**Pancreatic cancer: Animal model and molecular biology**

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**Abstract**
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**Animal models for pancreatic cancer**

*Direct Tumor Xenografts*

Direct xenografts of pancreatic cancer offer significant advantages over other models. These tumor-grafts retain original genetic traits and tumor characteristics unique to the cancers from which the grafts are derived. As clinical outcome is determined by such features, it was hypothesized that successful growth of xenografts reflects the biology of the source PDAC and predicts patient outcome after resection. As part of prospective study, tumors obtained at surgery were implanted within NOD/SCID mice. Patient/tumor factors and clinical endpoints (recurrence/death) were measured. Successful engraftment and postoperative recurrence and survival were primary endpoints. Establishment of direct PDAC tumor-grafts was attempted in 59 patients with a current overall engraftment rate of 70 percent. Xenografts accurately recapitulated original tumors. The experiments’ data demonstrates that engraftment of direct PDAC xenografts forms the basis of a novel in-vivo assay reflecting the biology of the original patient tumor that predicts postoperative metastatic recurrence and subsequent survival in individual patients independent of other clinical/pathologic factors. The results of such personalized biological assays allow for more accurate postoperative follow-up and potential for tailored treatment recommendations dependent on the status of tumor engraftment [1].

**Immunodeficient Animals**

Pancreatic adenocarcinoma is among the most resistant of human cancers yet specific mechanisms of treatment resistance remain poorly understood. The limited understanding of resistance to current therapies stems, in part, from a lack of clinically relevant model systems that reflect in vivo changes occurring within patient tumors following treatment. It was sought to identify consistent, differentially expressed genes between treatment-naive pancreatic tumors and those exposed to neoadjuvant therapy using a strict, direct xenograft model system. It was concluded that engraftment of human pancreatic tumors into immunodeficient mice prior to and following neoadjuvant therapy is possible and provides an in vivo platform for comparison of global gene expression patterns. Decreased TGBFR2 expression and increased IGFBP3 expression among all direct xenograft tumors derived from treated tumors relative to untreated tumors suggests a role in therapy resistance [2].

**Xenografts**

Pancreatic cancer is characterized by early metastasis, thus modeling of the progression to metastasis is needed. Few models allow examination of both primary human tumors and synchronous metastases. There is renewed interest in direct xenografts from resected PDAC. Most xenografts are isolated from primary tumors and fail to capture phenotypes present in loco-regional/distant disease. It remains unclear whether tumors established from metastatic sites will recapitulate critical features of metastases in PDAC relative to primary tumor. It was
reported findings in which it was successfully engrafted primary tumor (PT) and synchronous lymph node metastases (LN). As part of prospective study, tumors obtained at surgery were implanted within NOD/SCID mice. Xenografts from both PT and LN in same patient were processed using Illumina chip platform. Selected gene expression differences between PT and LN validated by RT-PCR and IHC. Xenografts derived from PT and LN recapitulated histology from original patient specimens including stromal elements and abundant tumor gland formation. When transcriptome of xenografts from PT and LN were compared, only about 500/48,000 (1%) individual genes were statistically different. Xenograft tumors from LN were >89-fold increased in MMP-7 mRNA levels and > 19-fold reduced in SMAD-4 mRNA levels as confirmed by RT-PCR. Changes were confirmed through IHC. Differential expression among signaling molecules in TGF-F, NF-GB and Wnt/F-catenin pathways were observed confirming known molecular alterations in metastasis. It was concluded that xenograft models incorporating both primary tumor and synchronous metastatic lesions from the same patients recapitulate critical histologic features of PDAC and reflect known molecular changes in PDAC progression (over-expression of metalloproteinases/inactivation of SMAD-4). It was identified differences in signaling pathways between xenografts derived from PT and LN. It was propose direct xenografts incorporating primary tumor and synchronous metastases to serve as a platform upon which to test therapeutic agents involving primary tumors and associated metastases, the latter of which determines patient outcomes[3].

**Fluorophore-Conjugated Anti-CEA Antibodies**

It has previously demonstrated that fluorophore antibody conjugates enhance the ability to detect pancreatic cancer (PC) in mouse models during laparotomy. One study was undertaken to determine if this technology could be used to improve staging laparoscopy for pancreatic cancer. Orthotopic and carcinomatosis mouse models of human PC were established using non-fluorescent BxPC-3 human PC cells in nude mice. One to two weeks post tumor implantation, mice were given a tail vein injection of Alexa 488-conjugated anti-CEA. It was found that the use of fluoroscens and fluorophore-labeled anti-CEA antibody permits rapid detection and accurate localization of primary and metastatic lesions of CEA-expressing human pancreatic cancer. The introduction of an LED light source permitting human carcinomas of different wavelengths without compromising background illumination. Development of this technology can serve as a novel tool in the staging and treatment of pancreatic cancer [4].

**Molecular biology in pancreatic cancer**

**Molecular Profiling Of Endoscopic Ultrasound (EUS)**

EUS-guided FNA is the preferred minimally-invasive method for obtaining a cytologic diagnosis in patients with presumed pancreas cancer. Molecular profiling of individual patients' tumors may then allow for targeted systemic therapy. It was sought to examine the feasibility of molecular profiling of previously-obtained paraffin-embedded cell blocks of pancreatic FNA specimens as proof of concept for a phase 2 trial of molecular profile-directed therapy. It was identified 9 paraffin-embedded FNA samples that had previously been obtained by EUS in patients with pancreas cancer. A commercially-available molecular profiling platform utilizing immunohistochemistry (IHC), FISH, PCR, sequencing and DNA microarray was performed. Seven of the 9 specimens had sufficient material for molecular analysis. Based on the profile, and on evidence from historical outcomes of tumors with similar profiles, a Target Now report was generated for each tumor detailing the chemotherapeutic and targeted agents that were or were not deemed to be associated with a potential clinical benefit. It was concluded that it is feasible to perform molecular profiling on paraffin-embedded cell blocks of pancreas FNA specimens[5].

