Molecular Characteristics of Pancreatic Carcinogenesis

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Cover: So far there is not an absolute cure for pancreatic cancer yet. However, *inch by inch* we are increasing our knowledge about this malignancy. *Because we know when we add up all those inches that is going to make the difference between winning and losing between living and dying*... Life's this game of *inches*. And so is football...

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Molecular Characteristics of Pancreatic Carcinogenesis

Moleculaire kenmerken van pancreas carcinogenese

(met een samenvatting in het Nederlands)

Proefschrift

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Co-promotor: Dr. A. Maitra

The inches we need are everywhere around us

Voor Pap en Mam

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General Introduction

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General introduction

Ductal adenocarcinoma of the pancreas (i.e., pancreatic cancer) is among the leading causes of cancer-related death in the Western countries. Annually, more than 34.000 patients are diagnosed with pancreatic cancer in the United States and almost the same number will die from this disease [1]. Although pancreatic cancer accounts for only about 3% of all malignant tumors, it nevertheless represents the fourth most common cause of death due to cancer. The peak incidence occurs in the seventh and eighth decade of life, with the average age of diagnosis being 60 to 75 years [2]. For all stages combined the overall 5-year survival rate of pancreatic adenocarcinoma is very poor, less than 5%, and the 1-year survival rate is around 20% [3;4].

The dismal prognosis is largely a reflection of the late diagnosis, because of lack of specific symptoms, in combination with the aggressive course of the disease. Most patients' initial symptoms are weight loss, abdominal pain, back pain, anorexia and malaise. In many cases obstructive jaundice is the presenting symptom, but this is also caused by benign diseases in the head of the pancreas region [5]. Radical resection of the tumor is the therapy of choice and at this moment the only serious option for cure, but more than 80% of patients have a carcinoma extending beyond the pancreas at the time of diagnosis, and only 10 to 20% of patients have lesions that are resectable. Studies from high volume centers with optimal staging report up to a 15-20% 5-year survival rate in patients undergoing surgical resection [6;7]. The reason of these unfortunately low numbers is the fact that even if curative surgery takes place, nearly all patients still develop local recurrence and/or distant metastases after the surgery. In addition, also conventional therapeutic modalities like chemo-radiation have had minimal impact, and the long-term survival of patients with pancreatic cancer has not improved in the last five decades [8-10]. Early detection seems to be the 'key-element' in the fight against pancreatic cancer. New tests that can diagnose early pancreatic cancers or better yet the precursors to these cancers are therefore needed.

Tumorigenesis

The past decade has witnessed an exponential growth in our understanding of the nature of pancreatic cancer. Nowadays, it is clear that pancreatic cancer is fundamentally a genetic disease, caused by inherited germline and acquired somatic mutations in cancer-related genes [11]. It is now recognized that, analogous to other epithelial cancers, pancreatic cancer does not arise de novo but follows a stepwise progression from normal cuboidal duct epithelium via histologically well-defined non-invasive precursor lesions, to finally invasive ductal carcinoma [12;13]. Briefly, in pancreatic cancer there are three common established precursor lesions: pancreatic intraepithelial neoplasias (PanINs), mucinous cystic neoplasms (MCN), and intraductal papillary mucinous neoplasms (IPMNs) [14]. These precursor lesions are associated with an accumulation of multiple specific and generalized molecular genetic alterations affecting a variety of cancer-causing genes. Currently, a compendium of alterations in tumor-suppressor genes, oncogenes, and genome-maintenance genes that are important in pancreatic cancer progression has been identified, including activating point mutations in the *KRAS* oncogene, and inactivation of the *p16INK4A/CDKN2A, p53, SMAD4/DPC4* tumor-suppressor genes [2;13]. For the discussion of these genes in more detail is referred to chapter 3.

Besides tumor-suppressor genes and oncogenes mutations, pancreatic cancer features aberrant signaling in morphogenic pathways such as the Hedgehog and Notch

pathways [15]. Both pathways are known as embryonic signaling pathways, which play an important role in multiple tissues during development *in utero*, and these pathways are for the most part turned off in adult somatic cells, including the exocrine pancreas. Recently, it has been shown that abnormal transcriptional activation of these pathways occur in both human and mouse models of pancreatic neoplasia [16-18]. A recent study of Vogelstein *et al,* suggests that pancreatic cancers are the result of genetic alterations in a large number of genes that function through a relatively small number of pathways and processes [19]. Therefore the best hope for therapeutic options may lie in the discovery of agents that target the effects of the altered pathways and processes, such as Hedgehog, Notch and others, rather than targeting individual gene components.

Familial pancreatic cancer

It is estimated that approximately 10% of the pancreatic cancers have a familial basis [20;21]. Having a first-degree relative with pancreatic cancer doubles the risk of developing pancreatic cancer, and the risk increases with increasing numbers of affected relatives [22]. A minority (~20%) of the patients with an inherited predisposition to pancreatic cancer harbor germline mutations in genes that are associated with cancer susceptibility syndromes, such as, *p16INK4A/CDKN2A, BRCA2, STK11/LKB1,* and *PRS11* or DNA mismatch repair genes (genome-maintenance genes) *hMLH1* and *hMSH2* [23]. For example, the familial atypical multiple mole and melanoma (FAMMM) syndrome is caused by germline mutations of *p16INK4A/CDKN2A*. Families with the FAMMM syndrome are not only at risk for melanoma but also for the development of pancreatic cancer [24;25]. Carriers of the germline *p16-Leiden* deletion have an estimated risk of 17% to develop pancreatic cancer by the age of 75 [26;27]. In the overwhelming majority of families, however, the underlying genetic cause is unknown, although genetic segregation analyses suggest that an autosomal dominant inheritance of a rare allele is most likely responsible for the familial predisposition [28].

MicroRNAs

MicroRNAs (miRNAs) are a novel class of small non-coding RNAs of about 18–24 nucleotides that regulate protein expression by posttranscriptional silencing [29]. MiRNAs control the gene expression posttranscriptional either via the degradation of the target mRNA or the inhibition of protein translation [30]. The relevance of this class of novel small RNA regulators has only become clear over the past few years. At this moment, approximately 400 mammalian miRNAs have been identified and recent evidence suggests the existence of many more that have yet to be characterized. Nevertheless, it was realized early on that miRNAs are fundamental regulators of cellular processes that have physiological significance, including development, cell proliferation, differentiation and apoptosis [31]. Hence, deregulation of various miRNAs is emerging as an important contributor to many diseases including cancer [32;33]. In humans, aberrant expression of miRNAs contributes to carcinogenesis by acting as tumor-suppressors ('TSGmiRs') or oncogenes ('oncomiRs') [34;35]. As several studies have highlighted, miRNA expression is deregulated in pancreatic cancer [36-38]. Consequently, understanding the aberrant expression of miRNAs in pancreatic cancer may give us new insights in the molecular basis of this cancer, and new biomarkers for –early-cancer diagnosis and cancer therapy. For instance, the recent availability of in vivo miRNA knockdown strategies ('antagomirs') might be useful as potential therapeutic option [39].

Axl

Receptor tyrosine kinases (RTKs) are transmembrane proteins which transduce signals from the extracellular environment to the cytoplasm and nucleus. In this manner, RTKs regulate normal cellular processes, including survival, growth, differentiation, adhesion, and motility. There are different families of RTKs which are mainly characterized by differences in the ligand-binding extracellular domains. The mammalian Axl RTK subfamily includes three closely related members: Axl, Sky and Mer, also known as the TAM family [40;41]. Axl, also called UFO, ARK, and Tyro7, was originally identified as a transforming gene in human leukaemia [42]. The ligand of Axl, Gas6 protein, is so named by virtue of the initial finding that the gene (growth arrest-specific gene 6) that encodes the protein is highly expressed in growth-arrested cells [43]. The Axl ligand Gas6 is a vitamin K-dependent protein with structural homology to the anticoagulation factor Protein S. Moreover, Gas6 is a common ligand for the RTK subfamily, activating all members, with the strongest affinity for Axl. The Gas6 protein interacts with the extracellular domain of the Axl-RTK, leading to increased receptor kinase activity and activation of the mitogen-activated protein (MAP) kinase pathway, or the phosphatidyl-inositol (PI)3-kinase pathway [44;45]. However the exact mechanism of the Axl/Gas6 axis is still not known, and appears complex.

In various human cancers, overexpression of Axl has been illustrated. It is suggested that Axl in tumors is involved in cell cycle re-entry, survival and tumorigenesis. Altered Axl expression has been reported in lung cancer, uterine cancer, breast cancer, ovarian cancer, prostate cancer, thyroid cancer, liver cancer, renal cell carcinoma, AML, CML, erythroid leukemia, megakaryocytic leukemia, melanoma, osteosarcoma, and glioblastoma [46]. As for gastrointestinal cancers, Axl is being overexpressed in esophageal, gastric and colon cancer [47]. The role of Axl in pancreatic cancer has never been documented yet.

