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Expression of the Ets-2 transcription factor in human breast cancer

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Ets proteins are downstream effectors of Ras-MAPK signaling pathways. Evidence from model systems suggests that Ets-2 may play a role in cellular transformation. This study investigated the *ex vivo* expression pattern of Ets-2 in a large cohort of human breast tissue in an attempt to establish a link between Ets-2 and the formation and progression of human breast cancer.

Reverse transcription PCR, Western blot analysis and immunohistochemical detection were used to compare expression of the Ets-2 transcription factor in human primary breast carcinomas, fibroadenomas and normal breast tissue. Levels were related to ER, PR, HER-2/*neu*, uPA, MMP-2 and MMP-9.

Ets-2 mRNA was detected in 69% of primary carcinomas (n=181), 63% of fibroadenomas (n=43) and 47% of normal breast tissue (n=43). Levels of Ets-2 mRNA were found to correlate with MMP-2 mRNA levels in the carcinomas ($p<0.05$; *Spearman Rank Correlation*). Two forms of the Ets-2 protein were detected, p52 (full length) and p54 (phosphorylated) using Western blotting. Both proteins were increased in primary carcinomas compared to fibroadenoma or normal breast tissue ($p<0.0001$, *Mann Whitney U-test*). These proteins were found to correlate with uPA protein levels in the carcinomas (52 kDa: $p<0.0001$, 54 kDa: $p<0.0005$; *Spearman Rank Correlation*). The Ets-2 protein was localised to the tumour cells in the primary carcinomas and to the epithelial cells surrounding the ducts and lobules in the fibroadenomas and normal breast tissue.

In conclusion, this is the first large scale study implicating a causal role for the Ets-2 transcription factor in human breast cancer.

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Expression of maspin in gastric cancer

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Background: Significant improvements in the analysis of genetic alterations for gastric cancer have been achieved by the recent progress in molecular biology. However, the informations are still insufficient for understanding common pathways for the development and progression of gastric cancer. For the purpose to seek for a candidate gene which regulates tumor progression and metastasis in gastric cancer, we investigated the gene expression profiles using oligonucleotide microarray. We further examined the protein expression of the maspin, which was selected for the lymph node metastasis related gene by the microarray, using immunohistochemistry.

Materials and Methods: Tumor tissue and adjacent normal tissue were obtained from a total of 21 patients with gastric cancer and total RNA was extracted. Total RNA was reverse transcribed to cDNA by using the T7-primer and then examined for gene expression profiles using Affymetrix chip U95Av2 chip set, which includes 12,000 human genes and EST sequences. Formalin fixed and paraffin embedded tumor tissue and lymph nodes were obtained from 34 patients with gastric cancer. The protein expression of maspin was investigated using immunohistochemistry.

Results: In microarray analysis, 25 genes were over-expressed and 2 genes were depressed at least 4-fold in tumor tissue. In further analysis according to lymph node metastasis, expression of maspin, as well as carcinoembryonic antigen and nonspecific cross reacting antigen, was significantly higher in tumors with lymph node metastasis than in those without it. The protein expression of maspin was not observed in normal mucosa. On the conctrary, maspin expression was observed in 29 of 34 tumor tissues. There was a significant correlation between the incidence of maspin positive tumor staining and the maspin mRNA expression levels.
Conclusions: Maspin was selected as lymph node metastasis relating gene in gastric cancer by oligonucleoide microarray and the over- expression of maspin protein was also confirmd by immunohistochemistry. These results suggest that maspin has a potential role for tumor metastasis in gastric cancer.

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A naturally occurring type III variant mutant EGF receptor confers a strong cytoprotective response to ionizing radiation

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Background: Many carcinomas and gliomas frequently express the variant form of EGFR, the type III variant (EGFRvIII), which is known to function as an oncoprotein. We explore the functional consequences of EGFRvIII on cellular responses following radiation and investigate the feasibility of adenovirus- mediated delivery of dominant- negative EGFR-CD533 to inhibit EGFRvIII.

Material and Methods: Chinese hamster ovary cells were used for mechanistical studies on EGFRvIII- mediated cellular radiation responses. To explore the importance of EGFRvIII as a modulator of radiation responses in malignant gliomas, EGFRvIII and EGFR-CD533 were expressed in vitro and in vivo in the cell line U-373 MG through transduction with adenoviral vectors. Radiation responses were studied at the molecular and cellular level including inhibition of the EGFRvIII function through AG-1478, effects on signal transduction cascades, clonogenic survival and apoptosis assay. In vivo studies were carried out with cells pre-transduced with adenoviral vectors before inoculation and with established xenograft tumors expressing intrinsic or adenoviral EGFRvIII and EGFR-CD533 after intratumoral infusions of the adenoviral vectors.Growth delay assays measured radiosensitivity.