**Adiponectin**

Epidemiologic and clinical studies clearly show that obesity portends a poor outcome in pancreatic cancer, though the mechanisms underlying this association are unclear. Adipokines such as adiponectin and leptin are metabolically active molecules released by adipocytes that may directly influence tumor growth. In a murine in vivo model of pancreatic cancer in obesity, it was observed a significant inverse correlation between tumor proliferation and circulating adiponectin concentration. It was therefore hypothesized that adiponectin and leptin would alter the intratumoral proliferation of murine pancreatic cancer cells. It was found that pancreatic cancer cell lines express adiponectin receptors, one cell line but not another express leptin receptors, exogenous leptin did not affect cellular proliferation and adiponectin, an adipokine that is found at higher concentrations in the lean phenotype, significantly decreased proliferation of murine pancreatic cancer cells in vitro. It was concluded that adiponectin, but not leptin alters the in vitro proliferation of a murine pancreatic cancer cell line[6].

**Aldehyde Dehydrogenase Activity**

Multiple studies in recent years have identified highly tumorigenic populations of cells that drive tumor formation. These cancer stem cells (CSCs), or tumor-initiating cells (TICs), exhibit properties of normal stem cells and are associated with resistance to current therapies. As pancreatic adenocarcinoma is among the most resistant human cancers to chemo-radiation therapy, it was sought to discriminate the presence and relative tumorigenicity of pancreatic cancer cell populations expressing the CSC markers CD133, aldehyde dehydrogenase (ALDH), CD44, and CD24. The cell line L3.6pl was examined for ALDH activity and CD133 cell surface expression using flow cytometry. The intensity and gross percentage of ductal carcinoma cells expressing ALDH1 were assessed visually and graded in a tissue
microarray (TMA) comprised of 106 untreated patient tumors. Multiple generations of direct xenograft tumors were established through transplantation of patient tumors into NOD/SCID mice and ALDH1 expression compared. Analysis by flow cytometry demonstrated high ALDH activity (ALDH$_{\text{high}}$) in 16 percent (median 14%) and CD133 expression in 0.2 percent (median=0.17) of L.3.6pl cells. Tissue microarray analysis confirmed ALDH1 expression in patient tumors with low, moderate, and high ALDH1 expression in 22 percent (23/106), 26 percent (28/106), and 22 percent (23/106) of examined patient specimens, respectively. Immunohistologic comparison of patient tumors and derived xenograft tumors demonstrated similar ALDH1 expression. Single cell suspensions prepared from multiple generations of direct xenograft tumors demonstrated conserved proportions of ALDH$_{\text{high}}$, ALDH$_{\text{low}}$, CD133+ and CD133- tumor cell populations as identified by flow cytometry. ALDH$_{\text{high}}$/CD44+/CD24+ cell populations comprised an average of only 0.015 percent (median 0.018 %) of all viable, human pancreatic cancer cells and an average of 0.48 percent (median 0.38 %) of all ALDH$_{\text{high}}$ cells. It was concluded that ALDH expression is heterogeneous among patients with pancreatic cancer and is recapitulated in serial generations of direct xenograft tumors in NOD/SCID mice. Cell populations enriched for high ALDH activity alone are sufficient for efficient tumor-initiation with enhanced tumorigenic potential relative to CD133-enriched and ALDH$_{\text{low}}$ cell populations in human pancreatic adenocarcinoma. ALDH$_{\text{high}}$/CD44+/CD24+ or ALDH$_{\text{low}}$/CD44+/CD24+ phenotypes do not appear to significantly contribute to tumor formation at low tumor cell inocula. Further efforts to identify novel therapeutic targets in pancreatic cancer should focus on ALDH$_{\text{high}}$ cell populations [7].

**Casein Kinase II**

The protein kinase casein kinase II (CK2) is a ubiquitously expressed serine threonine kinase and is believed to play a role in the survival of cancer cells. It was evaluated the inhibition of CK2 activity as a potential therapeutic strategy for pancreatobiliary cancer cell lines. It was found that inhibition of CK2 induces cell death in pancreatobiliary cancer cells by autophagy. It also sensitizes pancreatic cancer cells to TRAIL induced cell death. These results suggest that inhibition of CK2 holds great promise as a potential target for drug development [8].