DNA Damage Response

When DNA is damaged, cells activate a response pathway that arrests the cell cycle and induces the transcription of genes that facilitate repair. The failure of this response results in genomic instability, a mutagenic condition that predisposes to cancer. The existence of these oncogenic barriers slows or inhibits the progression of pre-neoplastic lesions to neoplasia. When a cell encounters damage that is more difficult to repair, the so called 'DNA Damage Response' (DDR) machinery, delays cell-cycle progression (a.k.a. cell cycle checkpoints) to provide the cell more time for repair of the lesions [48;49]. Even failing to repair the damage does not usually result into deleterious mutations; as such genetically altered cells are commonly eliminated from the proliferative pool. This can be accomplished through inducing either a permanent cell-cycle arrest known as cellular senescence or physical elimination of the potentially hazardous, genetically unstable cells through one of the several forms of programmed cell death, called apoptosis [50]. Cellular senescence, a state of irreversible growth arrest, can be triggered by multiple mechanisms including telomere shortening and DNA damage [51]. Thus the state of senescence protects against the development of cancer.

Cell cycle regulators such as those within the retinoblastoma (Rb) and p53 pathways are the gatekeepers that maintain the senescence program [52]. The regulatory proteins involved in the pRb (retinoblastoma protein) and p53 pathways are cyclin-dependent kinases (CDKs) and cyclin-dependent kinase inhibitors (CDKIs). Among the CDKIs, the genes *p14/ARF* and *p16INK4A/CDKN2A* have been identified as important in maintaining senescence. p16 negatively regulates the cell cycle through competitive binding of CDK4 and CDK6,

thereby inhibiting their binding to cyclin D1. This in turn, regulates the G1 checkpoint via CDK/cyclin-D phosphorylation of the pRb. Once the pRb is phosphorylated, the cell is allowed to pass through the G1 phase onto the S-phase [53]. The p16 protein is often inactivated in a variety of human cancers, including pancreatic carcinoma [54;55]. p14 is a protein encoded by the same *CDKN2a* gene which encodes p16. It does not only negatively regulates the cell cycle at G1/S and G2/M by inhibiting the MDM2 oncoprotein, thereby blocking the MDM2-mediated breakdown of p53 [56], but is also a marker of cellular senescence [57].

Another interesting pathway that is activated in response to DNA damage is the ATM-Chk2-p53 checkpoint pathway. The ATM (ataxia telangiectasia mutated) kinase plays a central role in the coordination of the DNA Damage Response [58]. ATM controls cell cycle arrest in the G1 and G2 phase and also prevents ongoing DNA synthesis. ATM controls G1 arrest by direct activation of p53, which induces transcription of the CDK inhibitor p21, resulting in G1 arrest [59]. Furthermore, activation of ATM results in the phosphorylation of Chk2 and γH2.AX. Additionally, phosphorylated Chk2 triggers downstream proteins such as activation/ stabilization of p53, leading to cell cycle arrest to repair DNA damage, or apoptosis if damage is too severe for proper repair [60].

Bartkova *et al,* showed that Chk2 phosphorylation occurred in colon adenomas and the early stage of urinary bladder cancer and decreased in advanced carcinoma [61]. They thus suggested that the ATM-Chk2-p53 checkpoint pathway was activated and delayed or prevented tumor progression in the early stage of tumorigenesis. Another study demonstrated the activation of the DNA Damage Response in precancerous lesions of lung and skin as indicated by phosphorylation of Chk2 [62]. It is postulated that in precancerous lesions, DNA damage activates p53, which, by inducing apoptosis or senescence, raise a barrier to tumor progression. Breach of this barrier by various mechanisms, most notably by p53 mutations, will impair the DNA Damage Response pathway and allows cancers to develop [63]. Indeed, aberrations of checkpoint-related genes, such as *ATM, Chk2,* and *p53* have been reported in the literature and their role as tumor-suppressors has been widely accepted [64;65].

Pancreatic cancer follows a multi-step tumor progression model with non-invasive precursor lesions. Despite the presence of clonal genetic abnormalities, only a minor fraction of these precursor lesions progress to invasive cancer during the lifetime of an individual. Probably the DNA damage that emerges in these precursor lesions turns on checkpoint mechanisms that induces cellular senescence. Examination of the ATM-Chk2-p53 checkpoint pathway could provide a better understanding of the *molecular characteristics of pancreatic carcinogenesis...*

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Outline of the thesis

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Outline of the thesis

As described in the general introduction, pancreatic ductal adenocarcinomas mostly are diagnosed at a late, incurable stage. The poor prognosis and late presentation of pancreatic cancer patients underscore the importance of an effective early detection strategy for patients with a high risk of developing pancreatic cancer. Since pancreatic cancer is basically a disease of the genes, the optimal marker needs to be sought in the molecular changes that occur relatively early in the stepwise tumor progression from normal epithelium to infiltrating carcinoma. Early detection seems to be the *sine qua non* for the fight against pancreatic cancer and the molecular genetic changes that govern this tumorigenesis may be the most promising and specific biomarkers that can be used for early diagnosis at a time that it is still a curable disease.

By the time a tumor is clinically detectable, an accumulation of generalized and specific genetic alterations has already taken place in the tumor. The review article in **chapter 3** focuses mainly on the molecular insights on pancreatic ductal adenocarcinoma and its precursor lesions, including insights gained trough experimental models of pancreatic carcinogenesis. It will discuss the oncogenes, tumor-suppressor genes and the genome-maintenance genes involved in pancreatic tumorigenesis in more detail.

Just as there is a progression in the colorectum from low grade adenoma, to high grade adenoma, to invasive cancer, so too is there a histologic and genetic stepwise tumor progression from PanIN-1, to PanIN-2, to PanIN-3, to finally invasive ductal adenocarcinoma of the pancreas. Most importantly, careful molecular analyses over the last two decades have unequivocally demonstrated that these consecutive PanIN lesions share many of the genetic alterations observed in the infiltrating cancers, underscoring their true precursor status. In **chapter 4** we describe some of the most common seminal alterations that are seen in PanIN lesions and probably contribute to the stepwise genetic progression model of pancreatic cancer.

Chapter 5 investigates a rare variant of pancreatic cancer, namely undifferentiated carcinoma with osteoclastic giant cells, also referred to as UCOCGC of the pancreas. An UCOCGC of the pancreas associated with the FAMMM syndrome due to the *p16-Leiden* deletion, is evaluated in this chapter. The nature of this neoplasm puzzled pathologists for decades until molecular analyses showed that these are fundamentally undifferentiated carcinomas with reactive osteoclast-like giant cells [1]. The case is the first one reported in association with this hereditary condition that carries a well defined increased risk for pancreatic cancer.

Aberrant expression of microRNAs (miRNAs) is commonly observed in pancreatic adenocarcinoma. In contrast, miRNA abnormalities in pancreatic cancer precursor lesions have not been documented yet. The purpose of **chapter 6** was to determine whether microRNAs are being misexpressed in one of the precursor lesions of pancreatic cancer. Relative expression levels of a panel of twelve miRNAs upregulated in pancreatic cancers were assessed in 15 microdissected non-invasive IPMNs *versus* matched normal pancreata, using quantitative reverse transcription PCR (qRT-PCR). Two significantly overexpressed miRNAs –miR-155 and miR-21– were further investigated on archival material of IPMNs, one of the precursor lesions of pancreatic cancer, and in pancreatic juice samples.

In **chapter 7** we looked at the Notch pathway in pancreatic tumorigenesis, a pathway that is functional in embryogenesis of the pancreas. Aberrant activation of the Notch signaling pathway is commonly observed in human pancreatic cancer. In a panel of human pancreatic

cancer cell lines, we outlined the expression of Notch pathway related ligands, receptors and target genes. In addition, we were interested whether the Notch signaling pathways has an important function in tumor maintenance. Therefore, we disrupted intracellular Notch signaling either genetically by RNA interference or pharmacologically by use of a gamma secretase inhibitor.

The aim of **chapter 8** was to identify the expression of the receptor tyrosine kinase Axl in pancreatic cancer and its significance during pancreatic carcinogenesis. So far, to our knowledge, this association has not been investigated in the human pancreas tissue yet. To this end, we performed immunohistochemistry on a panel of 99 archival pancreatic adenocarcinomas, and looked at clinicopathological features. We also examined whether pancreatic cancer cells expressed Axl, and if so, we have tried to elucidate the role of Axl signaling in these pancreatic cancer cells.

Finally, in **chapter 9** we studied the ATM-Chk2-p53 checkpoint pathway in PanIN lesions. The purpose of this study was to clarify the involvement of the DNA damage checkpoint pathway in the tumorigenesis and progression of PanINs. On tissue microarrays of an independent set of 58 PanIN lesions immunohistochemical labeling was performed using antibodies against phospho-γH2AX^{Ser139}, phosphoATM^{Ser1981}, phosphoChk2^{Thr68} and p53.

The thesis includes a **summary** in English and Dutch in **chapters 10 and 11**.

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Pancreatic Carcinogenesis

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Abstract
Pancreatic cancer is an almost universally lethal disease. Research over the last two decades has shown that pancreatic cancer is fundamentally a genetic disease, caused by inherited germline and acquired somatic mutations in cancer-associated genes. Multiple alterations in genes that are important in pancreatic cancer progression have been identified, including tumor-suppressor genes, oncogenes, and genome-maintenance genes. Furthermore, the identification of non-invasive precursor lesions of pancreatic adenocarcinoma has led to the formulation of a multi-step progression model of pancreatic cancer and the subsequent identification of early- and late genetic alterations culminating in invasive cancer. In addition, an increased understanding of the molecular basis of the disease has facilitated the identification of new drug targets enabling rational drug design. The elucidation of genetic alterations in combination with the development of high-throughput sensitive techniques should lead to the discovery of effective biomarkers for early detection of this malignancy. This review focuses mainly on the current knowledge about the molecular insights of the pathogenesis of pancreatic ductal adenocarcinoma.