Results: 2 Gy resulted in a 4.3-fold increase in EGFRvIII tyrosine phosphorylation in CHO cells.Importantly, this activation led to an immediate 8.5 fold and 3.2 fold activation of MAPK and Akt, respectively. Colony formation and apoptosis assays verified the enhanced relative radioresistance of cells in the presence of EGFRvIII. In vivo studies confirmed the radiation-induced activation of intrinsic EGFRvIII in xenograft tumors, which was completely inhibited through EGFR-CD533. Furthermore, EGFR-CD533 abolished the enhanced clonogenic survival as well as the increased tumorigenicity mediated by EGFRvIII. Growth delay assays demonstrated that EGFR-CD533 expression significantly enhanced radiosensitivity of tumors under conditions of intrinsic as well as adenoviral- mediated EGFRvIII expression.

Conclusions: This work will demonstrate that EGFRvIII is an important modulator of radiation responses conferring a stronger cytoprotective response than EGFR via the MAPK and PI3K pathway. EGFR-CD533 effectively inhibits EGFRvIII function. The dominance of EGFRvIII in cytoprotective responses to radiation needs to be addressed in any therapeutic approach disabling EGFR function for tumor cell radiosensitization.

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In vitro and in vivo efficacy of a HIF-1alpha-antisense oligonucleotide containing locked nucleic acids

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The purpose of this study was to obtain a specific down-regulation of hypoxia-inducible factor 1α (HIF-1α) using antisense oligonucleotides containing locked nucleic acids (LNA).

HIF-1αplays a central role in the mammalian cellular response to hypoxia and the protein is frequently found over-expressed in human cancers. This over-expression is associated with a poor prognosis, resistance to therapy and the formation of metastasis, which makes HIF-1α a potentially interesting molecular target for anticancer therapy.

LNA is a novel class of nucleic acid analogs containing a 2'-O, 4'-C-methylene bridge resulting in an unprecedented high binding affinity toward complementary DNA and RNA. The LNAs obey the Watson-Crick base pairing rules, shows a higher stability toward nucleases and are readily taken up by mammalian cells.

We designed and screened a series of 16-mer antisense oligonucleotides against HIF-1α containing LNAs and phosphorothioate DNAs. Cell cultures were treated for 6 hours with different concentrations of oligonucleotide and 0.5% Lipofectamine 2000 in the medium with or without 10% FCS. After treatments the cells were washed in PBS and medium containing 10% FCS was added. The cell cultures were then exposed to severe hypoxia in incubation bags, using the Anaerocult system, (Merck). Cells were harvested

for protein extraction after 20 hours of severe hypoxia and the expression of cellular protein was measured by densitometry of western blots.

Treatment of human glioblastoma multiforme cells U87 with a fluorescent (FAM-tagged) LNA/phosphorothioate gapmer confirmed a very efficient uptake (>95%) of oligonucleotide into the cells.

Using U87 cells we found that the most potent of the screened oligonucleotides was a 4 LNA, 8 phosphorothioate, 4 LNA gapmer (Cur813) targeting the 3'-coding region of the HIF-1α-gene. For further in vitro investigation of Cur813 we treated human glioblastoma cell lines U87 and U373 and the human prostate cancer cell line 15PC3 with the gapmer oligonuleotide. We were able to establish a dose-dependent down-regulation of hypoxia induced HIF-1α protein and the HIF-1-regulated protein Glut1 in all the cell lines using Cur813. Relative to untreated controls a down-regulation of 75-90% was obtained by 100 nM Cur813 and a down-regulation of 85-90% was obtained by 400 nM, the effect of the treatment varying between the three different cell lines.

Since the proposed molecular target was effectively inhibited without any apparent in vitro toxicity, we are currently investigating the in vivo efficacy of Cur813 in U373 xenografts on nude NMRI mice. Initial experiments have shown a significant inhibition of tumor growth by a 1 daily i.p. injection of 5 mg/kg Cur813 for 7 days.

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Identification and characterization of DEGA, a novel leucine-rich repeat family member differentially expressed in human gastric adenocarcinoma: effects on cell cycle and tumorigenicity.

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Using a cDNA microarray approach to search for genes involved in a variety of cancers, we discovered DEGA, a novel cDNA differentially expressed in human gastric adenocarcinomas. Cloning of this cDNA from A549 cells revealed a 2070 bp fragment containing an open reading frame of 1569 bp that encodes a 522 amino acid protein. This protein contains a signal peptide, five leucine-rich repeat motifs (protein-protein interaction domains) and an IgG and transmembrane domain, suggesting that it resides on the plasma membrane. Transfection of 293 cells with an EGFP fusion construct confirmed cell surface localization. Although the cytosolic portion of this protein does not possess signal transduction-related domains, approximately 1/5 of the cytosolic amino acids are either a serine or a threonine. Blast searches for sequence similarity to this protein revealed an exact match to AMIGO-2, a very recently identified, but functionally uncharacterized protein related to AMIGO, a leucine-rich repeat family member implicated in axon tract development (Kuja-Panula et al., JCB 160:963-973, 2003). Aside from being highly expressed in normal tissue of the breast, ovary, uterus, and lung, in this report, we show that DEGA/AMIGO-2 is differentially expressed in approximately 1/3 of tumor versus normal tissue from gastric adenocarcinomas patients. It is also expressed in some gastric adenocarcinoma cell lines (ex. AGS) as well as other cancer cell lines. When compared to empty vector controls, stable expression of an DEGA/AMIGO-2 anti-sense construct in the gastric adenocarcinoma cell line, AGS, led to an accumulation of cells in the G2/M phase of the cell cycle and a nearly complete abrogation of tumorigenicity in athymic (nu/nu) mice. Although we have not defined a precise role for DEGA/AMIGO-2, our data suggest that it may be an etiologic factor functioning as a signaling cell adhesion molecule to regulate the cell cycle as well as tumorigenesis in a subset of gastric adenocarcinomas.