**COX-2**

It is well demonstrated that prostaglandin E2 (PGE2), which product from arachidonic acid (AA), stimulates pancreatic cancer cell growth through EP2 and 4-receptors in cyclooxygenase-2 (COX-2) dependent manner. It is well demonstrated that COX-2 expression of pancreatic cancer cell lines are related with PGE2 production from arachidonic acid and those stimulate cell growth. This stimulation is suppressed by selective COX-2 inhibitor (Coxibs) and a selective EP4-antagonist (EP4-Ant). On the other hand, recent studies of lung, colon and prostate cancer defines a role of COX-2 in the over-production of ELRpositive CXC chemokines (ELR+CXCLs: CXCL5 and 8). ELR+CXCL show growth stimulation via CXC-receptor-2 (CXCR2) in those cancers. Therefore, COX-2 expression is thought that tightly related with CXCLs production; moreover ELR+CXCLs are the crucial mediators on PaCa growth, too. However, in pancreatic cancer, the relation of COX-2 dependent CXCL production has not been identifying. The aim of one study was to establish the profiles of COX-2 depending CXCLs production. COX-2 positive cell line, BxPc-3(B), KMP-4 (K4) and COX-2 negative cell lines, Mia-PaCa-2 (MP) and KMP-3 (K3) were analyzed for CXCL5 and 8 by ELISA and Immunofluorescence studies (IMF). The data demonstrate that the production of ELR+CXCL is correlated with COX-2 expression, moreover ELR+CXCLs and CXCR2 are highly expressed in COX-2 positive pancreatic cancer cell lines. The production of ELR+CXCL is enhanced by AA and PGE2, and is suppressed by Coxibs and EP4-Ant. COX-2 dependent Pancreatic cancer cell growth may have possibility that be correlated with CXCLs production and CXCLs dependent cell growth with autocrine or paracrine systems. Since, CXCLs production may be regulated by COX-2 dependent manner; Coxibs or EP4 receptor antagonist may become new approach for pancreatic cancer treatment and prevention [9].

**EGFR**

The combination of gemcitabine with the EGFR-tyrosine-kinase-inhibitor (EGFR-TKI) erlotinib translated into a significant improvement of overall-survival (OS), whereas the combination with anti-EGFR cetuximab failed to meet its primary end-point of improving OS. Although increase in tumor control rates and association between rash and outcome seen in the gemcitabine + erlotinib study suggested that therapeutic benefit is confined to a subset of patients, in both trials analysis of EGFR expression failed to reveal a correlation with outcome. However, previous studies showed a correlation of EGFR expression with grading and prognosis in PDAC patients who underwent only surgical treatment. Therefore, it was performed an immunohisto-chemical analysis of EGFR in 100 PDAC patients, enrolled between 2004 and 2008, who underwent PDAC resection and were treated with gemcitabine, in order to evaluate the correlation between histological grading and outcome. Furthermore, it was evaluated by uni-/multi-variate analysis the role of stage (I-II/III), lymph-node/neural infiltration (yes/no) and resection margin (R0/R1). EGFR staining was performed in from tissue microarray (TMA) sections obtained from paraffin-embedded tumor material (from 4 different tumor areas for each patient). EGFR staining was detectable at membrane and cytoplasmatic level in 84 percent and 82 percent of the patients, respectively. The values of EGFR expression, as evaluated by a total score from the analysis of both the number of positive cells and the staining intensity, ranged between 0 and 10 (median=7). EGFR expression was significantly higher in grade-3 PDAC. The histological differentiation resulted as a prognostic factor.
with median OS of 25 versus 11 months in patients with grade-1/2 and grade-3 tumors, respectively. Similarly, patients with grade-1/2 and grade-3 PDAC had median progression-free survival of 17 and 7 months, respectively. Among other possible prognostic factors, only lymph-node positive-status was correlated with significantly shorter OS and PFS. In the Cox proportional-hazards-model both grade-3 and lymph-node positive-status point out the independent predictive parameters for death and progression risk. In contrast, no correlation was observed between EGFR expression and both PFS and OS, as well as with stage, lymph-node/neutral infiltration and resection margin. In conclusion, EGFR expression correlated with PDAC grading, but it lacked predictive value with respect to outcome in gemcitabine-treated patients [10].

**ErbB3**

The tumor microenvironment plays a key role in PDAC progression and understanding the dynamic interactions between carcinoma cells and surrounding stromal elements is essential in the design of novel therapeutic interventions. It has previously been demonstrated that ErbB3 is an obligate heterodimer partner to the Epidermal Growth Factor Receptor (EGFR) which influences pancreatic cancer cell response to anti-EGFR therapy with erlotinib. Based on those findings we postulated the existence of ligand dependent Erb receptor signaling mechanisms that mediate interactions between PDAC cells and stromal cancer-associated fibroblasts (CAF). It was hypothesized that targeted disruption of CAF-mediated, ligand-dependent, EGFR and ErbB3 signaling cascades may provide a more rational approach to inhibition of tumor progression in PDAC. To test this hypothesis it was first established primary CAF cultures from surgically resected human PDAC specimens (n=15). Conditioned media (CM) from cultured CAF was rich in neuregulin-1 (NRG-1) a specific ErbB3 ligand that promotes PI3K mediated signaling and activation of AKT. In vitro, stimulation of pancreatic cancer cell lines (AsPC-1 and BxPC-3) with CAF-derived NRG-1 rich CM promoted tumor cell proliferation and activation of the ErbB3-Pi3K-AKT signaling axis. This effect was partially abrogated by treating AsPC-1 cells with a specific monoclonal ErbB3 antibody (MM-121). When combined with erlotinib, MM-121 completely abolished the activation of ErbB3-AKT cascade, a result that could not be achieved by either agent alone. In vivo, it was established a novel murine model of PDAC sub-cutaneous xenografts which included both CAF and AsPC-1 pancreatic cancer cells. The presence of CAF vigorously promoted pancreatic cancer cell tumorigensis as reflected by a significant increase in xenograft volume. CAF-containing AsPC-1 xenografts were found to be resistant to the anti-tumor effects of EGFR inhibition with erlotinib (treatment duration of 21 days). The anti-proliferative effects of MM-121 antibody treatment on AsPC-1 xenografts were tested with doses ranging from 75 mg to 600 mg every third day. It was observed a dose-dependent effect in the inhibition of tumorigenesis with complete inhibition of tumor proliferation for the 600 mg treatment group. Analysis of the xenografts demonstrated not only decrease in ErbB3/AKT activation but also an effect on total ErbB3 receptor levels further supporting the potential therapeutic effectiveness of this drug in PDAC. It was concluded that CAF-secreted NRG-1 promotes pancreatic cancer cell tumorigensis via ErbB3 and confers resistance to the anti-proliferative effects of erlotinib-induced EGFR inhibition. It was identified the NRG-1/ErbB3 axis as an attractive target in the attempt to disrupt the tumorigenic stromal-epithelial interactions within the PDAC microenvironment [11].