Epidemiology

Pancreatic cancer is a disease with a dismal outlook. In the United States approximately 34.000 patients are diagnosed with pancreatic cancer annually, and nearly an equal number will die from the disease, representing the fourth most common cause of cancer related mortality. Men and woman have an approximately equal risk [1]. Worldwide pancreatic cancer causes an estimated 213.000 deaths each year [2]. For all stages combined, the 1-year survival rate is around 20%, and the overall 5-year survival rate is less than 5%, despite even the most aggressive therapies currently available [1].

A number of risk factors have been identified [3]. Pancreatic cancer is predominantly a disease of the elderly. Pancreatic cancer is rare before the age of 40, and the median age at diagnosis is 73 years. Cigarette smoking is by far the leading preventable cause of pancreatic cancer [4]. Cigarette smoking doubles the risk of pancreatic cancer (Relative Risk = 2) [3]. Other risk factors include diets high in meats and fat, low serum folate levels, obesity, long standing diabetes mellitus, and chronic pancreatitis [3;5-7]. Approximately 10% of patients demonstrate a familial predisposition for pancreatic cancer, and a subset of these patients harbor germline mutations in *BRCA2, p16/CDKN2A, PRSS1, STK11/LKB1*, or the DNA mismatch repair genes (*see further Discussion below*). In the vast majority of patients with familial risk, however, the underlying genetic predisposition remains unknown.

Complete surgical resection remains the only curative treatment. Studies from high-volume centers with optimal staging report up to a 15%-20% 5-year survival rate in patients undergoing surgical resection [8;9]. The mortality rate is so high because pancreatic cancer usually only produces symptoms when it has already metastasized, and because there are no sensitive and specific tools to detect the disease at an earlier stage. Although multiple histological subtypes of pancreatic cancer have been described, the most common and deadliest form is pancreatic ductal adenocarcinoma [10]. Novel approaches to the management of patients with this aggressive disease are urgently needed.

Research over the last two decades has shown that pancreatic cancer is fundamentally a genetic disease, caused by inherited germline and acquired somatic mutations in cancer-associated genes. A compendium of alterations in tumor-suppressor genes, oncogenes, and genome-maintenance genes that are important in pancreatic cancer progression have now been identified (fig. 1). This review focuses mainly on the molecular insights on pancreatic ductal adenocarcinoma and its precursor lesions, including insights gained through experimental models of pancreatic carcinogenesis.

Precursor lesions of pancreatic cancer

Prior to a discussion on molecular genetics of pancreatic cancer, we will briefly discuss the current state of knowledge on precursor lesions of the pancreas. This is essential from the context of separating 'early' genetic changes (i.e., those associated with tumor initiation) from 'late' abnormalities (i.e. those associated with tumor progression). A recent review in *Pancreatology* has extensively discussed the histology and genetics of pancreatic cancer precursors (Singh *et al, Pancreatology* 2007) [11] and therefore, we will only discuss these in fleeting detail. Briefly, pancreatic intraepithelial neoplasias, PanINs, are classified into a four tier classification, including PanIN-1A, -1B, -2, -3, reflecting a progressive increase in histologic grade culminating in invasive neoplasia (fig. 2). The lowest grade PanIN lesions can be flat (1A) or papillary (1B), but are characterized by absence of nuclear atypia and retained nuclear polarity. PanIN-2 lesions have micropapillary features with evidence

of nuclear atypia and infrequent mitoses, while PanIN-3 lesions (a.k.a carcinoma-*in-situ*) demonstrate widespread loss of polarity, nuclear atypia, and frequent mitoses. In addition to microscopic PanIN lesions, there are now recognized macroscopic (cystic) precursor lesions of pancreatic adenocarcinoma –including Intraductal Papillary Mucinous Neoplasms (IPMNs) and Mucinous Cystic Neoplasms (MCNs). Akin to PanINs, the cystic precursor lesions also demonstrate a multistep histological and genetic progression to invasive neoplasia. Since IPMNs and MCNs can be detected by radiologic scans, they represent an opportunity to diagnose invasive pancreatic cancer before it can develop [11].

Tumor-suppressor genes

Tumor-suppressor genes are genes that promote tumor growth when inactivated. Tumor-suppressor genes are recessive, i.e. the two copies need to be mutated for loss of function, and they can be inactivated by a variety of mechanisms. First, by an intragenic mutation in one allele (copy of a gene) coupled with loss of the second allele; second, through a deletion of both alleles (homozygous deletion); and third, by hypermethylation of the promoter of the gene silencing gene expression. In sporadic cancers these alterations are both somatic mutations acquired during life, while patients with inherited forms of cancer inherit one mutant allele in the germline while the second allele is somatically mutated in the cancer cells.

The ***p16INK4A/CDKN2A*** gene located on the short arm of chromosome 9 (9p), is one of the most frequently inactivated tumor-suppressor genes in pancreatic cancer [12]. Remarkably, virtually all pancreatic carcinomas have loss of *p16INK4A/CDKN2A* function, in 40% of pancreatic cancer through homozygous deletion, in 40% by an intragenic mutation coupled with loss of the second allele, and in 15% by hypermethylation of the *p16INK4A/CDKN2A* gene promoter [12;13]. The protein p16 belongs to the cyclin-dependent kinase (CDK) inhibitor family and functions to prevent the phosphorylation of Rb-1 by cyclin-dependent kinases, and Cyclin D-Cdk4 and Cyclin D-Cdk6 complexes, which act as cell-cycle regulators [14;15]. Loss of *p16INK4A/CDKN2A* results in inappropriate phosphorylation of Rb-1, thereby facilitating progression of the cell cycle through the G1/S transition [16]. Thus, the p16/Rb pathway is inactivated in virtually all pancreatic cancers, leading to an inappropriate progression through the G1 phase of the cell cycle. Of note, in a small group of patients, inherited mutations of the *p16INK4A/CDKN2A* gene cause the Familial Atypical Multiple Mole Melanoma (FAMMM) syndrome, which is associated with an increased risk of developing melanoma and an increased risk of developing pancreatic cancer [17;18]. Particularly, the *p16*-Leiden deletion, a 19 bp deletion, is associated with an increased pancreatic cancer risk [19].

In addition, the homozygous deletions, which inactivate *p16*, can encompass adjacent genes, including the *MTAP, IFNA1* and *IFNB1* genes [20;21]. The *MTAP* gene is located approximately 100 kilo bases telomeric to the *p16INK4A/CDKN2A* gene on chromosome 9p21, and is frequently contained in the *p16INK4A/CDKN2A* homozygous deletions. As a result, *MTAP* function is completely lost in approximately 30% of pancreatic adenocarcinomas. This is a potentially promising finding, because it may have therapeutic implications [22]. The product of the *MTAP* gene, the enzyme methylthioadenosine phosphorylase plays an important role in the synthesis of adenosine [23]. Chemotherapeutic agents, such as L-alanosine, a purine biosynthesis inhibitor, have been developed, to

specifically target the selective loss of *MTAP* function in cancers, implicating that it might be effective against one third of the adenocarcinomas of the pancreas [22;23].

Mutation of the ***p53*** gene on chromosome 17p is the most common somatic alteration in human cancer. The p53 protein plays a central role in modulating cellular responses to cytotoxic stress by contributing to both cell-cycle arrest and programmed cell death. Loss of *p53* function during carcinogenesis can lead to inappropriate cell growth, increased cell survival, and genetic instability [24]. In pancreatic cancer, the *p53* tumor-suppressor gene is inactivated in 50-75% of the cases and occurs predominantly through single allelic loss coupled with an intragenic mutation of the second allele [25]. The loss of *p53* means that two critical controls of cell number (cell division and cell death) are deregulated in the majority of pancreatic cancers. Of interest, *14-3-3σ*, a *p53* regulated gene plays a role in signal transduction, apoptosis, stress response and cytoskeletal organization [26]. *14-3-3σ* is transcribed in response to DNA damage and in a number of cancers it is an important mediator of *p53* induced G2 arrest [27].

In addition, *p53* induced growth arrest is also achieved by transactivation of *p21*. *p53*-binding to DNA stimulates production of the protein p21, which negatively regulates the complex consisting of cyclin D and the cell division stimulating protein cyclin-dependent-kinase-2 [28], thereby preventing the cell from progressing from G1-S phase. This mechanism allows time for repair to damaged DNA. If *p53* mutated, it is not able to bind DNA, so p21 is not made available and abnormal growth can occur. Cell lines which lack wild type *p53* show a reduced or complete absence of p21 [29]. Loss of *p21* activity has been observed in approximately 30-60% of pancreatic tumor specimens [30-32]. Pancreatic cell lines and pancreatic tumors show a correlation between active *p53* and *p21* [33].

