD1, SW837). In a wild-type p53-expressing cell line we observed more than 20% decrease of cells proliferation by MTT test after transfection of anti-miR-21. Now we are testing attenuating effect of anti-miR-21 on CRC cells survival under conditions of p53-directed apoptosis induced by doxorubicin treatment. Simultaneously, we are evaluating also changes in invasive properties of anti-miR-21 transfected cancer cells by matrigel invasion assay. Our results suggest possible role of miR-21 in colorectal cancer pathogenesis. Supported by IGA MZ CR NR/9076 – 4 and project MZ0MOU2005

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Differential expression of annexin A1 modulates invasion of melanoma B16 cells

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Identification of proteins involved in melanoma dissemination should complete the knowledge of physiopathology and potentially the prognosis for patients with a primary tumour. We used the B16 mouse melanoma