**ERK**

The purpose of this communication is to present three novel advances in the field of gallstone pancreatitis pathogenesis. The introduction of a novel murine experimental model, the use of the adeno-associated viral (AAV) vector in the exocrine pancreas, and reduced mortality with specific inhibition of the MAP kinase Extracellular Regulated Kinase (ERK) were explored. Suitable experimental models of gallstone pancreatitis with systemic inflammation and mortality are limited. It was therefore developed a novel murine model of duct ligation-induced acute pancreatitis associated with multi-organ dysfunction and severe mortality. Laparotomy was done on C57/BL6 mice followed by pancreatic duct (PD) ligation, bile duct (BD) ligation alone, or sham operation. Only mice with PD ligation developed acute pancreatitis and had 100 percent mortality. Pulmonary compliance was significantly reduced after PD ligation but not BD ligation. Bronchoalveolar lavage fluid neutrophil count and IL-1b concentration, and the plasma creatinine level, were significantly elevated with PD ligation but not BD ligation. Pancreatic NF-kB (p65) and AP-1 (c-Jun) were activated within 1 h of PD ligation. PD ligation-induced acute pancreatitis in mice is associated with systemic inflammation, acute lung injury, multi-organ dysfunction, and death. The development of this novel model is an exciting and notable advance in the field. Using this model, it was shown that AAV-mediated in vivo expression of dominant negative (DN) ERK improves mortality. This is the first report of the use of the AAV vector for in vivo gene modulation in an exocrine pancreatic disease. AAV vectors have several advantages over adenoviral vectors for in vivo work primarily due to low immunogenicity. First, it was shown that 3e12 viral genome particles (VGP) of AAV8.GFP i.p. results in robust pancreatic expression of GFP in 1 week that peaks by 2 weeks and plateaus at 3 weeks (immunoblot). Immunohistochemistry confirmed GFP expression in acinar cells and H&E stain showed absence of neutrophilic infiltration. GFP expression was also seen in the liver but not the lung. To determine the effect of MAP kinase inhibition on mortality, it was injected mice with 3e12 VGP of either AAV8.DN.ERK or AAV8.DN.p38 alone, or AAV8.DN.ERK and AAV8.DN.p38 combined, and ligated the pancreatic duct after two weeks. Sham-operated controls had no mortality. Diseased-controls showed median mortality of 3.5 days and 100 percent mortality by 5 days. AAV8.DN.ERK pretreatment alone significantly
diminished the mortality with a median mortality of 5 days with 70 percent mortality by 5 day. Gene transduction was confirmed with RT-PCR and immunoblotting. AA8V.DN.p38 pretreatment alone showed no difference in survival, although it was confirmed gene transduction with RT-PCR and immunoblotting. Pretreatment with both AA8V.DN.ERK and AA8V.DN.p38 showed a trend towards even better survival (66 % mortality by 7 days) than AA8V.DN.ERK alone. It was concluded that using an original murine model and a novel gene modulation technique, it was shown that ERK plays an important role in disease pathogenesis and mortality, while p38 may have a possible contributory role [12].

Gli
Pancreatic ductal adenocarcinoma (PDAC) is the most common form of pancreatic cancer. Activating mutations in KRAS are present in almost all PDAC, and can be identified in early precursor lesions. Inactivation of TP53 is also commonly observed in advanced precursor lesions and in invasive and metastatic carcinoma. In addition, components of the Hedgehog signaling pathway, including the Gli transcription factors, are abnormally expressed during pancreatic tumorigenesis. Furthermore, it has been previously demonstrated that RNAi-mediated knockdown of Gli1 in PDAC cell lines results in the induction of apoptosis, suggesting that Gli activity is required in PDAC cells. However, the requirement for Gli activity during pancreatic cancer formation in vivo has not been tested. Therefore, using a R26-Gli3T allele that expresses a dominant repressor form of Gli3 from the ubiquitous Rosa26 locus, it was ascertained whether Gli activity is required during PDAC development in vivo. It was found that activity of the Gli transcriptional regulators is required for the development of Kras-driven pancreatic cancers [13].