As stated, p53 loss is a 'double threat,' because it results in both loss of cell cycle checkpoints, as well as deregulation of programmed cell death (i.e., apoptosis). It is now known that p53-induced apoptosis is mediated by activation of genes involved in the apoptotic pathway, for example genes such as *PUMA* (p53 upregulated modulator of apoptosis) and *Noxa*. *PUMA* and *Noxa* are activated in a p53-dependent manner following DNA-damage. Once activated; they bind to Bcl-2, localize to the mitochondria to induce cytochrome c release, and activate the induction of programmed cell death [34-36].

Finally, the mircroRNA miR-34a deserves mention (*MicroRNAs in general are discussed later*): miR-34a is a direct transcriptional target of *p53*. MiR-34a activation can recapitulate elements of *p53* activity, including induction of cell-cycle arrest and promotion of apoptosis, and loss of miR34a can impair *p53*-mediated cell death [37;38]. Chang *et al*, showed that reduced expression of miR-34a is a very frequent feature of pancreatic cancer cells [39].

DPC4 (Smad4) is a tumor-suppressor gene on chromosome 18q and is one of the most commonly inactivated genes in pancreatic ductal adenocarcinoma, detected in approximately 55% of the cases. Inactivation occurs either through homozygous deletion, in approximately 30%, or loss of one allele coupled with an intragenic mutation in the second allele in approximately 25% [40-42]. The transcription factor SMAD4 is an important regulator of the transforming growth factor β (TGF-β) signaling pathway [43]. Upon receptor activation SMAD proteins become phosphorylated and heterodimerize with Smad4 to transmit upstream signals to the nucleus and transactivate transcription of specific target genes [44].

Loss of *SMAD4/DPC4* interferes with the intracellular signaling cascades downstream from TGF-β and activin, resulting in decreased growth inhibition via loss of pro-apoptotic signaling or inappropriate G1/S transition [43;45]. The *SMAD4* gene is remarkable for two reasons. First, inactivation of the *DPC4* gene is relatively specific to pancreatic cancer, although it occurs with low incidence in other cancers, such as colon, breast, and ovarian or biliary tract carcinomas [46;47]. Secondly, immunohistochemical labeling for Smad4 protein expression mirrors *DPC4/ SMAD4* gene status in pancreatic cancers with rare exceptions [42]. Inactivation of *DPC4/ SMAD4* is uncommon in non-ductal neoplasms of the pancreas [10], and is rare in most extra-pancreatic malignancies [10;46]. Therefore, immunolabeling for loss of Smad4 is a convenient ancillary diagnostic marker in clinical specimens, including suspected metastases from an occult pancreatic primary.

Many other tumor-suppressor genes that are targeted at low frequency in pancreatic cancer (<10%) deserve mentioning. Mutations in the *LKB1/STK11* gene are the cause of the autosomal-dominant inherited Peutz-Jeghers syndrome. Patients with Peutz-Jeghers syndrome have an increased risk of pancreatic cancer and it is conceivable that *LKB1* acts as tumor-suppressor gene in pancreatic cancer as well [48;49]. Intragenic mutations and homozygous deletions of the *MKK4* gene occur in a small percentage of pancreatic cancers [50]. The *MKK4* gene encodes for a component of a stress-activated protein kinase cascade and has a function in apoptosis and growth control. Furthermore, *MKK4* is preferentially inactivated in subsets of pancreatic cancer metastases, suggesting that the protein product may function as a metastasis suppressor [51]. Other less frequently affected tumor-suppressor genes include the *TGF-β/ activin* signaling pathway receptors such as *TGFβR1* (*ALK5;* chromosome 9q), *TGFβR2* (chromosome 3p), *ACVR1β* (*ALK4;* chromosome 12q) [52] and *ACVR2* (chromosome 2q) [53;54]. The TGF-β type I receptor (*TGFβR1*) ALK-5 forms a hetero-dimer with the TGF-β type II receptor (*TGFβR2*) to mediate signaling of *TGF-β* ligands. A downstream component of this pathway includes *DPC4 (SMAD4)*. Signaling initiated after binding of *TGF-β* related ligands to their cognate receptors leads to heteromerization and nuclear translocation of the Smad proteins and the transcriptional activation of target genes [55;56]. *TGF-β* is a pleiotropic factor that regulates cell proliferation, angiogenesis, metastasis, and immune suppression. The involvement of the *TGF-β* pathway has been established in cancers of many organs including the breast, lung, colon and pancreas. *TGF-β* signaling is frequently attenuated in pancreatic cancer because of alterations in components of the pathway [57;58].

Oncogenes

Oncogenes are genes that contribute to oncogenesis when mutationally activated. In contrast to tumor-suppressor genes they act in a dominant fashion, i.e. mutation of one copy of the gene suffices for activation. Oncogenes can be activated through a variety of mechanisms including point mutations within the gene and amplification of the gene itself. A growing number of oncogenes have been identified that are targeted in pancreatic cancer.

The most common activating point mutation involves the ***KRAS2*** oncogene, on chromosome 12p, in over 90% of pancreatic ductal adenocarcinomas[59;60]. This is the highest fraction of K-ras alteration found in any human tumor type. Frequent mutation sites involve codons 12, 13 and 61, but in pancreatic ductal cancers the majority occur in codon 12. The *KRAS* gene product mediates signals from growth factor receptors and other signal inputs. Mutation of

KRAS results in a constitutive gain of function, because the RAS protein remains trapped in the activated state even in the absence of growth factor signals, which leads to proliferation, suppressed apoptosis and cell survival.

The RAS family proteins encode small GTP-binding cytoplasmic proteins [44]. The constitutively active RAS intrinsically binds to GTP and confers uncontrolled stimulatory signals to downstream cascades including Ras effectors. Activated *KRAS* engages multiple effector pathways, notably the RAF-mitogen activated protein kinase (*RAF-MAPK*), phosphoinositide-3-kinase (*PI3K*) and RalGDS pathways.

Mutant *KRAS* has been extensively investigated as a marker of pancreatic cancer because mutations are basically entirely limited to one codon, can be readily detected using molecular assays and are present in approximately 90% of pancreatic ductal adenocarcinomas. Unfortunately, *KRAS* mutations are not specific for invasive pancreatic cancer and they occur in patients with chronic pancreatitis, in individuals who smoke, and *in-situ* neoplasias from patients without pancreatic cancer [61;62].

The ***BRAF*** gene on chromosome 7q, is a member of the RAS-RAF-MEK-ERK-MAP kinase pathway, and is mutated in one-third of the pancreatic cancers with wild-type (normal) *KRAS* [63]. *BRAF,* a serine/threonine kinase located immediately downstream in RAS signaling, is a frequent mutational target in several cell lines and non-pancreatic primary cancers including 66% of melanomas and 10% of colorectal carcinomas [64;65]. Interestingly, *KRAS* and *BRAF* mutations are mutually exclusive and tumors with mutant forms of one of these two genes invariably retain wild-type copies of the other. The requirement of oncogenic *KRAS*- or *BRAF*- pathway related signal transduction appears to be critically important for most instances of pancreatic ductal carcinogenesis.

The **PI3K-kinase-AKT** pathway is a key effector of *RAS* dependent transformation of many cell types and plays a role in cell survival, cell proliferation and other growth-related processes [66]. Activated PI3K results in phosphorylated phosphatidylinositides (PIP3), a step inhibited by product of the tumor-suppressor gene, *PTEN*. PIP3 in turn phosphorylates and activates AKT [29]. Recently, activating mutations of *PIK3CA*, the gene encoding PI3K, have been reported in a subset of pancreatic cancer precursors, specifically in intraductal papillary mucinous neoplasms (IPMN) [67]. Even in the absence of mutations, the *PI3K/AKT* pathway is constitutively active in the majority of pancreatic cancers [68]. This might be due the aberrant expression of their natural antagonist *PTEN* [69]. Although *PTEN* is not mutated in pancreatic cancers, the reduction of its expression may give pancreatic cancers cells an additional growth advantage [70]. Furthermore, amplification or activation of *AKT2* kinase, a major target of the *PI3-K* complex, occurs in up to 60% of pancreatic cancers [71-74] supporting the participation of an activated *PI3-K-AKT* axis in this disease.

A third downstream pathway activated trough *RAS* is the **RalGDS** pathway. *RalGDS* is one of several known Ras-regulated guanine-nucleotide exchange factors, or that function by activating Ral A and Ral B GTPases [75]. Recently, *RAL A* was shown to be activated in a variety of pancreatic cancers, and knockdown of *RAL A* suppressed tumorigenicity of RAS-transformed human cells [76]. In the same studies, knockdown of *RAL B* had no effect on tumor initiation, but suppressed tumor progression (i.e., metastases), suggesting divergent roles for the two RAL proteins in the context of pancreatic neoplasia. Whether or not these signaling moieties can be utilized as therapeutic targets remains to be determined.