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MSD-EACR Research Award. Celecoxib activates a novel mitochondrial apoptosis signaling pathway

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Background: The cyclooxygenase-2 inhibitor Celecoxib has been shown to inhibit tumor cell growth independently from its capacity to block the COX-2 enzyme and to increase the efficacy of ionizing radiation in experimental settings. The growth inhibitory effects of celecoxib had been attributed to its pro-apoptotic action.

Methods: To gain insight into the mechanisms of celecoxib induced apoptosis and to differentiate between death receptor and mitochondrial pathways the activation of caspases-9, -8 and -3, the cleavage of the caspase-3 substrates PARP and ICAD as well as the induction of mitochondrial alterations and of nuclear changes were tested in Jurkat T- and BJAB B-lymphoma cells with defects in either pathway as well as in embryonic fibroblasts of Apaf-1 expressing and Apaf-1 knock out mice. For comparison, apoptosis was also induced by death receptor stimulation and irradiation.

Results: Celecoxib induced dose and time dependent apoptosis in Jurkat and BJAB cells. Activation of caspases-9, -8 and -3 was detectable in parallel with cleavage of PARP and inactivation of ICAD. The celecoxib action was associated with a breakdown of the mitochondrial membrane potential and release of cytochrome c. Lack of FADD, overexpression of a dominant negative FADD, lack of caspase-8 and treatment with caspase-8 specific inhibitors had no influence celecoxib induced apoptosis. In contrast, overexpression of a dominant negative caspase-9 mutant or inhibition of caspase-9 interfered with celecoxib induced cell death. Similarly, lack of Apaf-1 expression abrogated Celecoxib induced apoptosis. Surprisingly, overexpression of anti-apoptotic members of the Bcl-2 protein family did not abrogate caspase-9, -8, and -3 activation, PARP cleavage, breakdown of the mitochondrial membrane potential and release of cytochrome c upon treatment with celecoxib, while inhibiting radiation induced apoptosis.

Conclusion: We conclude that celecoxib induces apoptosis via a novel caspase-9 and Apaf-1 dependent, but Bcl-2 and death receptor independent mechanism.

Adult leukemia

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Outcome and patterns of failure in solitary plasmacytoma: a multicenter rare cancer network study on 258 patients

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A series of 258 adult patients with bone (n = 206) or extramedullary (n = 52) solitary plasmacytoma, consecutively treated between 1977 and 2001, was collected in a retrospective multicenter Rare Cancer Network study. Median age was 60 years (18-95), and male to female ratio was 1.87. Staging work-up included bone-marrow assessment (all patients), serum immunoglobulins (n = 149), immunosubtraction (n = 161), standard X-rays (n = 191), CT-scan (n = 163), MRI (n = 85), and bone scintigraphy (n = 73). HIV test was performed in only 7 patients, and all were negative. Inclusion criteria included solitary plasmacytoma in 18-year or older patients without evidence of multiple myeloma. Histopathologic diagnosis was obtained in all patients including biopsy in 160, partial resection in 85, and complete resection in 9 patients. Most (n = 215) of the patients benefited from RT alone; 34 had chemotherapy (mostly melphalan and prednisone) and RT, 8 had surgery alone, and one patient died before starting the RT. External RT volume included the clinical tumor volume with a sufficient margin. The median RT dose was 40 Gy (20-66) in 20 fractions (4-50) using 2 Gy (1.25-5) per fraction during median 29 days (4-74). RT was delivered using megavoltage photons in all patients. The median follow-up period was 56 months (7-245). Median time to multiple myeloma development was 21 months (2-135). One hundred seventeen (45%) patients developed multiple myeloma with a 5-year projected probability of 45%. The 5-year probability of overall survival, disease-free survival (DFS), and local control was 74%, 50%, and 86%; respectively. Six out of 9 patients treated with surgery alone presented a local relapse compared to 10 (10%) out of 105 treated with RT < 40 Gy or 21 (15%) out of 144 treated with RT > 40 Gy. In univariate analyses (logrank test), significant factors favorably influencing the survival were younger age (60 years or younger), extramedullary localization, and tumor size (< 4 cm). For DFS, in addition to the above-mentioned parameters, treatment with RT was a favorable factor as well. Local control was better in small tumors (< 4 cm). Unfavorable factors for myleoma development were older age (> 60 years) and bone localization. Multivariate analysis (Cox model) revealed that the best independent factors predicting the outcome were younger age and tumors < 4 cm. Bone localization was the only independent predictor for multiple myeloma development. In this multicenter retrospective study, extramedullary solitary plasmacytoma was found to have the best outcome,