Hedgehog
A recent shift in the understanding of the role of the hedgehog (Hh) signaling pathway in PDAC focuses on the tumor microenvironment. Pathway inhibition affects the stroma and increases tumor vascularity. However, in non-tumor models Hh signaling is proangiogenic. Thus the aim of one study was to determine the effects of Hh pathway inhibition on the tumor cells and their stroma, as well as on tumor angiogenesis in a human PDAC xenograft model. Mice bearing three different subcutaneously xenografted human PDAC (n=5/group) were treated with 5E1, an antibody inhibiting the Hh ligand SHH. After treatment for seven days, tumor growth, viability, Hh pathway activity, mean vascular area and mesenchymal content were evaluated. Moreover, the expression of 76 pro- and antiangiogenic factors was assessed in the tumor cells and in their stroma using species-specific probes and nanostrings. The in vivo findings were further assessed using an in vitro sprouting angiogenesis assay. In vivo targeted inhibition of the Hh pathway in the stroma all three PDAC cancers was achieved and confirmed by knock down of the downstream targets of this pathway by real time PCR. In two of the three PDAC xenograft lines, Hh pathway inhibition affected tumor growth, viable gland density (a measure of viable tumor cells) tumor stromal content and tumor vascularity. The third cancer did not respond to therapy and was noted to have increased tumor vascularity. Nanostring evaluation revealed an upregulation of the antiangiogenic and proapoptotic thrombospondin-2 in the mesenchyme of the responsive tumors. In contrast the non-responsive tumor did not show a thrombospondin-2 up-regulation, but instead the tumor cells upregulated the proangiogenic factor VEGF-A. Using the in vitro sprouting-based angiogenesis assay, the Hh ligand SHH alone specifically increased VEGF-A-induced sprouting, but was not antiangiogenic alone or in the presence of other angiogenic factors such as b-FGF. Moreover, sprouting caused by Hh-responsive mesenchymal cells was enhanced in the presence of SHH. The addition of recombinant thrombospondin-2 abolished this effect. Antibodies to thrombospondin-2 were able to rescue the effect of the addition of anti-SHH. Inhibition of Hh signaling in PDAC affects both the tumor and its stroma. In contrast to previous reports, Hh pathway inhibition reduced tumor vascularity, suggesting that Hh plays a role in the maintenance or formation of the tumor vasculature. The molecular basis of this effect seems to be the repression of the mesenchymal antiangiogenic thrombospondin-2 by SHH. The reduction of tumor growth and viability seen in the epithelial compartment may in part be caused by the effects of SHH on the mesenchyme and vasculature [14].

Heme Oxygenase
Acute pancreatitis is a severe and frequently a life-threatening disease, which can lead to pancreatic necrosis, acute lung injury, SIRS and MODS. The inducible enzyme heme oxygenase-1 (HO-1) is an anti-oxidative, anti-inflammatory, and cytoprotective enzyme that is induced in response to cellular stress. The HO-1 promoter contains (GT)n dinucleotide repeats and is highly polymorphic in the population. The presence of longer repeats have been shown to be associated with lower levels of HO-1 expression in vitro and is associated with many diseases in vivo. In one study, it was hypothesized that the number of GT repeats in HO-1 promoter can influence the occurrence of acute pancreatitis due to its protective function. Patients with acute pancreatitis are more likely to have long repeats than controls. Acute pancreatitis (n=131) patients and age- and sex-matched healthy controls (n=33) were studied. Peripheral blood samples from pancreatitis patients were collected on admission. Genomic DNA was extracted from the blood samples of patient and control groups. The data demonstrate a strong bias toward longer alleles among patients with acute pancreatitis. Thus, polymorphism of the GT repeats in the HO-1 promoter region may be a risk factor for developing acute pancreatitis. Further studies are now underway to analyze the pancreatic levels of HO-1 protein in acute pancreatitis patients and controls and to determine whether the presence of the short alleles facilitate HO-1 upregulation and consequently promote its
hENT
Expression of intratumoral dihydropyrimidin dehydrogenase (DPD) and human equilibrative nucleoside transporter 1 (hENT1) has been reported to be associated with chemosensitivity to fluoropyrimidines and gemcitabine, respectively. The aim of one study was to investigate prognostic impact of intratumoral DPD and hENT1 expression on patients treated with adjuvant gemcitabine plus S-1 (GEM+S-1) chemotherapy after surgical resection for pancreatic adenocarcinoma. Intratumoral DPD and hENT1 expression was investigated by immunohistochemistry for 86 pancreatic adenocarcinoma patients who received adjuvant GEM+S-1 chemotherapy after surgical resection. Association between clinicopathological factors including DPD and hENT1 expression and survival was evaluated by univariate and multivariate analyses. Furthermore, all 86 patients were classified into three groups according to the number of favorable factors related to DPD and hENT1 expression: 2 favorable factors (low DPD and high hENT1), 1 favorable factor (low DPD and low hENT1 or high DPD and high hENT1), and 0 favorable factors (high DPD and low hENT1). Association between this combined DPD/hENT1 classification and survival was also evaluated. High DPD and hENT1 expression was observed in 35 (41 %) and 63 (72 %) patients, respectively. According to the combined DPD/hENT1 classification, 37 (43 %), 41 (48 %), and 8 (9 %) patients had 2, 1, and 0 favorable factors, respectively. Five-year disease-free survival (DFS) and overall survival (OS) rates for all 86 patients were 26 percent and 29 percent, respectively. Univariate analysis revealed that patients with low DPD expression experienced significantly longer DFS and OS than those with high DPD expression, and that patients with high hENT1 expression experienced significantly longer DFS and OS than those with low hENT1 expression. In addition, the combined DPD/hENT1 classification was also significantly associated with DFS and OS. Sub-analysis between pairs of patient groups revealed that patients with 2 favorable factors experienced significantly longer DFS and OS than those with 1 favorable factor, and experienced significantly longer DFS and OS than those with 0 favorable factors. Patients with 1 favorable factor experienced significantly longer DFS and OS than those with 0 favorable factors. Multivariate analysis revealed that both DPD and hENT1 expression was independently associated with DFS and OS combined DPD/hENT1 classification was also independently associated with DFS and OS. It was concluded that DPD and hENT1 expression can predict survival of pancreatic adenocarcinoma patients treated with adjuvant GEM+S-1 chemotherapy, and the combined DPD/hENT1 classification enables the stratification of these patients based on their likelihood of survival [16].