The mammalian ***Hedgehog*** family of secreted signaling proteins –comprised of Sonic, Indian, and Desert Hedgehog (*Shh, Ihh*, and *Dhh*)- regulates the growth and patterning of many organs, including the pancreas, during embryogenesis [77]. The Hedgehog pathway is under negative regulation by the Patched (PTC) tumor-suppressor protein that inactivates the Smoothed (SMO) protein. The Hedgehog ligands engage the PTC transmembrane protein, disrupting the inhibition of SMO and thereby enabling signaling transduction to the *GLI* family of transcriptional regulators [78]. Loss of *PTC,* activating mutations in *SMO* and overexpression of GLI and Hedgehog proteins are associated with a variety of cancers [79]. Activation of the Hedgehog pathway has been implicated in both the initiation of pancreatic ductal neoplasia and in the maintenance of advanced cancers [80]. The expression of the *Hedgehog* ligands, the transcriptional target gene Patched (*PTC*), and the essential pathway component Smoothened (*SMO*) is undetectable in normal human pancreatic ducts. In contrast, a relative increase in the expression of these proteins is observed during pancreatic ductal tumorigenesis [78;81;82]. Moreover, it has been confirmed that the Hedgehog pathway plays a role in metastases. Inhibition of Hedgehog signaling has been shown to reduce the incidence of systemic metastasis in pancreatic adenocarcinoma xenografts [83]. Recently, Ji *et al.* showed that there is a cross-talk between oncogenic *KRAS* and the Hedgehog signaling pathway in pancreatic cancer cell lines. Their studies suggest that oncogenic *KRAS* through the *RAF/MEK/MAPK* pathway suppresses GLI1 protein degradation and consequently plays an important role in activating Hedgehog signaling pathway in the absence of additional *Hedgehog* ligand during pancreatic tumorigenesis [84].

The ***Notch*** signaling pathway is another pathway which is important in directing cell fate and cell proliferation during embryonic development. Later in life, the Notch signaling pathway plays a critical role in maintaining the balance among cell proliferation, differentiation, and apoptosis [85]. In mammals, this signaling pathway involves interaction of the membrane-bound Notch receptors (Notch 1-4) and Notch ligands (Delta-like, and Jagged) on adjacent cells [85;86]. The function of Notch signaling in tumorigenesis can be either oncogenic or antiproliferative, and the function is context dependent. In a limited number of tumor types, including human hepatocellular carcinoma and small cell lung cancer, Notch signaling is antiproliferative rather than oncogenic. However, most of the studies show an opposite effect of Notch in many human cancers including pancreatic cancer [87]. In the normal adult pancreas, Notch and its ligands are expressed at low levels. Interestingly, aberrant expression of its ligands, expression of mutant Notch1 oncoprotein, and abnormal expression of transcription targets of Notch signaling can be observed in early stages of pancreatic tumorigenesis as well as in invasive pancreatic cancer [88].

Several other oncogenes that are targeted in pancreatic cancer by amplifications deserve mentioning. First, the *AKT2* gene on chromosome 19q, is a downstream effector of the *PI3K/AKT* pathway, and is amplified in 10-15% of pancreatic cancers [73;89]. *AKT2* can be activated by stimuli such as platelet-derived growth factor, basic fibroblast growth factor, and insulin trough the *PI3K/AKT* pathway, suggesting this pathway's importance in this tumor type [72]. Secondly, the *AIB1* gene on chromosome 20q is amplified in approximately 60% of pancreatic cancers [90]. The nuclear receptor co-activator *amplified in breast cancer 1 (AIB1/ SRC-3)* belongs to the p160/ steroid receptor co-activator family (*SRC*) [91]. *AIB1* amplification and/ or overexpression is not only detected in hormone-sensitive tumors, such

as breast, prostate and ovarian, but it is also found in non-steroid-targeted tumors such as pancreatic cancer, colorectal carcinoma and hepatocellular carcinoma [92]. Thirdly, the *MYB* gene on chromosome 6q, is amplified in 10% of pancreatic carcinomas [93]. Abnormalities in the locus of the human *MYB* gene have been observed in several human cancers. In a majority of these tumors, these abnormalities seem to be accompanied by an amplification of the *MYB* gene followed by enhanced transcription [94].

Genome-maintenance genes

Genome-maintenance genes are those that function to identify and repair damage to DNA. When a genome-maintenance gene is inactivated, DNA damage is not repaired efficiently and DNA mutations accumulate. If these mutations occur in cancer-associated genes they can contribute to tumorigenesis [90]. Although gross chromosomal abnormalities are frequent in pancreatic ductal adenocarcinomas, genetic instability also occurs through DNA mismatch repair defects [95]. The DNA mismatch repair genes ***hMLH1*** and ***hMSH2*** are examples of genome maintenance genes targeted in pancreatic cancer [96]. When one of these genes is inactivated, DNA changes occur leading to 'microsatellite instability' (MSI). MSI is associated with poor differentiation, lack of *KRAS2* and *p53* mutations, and germline mutations of this gene are associated with the human nonpolyposis colorectal cancer syndrome (HNPCC) [96-98]. Approximately 4% of pancreatic cancers have MSI and these cancers have a specific microscopic appearance called 'medullary type', which includes a syncytial growth pattern, pushing borders and lymphocytic infiltrate [96].

The causative genes of Fanconi anemia, *FANCC* and *FANCG,* also play a role in pancreatic tumorigenesis [99]. Fanconi anemia is a hereditary cancer susceptibility disorder, with the occurrence of hematologic abnormalities or acute myelogenous leukemia at an early stage, usually leading to death before the age of 20. Patients who survive into adulthood often develop solid tumors [99]. The *BRCA2* gene represents Fanconi complementation group D1 and is thought to aid DNA strand and interstrand crosslinking repair. *BRCA2* has therefore been categorized as genome-maintenance gene rather than a standard tumor-suppressor. In ductal pancreatic cancers 7% to 10% harbor an inactivating intragenic inherited mutation of one copy of the *BRCA2* gene, accompanied by loss of heterozygosity [100;101]. Of interest, it has been shown that the presence of *BRCA2*/ Fanconi anemia gene mutations in pancreatic cancer may make them particularly sensitive to chemotherapeutic agents that cause DNA crosslinks such as Mitomycin C, because these cancers are unable to repair DNA interstrand crosslinks [102].

Growth factors

Several of the genes known to be overexpressed in pancreatic cancer include growth factors and their receptors. Growth factors are the proteins that control cell differentiation and proliferation. Disturbances in growth-inhibition and an abundance of growth-promoting factors give cancer cells a distinct growth advantage, which clinically results in rapid tumor progression. The epidermal growth factor receptor (**EGFR**) is overexpressed and plays a distinct role in pancreatic cancer. The four receptors of the EGF family are membrane spanning glycoproteins composed of an amino terminal extracellular ligand-binding domain, a hydrophobic transmembrane region and a cytoplasmic domain that contains both the tyrosine kinase domain as well as the receptor [103]. The classical EGF receptor is also known as HER1 or ErbB-1.

The remaining three receptors are designated HER-2/Neu (ErbB-2), HER-3 (ErbB-3), and HER-4 (ErbB-4). *HER-2/Neu* overexpression is most prominent in well-differentiated ductal adenocarcinoma, as well as in the early stage precursor lesions, and appears to correlate with the grade of dysplasia in the precursor lesions [104;105]. In pancreatic cancer, *HER-2/neu* amplification has been observed with a variable incidence of 10-60% [106;107]. In addition, increased levels of fibroblast growth factor (FGF), FGF-receptor, insulin-like growth factor I (IGF-I), IGF-I receptor, nerve growth factor (NGF), and vascular endothelial growth factor (VEGF) are also reported in pancreatic cancer [108;109].

Tumor growth requires accompanying expansion of the host vasculature with tumor progression, which is often correlated with vascular density. Vascular endothelial growth factor (VEGF) is the best-characterized inducer of tumor angiogenesis. Interestingly, Delta-like ligand 4 (Dll4), a Notch ligand, is dynamically regulated by VEGF [110]. Several studies demonstrated that Dll4 may act downstream of VEGF as a 'brake' on VEGF-mediated angiogenic sprouting [111]. Dll4, a transmembrane ligand for the Notch family of receptors, is induced by VEGF as a negative feedback regulator and acts to prevent overexuberant angiogenic sprouting [112].

Table 1: Frequency of selected tumor-suppressor genes, oncogenes and genome-maintenance genes.

Gene mutations	Incidence in Pancreatic Adenocarcinoma %
p16	80-95%
p53	50-75%
DPC4	45-55%
K-RAS	75-90%
BRAF	5-10% (estimated)
hMLH1, hMSH2	4%
BRCA2	7-10%

Telomere shortening

Defective telomeres may be the major cause of the chromosomal instability observed in many cancers and in the vast majority of pancreatic cancers [113]. Telomeres are structures at the end of linear chromosomes that normally function to protect the terminal sequences and prevent the ends of chromosomes from joining aberrantly [114;115]. Telomeres serve as protective 'caps' and are composed of short repeated DNA sequences and associated proteins. It appears that telomeres become abnormally short very early in the development of pancreatic neoplasia [114]. These shortened telomeres can presumably lead to the abnormal fusion of chromosome ends and in this fashion to chromosome instability, promoting further neoplastic progression in these cells [90]. Such a chromosome fusion leads to so-called anaphase bridges during mitosis [116]. These anaphase bridges frequently break during cellular replication, generating unstable chromosome ends that are subject to abnormal fusion events and subsequent chromosomal rearrangements [117]. This process, called breakage-

fusion-bridge cycles, has been observed in pancreatic cancers and is believed to be one of the major causes underlying loss of function of tumor-suppressor genes and the gain of function of oncogenes as described earlier [90]. In most instances, cells harboring this degree of genomic instability are eliminated through activation of *p53*. However, chromosomal rearrangements likely persist in cells with *p53* mutations, and these cells will then quickly accrue further genomic alterations [118]. Thus telomere dysfunction and *p53* loss cooperate to promote the development of carcinomas in multiple tissues [79]. Chromosomal instability provides a tumor with the genetic diversity to overcome certain barriers in carcinogenesis. However, ultimately, chromosomal instability might prove counterproductive to tumor growth, which may explain why neoplasms seem to acquire mechanisms to elongate their telomeres at later stages in the development of a malignancy, often through the reactivation of the enzyme telomerase, or through alternate lengthening of telomeres (ALT) [119].

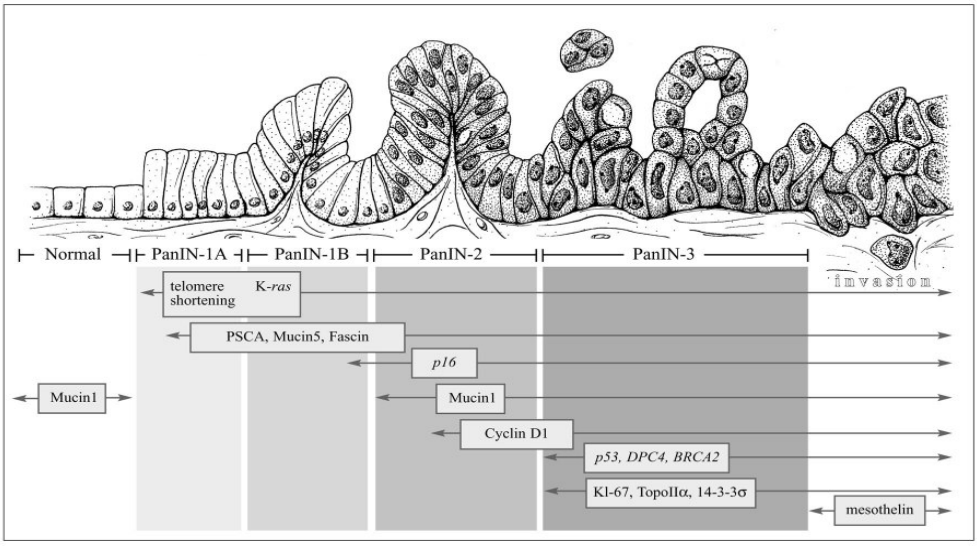

Figure 1: Progression model of pancreatic ductal adenocarcinoma from normal (left) to carcinoma (right). The histological progression is associated with the accumulation of specific genetic alterations. Reprinted with permission from Maitra *et al*. [136].

Familial pancreatic cancer

In the majority of cases, cancer is a multifactorial disorder in which genetic and environmental factors interact to initiate carcinogenesis. However, in a minority, the disease follows a familial pattern of transmission, suggesting a hereditary cancer syndrome. Characterization of the genetic mutations segregating in such families has helped to elucidate the molecular events that underlie tumorigenesis in the more common multifactorial form of the disease. Elucidation of the mechanisms of hereditary colorectal cancer and breast/ovarian cancer syndromes represents some of the greatest triumphs of the last century in the field of cancer genetics.

It has been estimated that 10% of pancreatic cancers have a familial basis [120;121]. Having a first degree relative with pancreatic cancer doubles the risk of developing pancreatic cancer [122], and the risk increases with increasing numbers of affected relatives [123]. Segregation analyses have suggested that an autosomal dominant pattern of inheritance is

the most parsimonious genetic model for this increased risk, [124] but the gene responsible for the familial aggregation of pancreatic cancer in the majority of cases has not yet been identified [125]. In different countries familial pancreatic cancer registries have been established to investigate the epidemiology and genetic background of these families, and to organize the screening programs for high-risk relatives and for follow-up. The largest such registry, the National Familial Pancreas Tumor Registry (NFPTR) is located at the Johns Hopkins Medical Institutions, Baltimore, MD, USA. (http://pathology2.jhu.edu/pancreas/nfptr.cfm) [125].

To date, at least five hereditary disorders that significantly increase the risk of pancreatic cancer have been described. These include familial breast /ovarian cancer syndrome (caused by inherited mutations in the *BRCA2* gene), the familial atypical multiple mole melanoma (FAMMM) syndrome (caused by germline mutations in the *p16* gene), the Peutz-Jeghers syndrome (caused by inherited mutations in the *STK11/LKB1* gene), hereditary pancreatitis (caused by germline mutations in the *PRSS1* gene), and hereditary nonpolyposis colorectal cancer syndrome (HNPCC) caused by mutations in *hMLH1* or *hMSH2*.

Familial breast /ovarian cancer syndrome is associated with an increased risk of breast cancer in men and women, and a subset of these families also harbor an increased risk for pancreatic cancer [126]. Germline mutations of the *BRCA2* gene, residing on 13q12-13, are identified in 4-17% of familial pancreatic cancer, with a particular propensity for occurring in families of Ashkenazi Jewish heritage [100;127]. As mentioned earlier, the protein product of the *BRCA2* gene has been shown to interact with protein products of several of the Fanconi anemia genes and to function in the repair of double-strand DNA breaks [99].

The FAMMM syndrome is an autosomal dominant disorder characterized by the familial occurrence of multiple melanocytic naevi, atypical naevi, and an increased risk of both melanoma and pancreatic cancer [128;129]. Familial atypical multiple mole melanoma can be caused by germline mutations in the *p16/CDKN2A* gene on chromosome 9p. The mutation carriers of the germline *p16-Leiden* mutation have an estimated risk of 17% to develop pancreatic cancer by the age of 75 years [19;130].

The Peutz-Jeghers syndrome is a rare, autosomal dominant condition characterized by the development of hamartomatous gastrointestinal polyps, mucocutaneous pigmentation and high lifetime risk of developing cancer, affecting both gastrointestinal and extra-gastrointestinal sites. The lifetime risk of developing pancreatic cancer is approximately 36% [131]. In 50% of families the pathogenesis is caused by germline mutations occurring in the *STK11/LKB1* gene [48;132].

Hereditary pancreatitis is characterized by the familial occurrence of pancreatitis with an early age of onset [133]. Germline mutations in the *PRSS1* gene cause an autosomal dominant form of the disease, whereas germline mutations in *SPINK1* lead to an autosomal recessive pattern of inheritance. An estimated 40% of patients with familial pancreatitis will develop pancreatic cancer by the age of 70 years [134].

HNPCC has an autosomal dominant pattern of inheritance and affects approximately 1 in 200 persons and is associated with multiple forms of cancer, most importantly colorectal, but also gastric, endometrial, and pancreatic cancer [135]. As discussed before, HNPCC is caused by mutations in one of the DNA mismatch repair genes. The group of individuals with a known predisposing familial syndrome, and with a history of familial pancreatic cancer would be among the first to benefit from screening tests for early detection of pancreatic cancer.

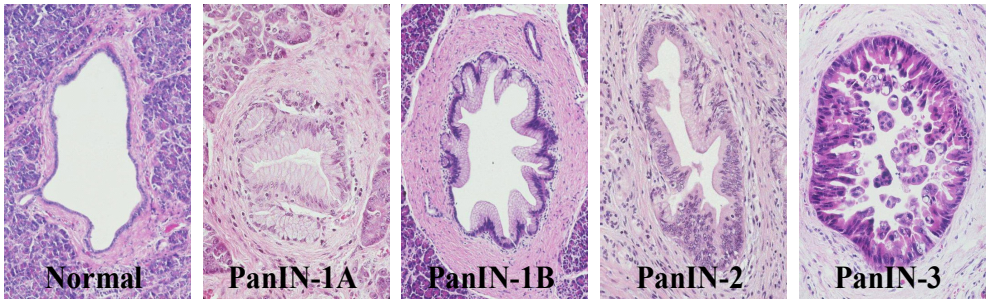

Figure 2: Consecutive pancreatic intraepithelial neoplasia (PanIN) lesions with progressive histological changes from normal to PanIN-3 (With permission from *http://pathology.jhu.edu/pancreas_panin)*