Gemcitabine is a nucleoside analogue used for the treatment of pancreatic cancer. Human equilibrative nucleoside transporter (hENT1) protein transports gemcitabine into cells. Previous studies reported that hENT1 expression of the tumor could predict response to gemcitabine-based adjuvant therapy in resected pancreatic cancer patients. However, there have ever been no reports describing the impact of hENT1 expression on the preoperative gemcitabine-based chemoradiotherapy (Gem-CRT). One study aimed to determine the relationship between intratumoral expression of hENT1 and the outcome of Gem-CRT in patients with T3/T4 pancreatic cancer. From February 2005 to July 2010, 93 patients with histologically or cytologically proven pancreatic adenocarcinoma had been enrolled for a Gem-CRT protocol: 57 patients with T3 and 36 with T4, whose tumor extension was determined by MDCT. The hENT1 expression was analyzed by immunohistochemical staining in the resected specimens from 55 patients who received curative-intent resection after Gem-CRT. Scoring for hENT1 was on the basis of relative intensities of staining of the pancreatic tumor with reference to the normally present hENT1 staining of cell membranes within the islets cells. The expression of 5-fluorouracil (5-FU) metabolic factors, thymidylate synthase (TS) and dihydropyrimidine dehydrogenase (DPD, were also stained immunohistochemically. According to our original definition of clinical efficacy for Gem-CRT (over 50 % reduction of serum CA19-9 level with PR or SD by RECIST criteria), 29 (53 %) of 55 patients were classified into the effective group. Residual tumor status included R0 (67 %), R1 (27 %) and R2 (5 %). hENT1 expression was positive in 39 (70 %) and negative in 16 (30 %). TS expression was strong in 29 (53 %) and DPD was strong in 40 (73 %). Positive hENT1 expression was significantly associated with clinical efficacy for Gem-CRT. Interestingly, the patients with positive hENT1 expression had significantly longer overall survival (OS) and recurrence-free survival (RFS) times than those with negative expression. Furthermore, the patients showing both clinically effective and hENT1 positive (25 patients) had significantly better prognosis than the other 30 patients. On the other hand, TS and DPD were not significantly associated with clinically efficacy and survival. The multivariate analysis for OS revealed that the positive hENT1 expression and R0 resection were significant prognostic factors. The data provides the evidence that hENT1 expression in pancreatic adenocarcinoma strongly influenced treatment outcome of preoperative Gem-CRT [17].

Hepatocyte Nuclear Factor (HNF)
Ampullary cancer (AC) is associated with a favorable prognosis compared to other periampullary carcinomas. Aside from other prognostic factors, the histological origin of AC may determine survival. Specifically, the pancreatobiliary subtype of AC displays worse prognosis compared to the intestinal subtype. However, knowledge of inherent molecular characteristics of different periampullary tumors and their effects on prognosis has been limited. Gene expression profiling was used in order to screen for differential gene expression between six
PDAC cases and 12 AC cases. Among others, hepatocyte nuclear factor 4-alpha (HNF4-alpha) mRNA over-expression was observed in AC cases. Nuclear HNF4-alpha protein expression was assessed using tissue microarrays consisting of 99 individual AC samples. Correlation of HNF4-alpha expression with clinicopathological data and survival was calculated. HNF4-alpha mRNA is 7.6 fold up-regulated in AC compared to that in PDAC. Bioinformatics analyses indicated its key role in dysregulated signaling pathways. Nuclear HNF4-alpha expression correlates with histological subtype, grading, CDX2 positivity, MUC1 negativity and presence of adenomatous components in the carcinoma. The presence of HNF4-alpha is a univariate predictor of survival in AC mean survival 50 versus 119

s of this approach. Growth of HuR

re healthy and no noticeable toxicity was

over a 96 percent reduction in HuR mRNA compared to control cells as detected by qPCR when RNA was extracted from pancreatic cancer cells after 48 hours post-transfection. Accordingly, the amount of HuR protein was markedly decreased as accessed by immunoblot assays. Reduction in HuR expression caused a dramatic decrease in anchorage-independent growth in soft agar. In the pre-clinical studies, MiaPac2 xenografts treated with HuR siRNA had a 1.6-fold increase in tumor volume after 4 intratumoral injections as compared to 2.8- and 3-fold increases in Luc siRNA- and PBStreated control tumors, respectively. Statistical comparisons of growth between Luc siRNA and HuR siRNA treated mice include a p value of 0.009 at day 3; and a p value of 0.031 at day 17. HuR protein expression from the tumors treated with HuR siRNA-nanotherapy was significantly diminished compared to the control groups. Of note, mice treated with HuR siRNA were healthy and no noticeable toxicity was detected. It was concluded that in vitro, HuR reduction in pancreatic tumor cells caused a marked decrease in anchorage-independent growth in soft agar, a tumorigenic property. Nanoparticle delivery of HuR siRNA to MiaPaCa2-generated xenografts dramatically decreased the amount of HuR protein in tumors and significantly suppressed tumor growth. These are the first proof-of-principle data that silencing HuR in pancreatic tumors, even as a monotherapeutic strategy, may be a promising therapeutic approach. Studies combining HuR inhibition with other chemotherapies are ongoing as are further pre-clinical studies in order to translate this therapeutic strategy rapidly to the clinic [19].