Mouse models of pancreatic cancer

Although the pancreas was the first organ where transgenesis was attempted over two decades ago [137], the development of a mouse model that faithfully recapitulates the multistep progression of human pancreatic adenocarcinoma has been elusive. In 2003, Tuveson *and colleagues* developed a mouse model of pancreatic neoplasia by conditional mis-expression of mutant *KRAS* in the pancreas from its endogenous promoter [138]. The bi-transgenic mice express a 'knock-in' *Kras*^{G12D} upon Cre-mediated recombination and removal of a lox-STOP-lox (LSL) allele within the *Pdx1* expression domain. Pdx1 is a transcription factor that is expressed in the developing pancreas and foregut, restricting mutant *KRAS* expression to these organs. The Pdx1-Cre; LSL-Kras^{G12D} mice develop the entire histologic compendium of murine PanIN (mPanIN) lesions observed in the cognate human disease, and in a subset of mice, develop invasive pancreatic carcinomas as well. Subsequent models have utilized additional cooperating mutations with Kras (for example, an oncogenic Trp53^{R172H} allele or bi-allelic deletions of INK4a/Arf) –these compound transgenic mice develop metastatic pancreatic cancers with near universal penetrance, and represent biologically relevant models of advanced pancreatic cancer in humans [139-141]. Several important lessons have been learnt from these newly developed mouse models of pancreatic cancer. First, these studies indicate the likely absolute requirement of mutant *Kras* in order to initiate pancreatic neoplasia along the mPanIN pathway, which might also explain the extremely high frequency of *KRAS* abnormalities in human PanIN lesions and pancreatic cancer [142]. Thus, mis-expression of other oncogenes by themselves do result in pancreatic 'cancer' in mice (for example, aberrant expression of the Hedgehog transcription factor GLI2) [82], but it is only upon co-expression with mutant *Kras* do these mice develop cancers preceded by mPanINs. Second, the expression of mutant *Kras* from its *endogenous promoter* appears to be a prerequisite as well, since earlier models of transgenic *Kras* expression have resulted in cancers of acinar histogenesis without mPanIN formation [143]. Third, these mouse models have helped elucidate some insights into the putative cell-of-origin of pancreatic cancer. For example, recent studies by Guerra *and colleagues* have demonstrated that mPanINs and adenocarcinomas can be reproduced in the pancreas of adult mice by conditional misexpression of mutant Kras to the Elastase-expressing acinar/ centroacinar compartment [144]; the one caveat is that the mature acinar/ centroacinar compartment appears to be resistant to the oncogenic transformation unless accompanied by an ongoing injurious stimulus (i.e., chronic pancreatitis). These studies provide remarkable experimental

reiteration to the long-standing epidemiological associations between chronic pancreatitis and an increased incidence of pancreatic cancer [3]. They also underscore the possibility that the moniker of 'ductal' adenocarcinoma might not reflect the true histogenesis of these cancers, at least in the context of murine pancreatic neoplasia. Fourth, and not the least, the development of these models have provided an unprecedented opportunity to explore pre-clinical diagnostic and therapeutic strategies in autochthonous models not afforded by short-term xenograft studies. For example, the cancers developing in these mice recapitulate not only the morphology of the cognate human disease, but also many of the oncogenic signaling pathways like EGFR, Notch and Hedgehog [138;141]. Small molecule inhibitors targeted against these pathways can now be tested in the transgenic models prior to clinical trials. There is little doubt that the development of these models has fulfilled a critical lacuna on the field of pancreatic cancer research.

Molecular biomarkers and therapy

The gene expression patterns in pancreatic cancer have been studied using multiple platforms. A decade ago, gene expression was studied through analysis of the product of one gene at a time. Currently, gene expression patterns can be studied using technologies that assay nearly the entire genome simultaneously. Examples of such technologies that have been applied to pancreatic cancer include serial analysis of gene expression (SAGE), cDNA arrays and oligonucleotide arrays [145-148]. The protein products of differentially expressed genes have proven useful as diagnostic markers in tissue biopsies, as serum markers, and as therapeutic targets. For example, prostate stem cell antigen (PSCA) and mesothelin were identified to be overexpressed in the majority of pancreatic cancers by serial analysis of gene expression (SAGE), and immunolabeling for these two proteins can be used to aid in the interpretation of challenging pancreatic biopsies [149;150]. Similarly, osteopontin was identified as overexpressed in pancreatic carcinoma using oligonucleotide microarrays, and serum osteopontin levels have a sensitivity of 80% and a specificity of 97% for pancreatic cancer [151].

Recently, micro-RNAs (miRNAs), a novel class of 18–23 nucleotide non-coding RNAs, have gained attention as another family of molecules involved in cancer development. Current evidence has illustrated that miRNAs are misexpressed in various human cancers, and further indicates that miRNAs can function as tumor-suppressors ('TSGmiRs') or oncogenes ('oncomiRs') [152;153]. Upon binding to their target RNAs, miRNAs cause posttranscriptional gene silencing by either cleaving the target mRNA or by inhibiting the translation process [154].

As several studies have highlighted, miRNA expression is deregulated in pancreatic cancer. A miRNA signature of pancreatic cancer has been elucidated, and it includes the upregulation of miR-21, miR-155, miR-221 and miR-222 [155;156]. Moreover, Mendell *et al* found that miR-34a is frequently lost in pancreatic cancer cell lines [39]. These studies demonstrate that miRNAs may become useful biomarkers for pancreatic cancer diagnostics. In addition, these aberrantly expressed miRNAs might be useful as potential therapeutic targets, with the recent availability of *in vivo* miRNA knockdown strategies ('antagomirs') [157].

The revolution in our understanding of the genetics of cancer and the exploration of gene expression on a large scale has brought with it the hope that novel therapies can be developed specifically exploiting the genetic deletions and resultant absolute biochemical

deficiencies present in pancreatic cancer. Two promising examples of therapies using a specific biochemical difference, including Mitomycin C for pancreatic cancers harboring *BRCA2* gene mutations and L-Alanosine, a purine biosynthesis inhibitor, for pancreatic cancers with loss of *MTAP* function were already mentioned above.

The down-regulation of Notch signaling could also be a novel therapeutic approach for pancreatic cancer. Numerous studies have proposed inhibition of Notch signaling as a strategy for cancer treatment, such as with the pharmacological block of γ-secretase enzyme with small molecule inhibitors, which has a striking anti-neoplastic effect in Notch expressing transformed cells *in vitro* and in xenograft models [158]. Inhibitors of γ-secretase prevent the second ligand induced proteolytic cleavage of the Notch receptor, thereby blocking the Notch signaling pathway. Importantly, in pancreatic cancer cells it has been shown that down-regulation of Notch1 inhibits cell growth and induces apoptosis [87]. In other compartments of the gastrointestinal tract, notably the colorectum and the esophagus, regression of tumorigenesis is observed after chemical inhibition of Notch [159;160].

Furthermore, developmental signaling pathways, like the Hedgehog signaling pathway, have emerged as therapeutic targets in pancreatic cancers [161]. This pathway is aberrantly activated in the majority of pancreatic ductal adenocarcinomas [78]. Drugs such as cyclopamine which specifically inhibit the hedgehog pathway, have been shown to be effective in xenograft models of human pancreatic cancer in treated mice [81]. Interestingly, the realization of cross-talk between *RAS/MAPK* and Hedgehog signaling pathways in pancreatic carcinomas also suggest that targeting the *RAS* and Hedgehog pathways synergistically may represent a new therapeutic strategy [84]. Additionally, there are a few promising agents on the therapeutic horizon, being tested in clinical trials, like *Bevacizumab*, the monoclonal antibody against vascular endothelial growth factor (VEGF), which targets tumor vascularization and *Cetuximab,* the monoclonal antibody against the epidermal growth factor receptor (EGFR) [162]. Of note, *Trastuzumab* (Herceptin®) is a humanized monoclonal antibody that acts on the HER2/neu (erbB2) receptor, a member of the EGFR family, and shows profound beneficial results with breast cancer patients whose tumors overexpress this receptor [103]. Whether *Trastuzumab* will be as effective a form of treatment in pancreatic cancer as it appears to be in breast cancer, is currently the focus of several studies [163;164].

Future perspectives

Intensive research over the last two decades has shown that pancreatic cancer is fundamentally a genetic disease, caused by inherited germline and/or acquired somatic mutations in cancer-associated genes. It has uncovered multiple alterations in many genes that are important in pancreatic cancer progression. In addition, an increased understanding of the molecular basis of the disease has provided the identification of new drug targets enabling rational drug design, and facilitated the production of animal models of the disease on which such therapies can be tested.

Pancreatic ductal adenocarcinoma is nevertheless still one of the most lethal cancers of all human malignancies. The poor prognosis and late presentation of pancreatic cancer patients emphasize the importance of early detection, which is the *sine qua non* for the fight against pancreatic cancer. It is hoped for the future that the understanding of genetic alterations in combination with the development of high-throughput sensitive techniques will lead to the rapid discovery of an effective biomarker.

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4

Morphogenesis of Pancreatic Cancer: role of Pancreatic Intraepithelial Neoplasia (PanINs)

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Abstract

Introduction: Pancreatic ductal adenocarcinoma (i.e., pancreatic cancer) is an almost universally lethal disease. The identification of precursor lesions of pancreatic cancer provides an opportunity for early detection, and potential therapeutic intervention, prior to the development of invasive cancer.

Discussion: It is now established that pancreatic cancers do not arise *de novo,* but rather exhibit a sequential histological and genetic progression of precursor lesions culminating in frank, invasive neoplasia. Pancreatic Intraepithelial Neoplasia (PanIN) is the most common non-invasive precursor lesion of pancreatic cancer. The development of a consensus nomenclature scheme for PanINs has facilitated research into pancreatic cancer precursors, and enabled standardization of results across institutions.

Conclusion: PanINs harbour many of the molecular alterations observed in invasive pancreatic cancer, confirming their status as true non-invasive precursor lesions. Recently developed genetically engineered mouse models of pancreatic cancer also demonstrate the stepwise PanIN progression model, underscoring the commonalities in pancreatic neoplasia between mouse and man.