**Human Antigen R (HurR)**

HuR is an mRNA binding protein that is both activated and localized to the cytoplasm upon pancreatic cancer associated stressors (e.g. chemotherapy and hypoxia). In normal cells, the HuR network is utilized to protect cells from harmful and natural stimuli. In pancreatic cancer cells, HuR post-transcriptionally regulates core pathways involved in the tumorigenesis process, such as invasion and growth. It was therefore hypothesized that silencing HuR may be detrimental to the growth and survival of pancreatic cancer cells. Thus, it was designed a highly specific therapeutic strategy to suppress HuR expression in pancreatic cancer cells. It was first transfected pancreatic cancer cell lines growing in culture to test a siRNA oligo designed to target HuR mRNA specifically. Efficiency of siRNA silencing of HuR in transfected cells was evaluated by immunoblot and qPCR assays. Anchorage independent growth in soft agar (a hallmark of malignancy) of pancreatic cancer cell lines (MiaPaCa2, CAPAN1, and PL5 lines) transfected with HuR siRNA was evaluated 2-3 weeks post-transfection. Nanoparticle delivery of HuR siRNA into representative pancreatic cancer cell xenografts (MiaPaCa2 cells) in mice was used for pre-clinical analysis of this approach. Growth of HuR siRNA-treated mouse tumors was measured over a 2-week period and compared to the growth of tumors in control groups (PBS-treated and Luc siRNA-treated). HuR expression in resected tumors was measured by immunoblot analysis. The HuR siRNA oligo was highly efficient and specific. siRNA HuR transfection caused over a 96 percent reduction in HuR mRNA compared to control cells as detected by qPCR when RNA was extracted from pancreatic cancer cells after 48 hours post-transfection. Accordingly, the amount of HuR protein

Apoptosis has been identified as a core signaling pathway disrupted in pancreatic ductal adenocarcinoma (PDA) tumorigenesis. Death Receptor 5 (DR5, TRAILR2) is a membrane bound protein that initiates the extrinsic apoptotic pathway upon ligand exposure and is currently being explored as a “druggable” target in multiple cancers including PDA. Identifying a mechanism that regulates DR5 in the tumor microenvironment (e.g. hypoxia, chemotherapeutic exposure) is critical for optimizing DR5 based-therapies. Human antigen R (HuR), an RNA binding protein, controls post-transcriptional gene expression by binding to specific regions of 3'and 5' UTRs of mRNA target genes. Previously, HuR, a pro-survival molecule, has been shown to play an important role in the intrinsic apoptotic pathway. It was identified DR5 mRNA as a HuR target in PDA cells and explored the significance of HuR’s role in functionally regulating the extrinsic apoptotic pathway in PDA cells. It was also explored HuR as a modulator of DR5-targeted therapy for the treatment of PDA. Ribonucleo-protein immunoprecipitation (RNP-IP) assays were performed on PDA cells using HuR antibody (Ab) compared to a control (IgG Ab) under stress conditions, 3 hours with 1 microM of the standard of care drug for PDA, gemcitabine; and 75 microM of a PARP inhibitor (PARPi). mRNA was converted to cDNA using RT-PCR, and then analyzed by qPCR. HuR silencing was done with HuR siRNA. Immunohistochemistry was performed to measure HuR and DR5 expression in patient samples. DR5 mRNA was validated as a HuR target with a 6 fold greater binding to HuRm compared to the control. Strikingly, HuR binding to DR5 increases 12- and 24-fold upon treatment with gemcitabine and the PARPi
respectively. Silencing HuR expression, through siRNA transfections, leads to an increase of DR5 protein expression at 24 and 48 hours in multiple PDA cell lines. Additionally, silencing of HuR significantly enhances the action of a DR5-specific monoclonal antibody (0.8 microg/mL) against PDA cells within 36 hours (a 20 % detected increase in cell death compared to control cells), most likely due to an enhanced availability of the DR5 receptor. Finally, in a training set of PDA clinical specimens, it was found a significant inverse correlation between high/lower HuR cytoplasmic expression and low/high DR5 levels. In over 80 percent (26 of 31) of the specimens HuR cytoplasmic levels inversely correlated with DR5 expression levels, providing further evidence that elevated cytoplasmic HuR is repressing DR5 protein levels in patient tumor cells. In sum, it was shown that “activated HuR” represses DR5 protein expression in PDA cells. Therefore, it was concluded that low cytoplasmic HuR levels allow for greater availability of the target DR5, and will thus accordingly enhance the efficacy of DR5-targeted therapy. Thus, manipulating and/or utilizing HuR expression levels may serve as a clinically informative tool for optimizing DR5-targeted therapy [20].

Twelve core signaling pathways with 540 overexpressed individual genes have recently been identified as critical for the development of pancreatic ductal adenocarcinoma (PDA). The mechanism of over-expression for nearly all (99 %) of the identified up-regulated genes in pancreatic tumorigenesis is unknown. It was explored the hypothesis that post-transcriptional gene regulation may be a powerful alternative process in which these up-regulated genes werebeing disrupted. A key component of this regulatory process is Human antigen R (HuR), which can modulate gene expression by binding to mRNAs that encode for tumor promoting proteins in cancer cells. Previously, it was discovered that HuR is a key marker for poor pathologic features in PDA and is a predictive marker for gemcitabine-based chemotherapy. It was identified 60 putative targets (11 %) for HuR regulation among the over-expressed genes in PDA. In comparison, genetic and epigenetic alterations contribute only 1 percent and 1.8 percent, respectively, to the proposed mechanisms by which these 540 genes are disrupted in pancreatic cancer. Ten of the 60 putative target genes which are a part of 5 of the 12 core signaling pathways in PDA, including K-Ras, were experimentally validated as specific HuR targets. It was concluded that HuR is an unprecedented regulatory protein of at least 5 critical signaling pathways in pancreatic cancer [21].

**Interferons**

Adjuvant therapy (chemotherapy with or without radiotherapy) may improve long-term survival and over the last few years the benefits of this approach have become clearer in pancreatic cancer. Still, the optimal choice of treatment remains controversial. There have been several studies addressing adjuvant and neoadjuvant interferon (IFN) therapy for pancreatic carcinoma, some with promising and some with disappointing results. However, it has never been investigated whether or not there is a clear rationale for those interferon therapies. The aim of one study was to explore the possibilities of interferon therapy in the treatment of pancreatic and cancer. It was evaluated the anti-tumor activity of type I IFNs in 9 different human pancreatic adenocarcinoma cell lines. There was a wide variation in responsiveness to IFNs amongst the panel of human pancreatic cancer cell lines. IFN-beta is a significantly more potent growth inhibitor in human pancreatic cancer cell lines than IFN-alpha. There is a positive correlation between the sensitivity to IFN-alpha and the expression of the IFNAR2c receptor. This correlation was not found for responsiveness to IFN-beta. It is possible that the growth inhibitory effect of IFN-beta not only takes place via the IFNAR2c receptor, but that there are more mechanisms of action responsible for its potent growth inhibitory effect. Still, the expression of the IFNAR2c in pancreatic carcinomas can be of predictive value in the responsiveness for IFN-alpha therapy [22].

**Metastatic-Protective Stromal Factor in Pancreatic Cancer**

The majority of patients who undergo resection of PDAC for curative intent develop metatstic recurrence. Since the progression from primary malignancy to metastasis requires sequential genetic changes, it was hypothesized that comparison of the transcriptome of PDAC tumors with high versus low metastatic potential would identify candidate markers of metastasis. To test our hypothesis, subclones of increasing metastatic potential (C5, C5LM2, FG, and L3.6) were developed from poorly metastatic parental pancreatic cancer cell lines (PANC1 and COLO357). It was concluded that using an unbiased hypothesis-generating gene expression array, it was identified a stromal marker whose expression is associated with low metastatic potential and improved survival and may act as a protective factor [23].

**Nicotine**

Nicotine, an addicting component of an established risk factor (tobacco) for pancreatic cancer is identified as a tumor promoter potentially acting through activation of oncogenes such as Src, a non-receptor protein tyrosine kinase. Recent studies have shown that the Id1 transcription factor (inhibitor of DNA binding/differentiation) is induced by nicotine in NSCLC cells. Id1 has been shown to play a role in tumor progression by promoting angiogenesis, invasion, and chemoresistance. Further, Src and its family of non-receptor protein tyrosine kinases (SFKs) play a role in angiogenesis, tumor progression, and chemoresistance. Based on these observations on NSCLC cells, it was hypothesize that nicotine-induces Id1 expression in a Src-dependent fashion and promotes tumorigenesis and chemoresistance. In a cell model it was demonstrate that Id1, by a nicotine promoting Src-dependent pathway, contributes to signaling pathways involved in tumor progression and establishment of a chemoresistant phenotype in pancreatic cancer [24].
Receptor for Advanced Glycation End-Products (RAGE)
Damage-associated molecular pattern molecules (DAMPs) and their receptors have been shown to contribute to the progression of neoplasia via recruitment of inflammatory cells and stimulation of angiogenesis and reparative cellular proliferation. RAGE is a well-characterized DAMP receptor which recognizes such danger molecules as heat-shock and s100 family proteins, high mobility group box-1 (HMGB-1) and double stranded DNA, and plays an integral part in the immune cell response to danger signals. Knock-down of RAGE with targeted shRNA in pancreatic cancer cell lines increases susceptibility to cytotoxic insults through upregulation of autophagy. It was hypothesized that eliminating RAGE in a murine model of pancreatic carcinogenesis would slow progression of intraepithelial neoplastic lesions and decrease the genesis of invasive cancers by preventing autophagy and enhancing apoptosis of dysplastic epithelial cells. It was found that eliminating RAGE expression in a Kras-driven mouse model of pancreatic neoplasia decreases progression of PanIN lesions, through increased apoptosis and decreased autophagy. Decreasing RAGE expression in human pancreatic cancer may increase the benefit of current cytotoxic chemotherapies [25].

Retinoblastoma Suppressor Gene
Retinoblastoma tumor suppressor binding protein, Rb, is a major regulator of the mammalian cell cycle progression. Inactivation of Rb by a cascade of phosphorylation events leads to its inactivation, facilitating release of transcriptionally active E2F and S-phase entry. Rb phosphorylation is mediated mainly by CDK, but it was observed Raf-1 could bind and phosphorylate Rb early in the cell cycle, facilitating phosphorylation events. Although previous reports have suggested that K-Ras, which is mutated in over 80 percent of pancreatic cancers, activates MAPK through Raf/Mek signaling, inhibition of this pathway have proven clinically unsuccessful. It was demonstrated that increased binding of Raf-1 to Rb, independent of the Mek/MAPK pathway, has contributed to tumor progression and inhibition of this interaction might be a suitable strategy for cancer therapies. It was now examined whether targeting Rb-Raf-1 interaction with a small molecule inhibitor RRD-251 is a viable strategy against pancreatic cancer. In pancreatic cancer cell lines it was demonstrated that a disruption of the Rb-Raf-1 kinase interaction with RRD-251 significantly affects the malignant properties of pancreatic cancer cells [26].

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