Introduction

Pancreatic cancer is a disease with a dismal prognosis. In the United States approximately 34.000 patients are diagnosed with pancreatic cancer annually, and nearly an equal number will die from their malignancy. Worldwide pancreatic cancer causes an estimated 213.000 deaths each year [1]. For all stages combined, the 1-year survival rate is around 20%, and the overall 5-year survival rate is only 5%, despite the availability of improved surgical and medical avenues [2;3].

The high mortality rate for pancreatic cancer is primarily due to the advanced stage at which the neoplasm is diagnosed, and because there are no sensitive and specific tools to detect the disease at an earlier stage. More than 80% of the patients with pancreatic cancer have locally advanced or distant metastastic disease at the time of diagnosis, rendering their malignancies surgically inoperable. Currently, surgical resection remains the only curative treatment. Studies from high-volume centres with optimal staging report up to a 15%-20% 5-year survival rate in patients undergoing surgical resection [4;5]. Even if pancreatic cancer is diagnosed early and surgical resection with curative intent is performed, nearly all patients develop local recurrence and/ or distant metastases following surgery and eventually succumb to the debilitating effects of metastatic growth [6]. Unfortunately, conventional therapeutic modalities like chemo-radiation have had minimal impact, and the long-term survival of patients with pancreatic cancer has not improved in the last five decades [7;8].

Improved patient survival has been achieved in a variety of epithelial neoplasms (e.g., colorectal, lung, breast, cervix and prostate cancer), largely because of identification of cancers at their primary anatomic sites at an early, often pre-invasive stage [9;10]. At this moment, however, there is no equivalent of a 'Pap smear' or a 'PSA test' for pancreatic cancer, which can conveniently detect early neoplasia. Nevertheless, it is now recognized that, analogous to other epithelial cancers, pancreatic cancers do not arise *de novo*, but rather undergo a stepwise progression through histologically well-defined non-invasive precursor lesions, culminating in frank, invasive neoplasia. Although putative precursor lesions of pancreatic cancer were first documented over a century ago [11], it was only in the latter half of the last century that multiple lines of evidence began to coalesce associating invasive pancreatic cancer with these lesions. For example, meticulous autopsy studies confirmed that the prevalence of what are now recognized as precursor lesions increased with age, thus paralleling the frequency of invasive pancreatic cancer. Similarly, most surgically resected pancreata harbouring invasive cancer also tend to demonstrate non-invasive intra-ductular lesions in the surrounding parenchyma, suggesting an etiologic association [12-14]. Most importantly, careful molecular analyses over the last ten years have unequivocally demonstrated that these precursor lesions share many of the underlying genetic alterations observed in the infiltrating cancer, underscoring their precursor status [15-17].

By the late 1990s, over 70 different terminologies were in use to describe these non-invasive ductal lesions, leading to considerable difficulties in comparing inter-institutional studies. Therefore, there was a dire need for the establishment of an international nomenclature scheme for precursor lesions of pancreatic adenocarcinomas. In 1999, the National Cancer Institute hosted a Pancreatic Cancer Think Tank at Park City, Utah, from which meeting emerged a consensus nomenclature scheme for precursor lesions of pancreatic cancer. The 'Pancreatic Intraepithelial Neoplasia' (PanIN) scheme for classifying these lesions, first proposed by Klimstra and Longnecker, has since become a gold standard at academic centers worldwide [18;19].

Histology

The detailed histopathological grading of PanIN lesions and their distinction from other neoplastic and non-neoplastic conditions in the pancreas have been described elsewhere [18;19]. The reader is also directed to a freely accessible 'teaching site' on the World Wide Web for this purpose, located at http://pathology.jhu.edu/pancreas_panin. Briefly, PanINs are microscopic lesions in the smaller (less than 5 mm) pancreatic ducts. PanINs can be papillary or flat, and they are composed of columnar to cuboidal cells with varying amounts of mucin. PanINs are classified into a four tier classification, including PanIN-1A, -1B (low-grade PanIns), Panin-2 (intermediate grade PanIns), Panin-3 (high-grade PanIN), reflecting a progressive increase in histologic grade culminating in invasive neoplasia. The lowest grade PanIN lesions can be flat (1A) or papillary (1B), but are characterized by absence of nuclear atypia and retained nuclear polarity. PanIN-2 lesions are architecturally slightly more complex than PanIN-1 lesions, and they have more nuclear changes including loss of nuclear polarity, nuclear crowding, variation in nuclear size (pleomorphism), nuclear hyperchromasia, and nuclear pseudostratification. Mitoses are rarely seen. In contrast, PanIN-3 lesions, also referred to as 'carcinoma-*in-situ*', demonstrate widespread loss of polarity, nuclear atypia, and frequent mitoses. However, as a pre-invasive lesion, PanIN-3 is still contained within the basement membrane [18;19]. As discussed above, PanINs are often present in the pancreatic parenchyma adjacent to infiltrating adenocarcinomas, and several case reports have documented patients with high-grade PanINs in the remnant pancreas, who later developed an infiltrating pancreatic cancer [15]. In summary, just as there is a progression in the colorectum from adenoma, to adenoma with dysplasia, to invasive cancer, so too is there histologic and genetic progressions from PanIN-1, to PanIN-2, to PanIN-3, to invasive ductal adenocarcinoma in the pancreas [20].

It is important to note that PanINs are the most common, albeit not the only, recognized precursor lesions for pancreatic cancer. Two 'macroscopic' precursor lesions (so-called because they present typically as radiologically detectable cysts in the pancreas [21]) are Intraductal Papillary Mucinous Neoplasm (IMPNs), and Mucinous Cystic Neoplasms (MCNs). IPMNs are mucin-producing epithelial neoplasms, which arise within the main pancreatic duct or one of its branches, and that often, although not always, have a papillary architecture [19;22]. By definition, IPMNs involve the larger pancreatic ducts. Those that involve the main pancreatic ducts are designated 'main duct type,' while those that involve the secondary branches of the main pancreatic duct are designated 'branch duct type' [18;19;23]. Two features characterize MCNs at the light microscopic level. First, the cysts are lined by columnar, mucin-containing epithelium. Second, the underlying stroma has the appearance of ovarian stroma, and in fact, expresses hormonal receptors like estrogen and progesterone [24;25]. Similar to PanINs, the cystic precursor lesions also demonstrate a multi-step histological and genetic progression to invasive neoplasia, but will not be discussed within the scope of the current review.

As discussed above, the strongest evidence establishing the precursor lesional status for PanINs has been derived from comparative molecular analyses with invasive pancreatic cancer. Herein, we discuss some of the most common seminal alterations that are seen in PanIN lesions, and likely contribute to the stepwise genetic progression model of pancreatic cancer.

Oncogene mutations in PanIN lesions

Oncogenes can be activated through a variety of mechanisms including point mutations within the gene and amplification of the gene itself. A growing numbers of oncogenes have been identified that are targeted in pancreatic cancer. The most common activating point mutation involves the *KRAS* oncogene, on chromosome 12p, in over 90% of pancreatic ductal adenocarcinomas [26;27]. This is the highest fraction of RAS alteration found in any human tumor type. Frequent mutation sites involve codons 12, 13 and 61, but in pancreatic ductal cancers the majority occur in codon 12 [28]. The *KRAS* family proteins encode small GTP-binding cytoplasmic proteins, and regulate cell-cycle progression via the mitogen-activated protein kinase (MAPK) and AKT cascades [29]. Activating mutations impair the intrinsic GTPase activity of the *KRAS* gene product, resulting in a protein that is constitutively active in intracellular signal transduction [30]. Mutations of the *KRAS* gene are one of the earliest genetic abnormalities observed in the progression model of pancreatic cancer, demonstrable in approximately 36%, 44%, and 87% of cancer-associated PanIN-1A, PanIN-1B and PanIN-2/3 lesions [31]. The frequency of *KRAS* gene mutations is somewhat lower (~10%) in PanIN lesions arising in the backdrop of chronic pancreatitis [32]. Of note is given that PanIN lesions and an adenocarcinoma within the same pancreas may harbour different *KRAS* gene mutations, suggesting that some precursors evolve as independent clones from the one that eventually progress to the invasive cancer [33]. The high frequency of *KRAS* gene mutations in human PanINs supports its role as an initiating event for pancreatic cancer formation. This fact has been reiterated in several recent animal models *(see discussion below)*, where expression of mutant Kras is a prerequisite for the development of ductal pre-neoplasia and cancer [34;35]. In addition to its role in pancreatic cancer initiation, constitutive RAS signaling appears to be required for pancreatic cancer maintenance as well [36].

Tumor-suppressor gene mutations in PanIN lesions

Tumor-suppressor genes are genes that promote tumor growth when inactivated. Tumor-suppressor genes are recessive, that means that two copies need to be mutated for loss of function, and they can be inactivated by a variety of mechanisms: first, by an intragenic mutation in one allele (copy of a gene) coupled with loss of the second allele; second, by deletion of both alleles (homozygous deletion); and third, by hypermethylation of the promoter of the gene, thus silencing gene expression. In sporadic cancers these alterations are both somatic mutations acquired during life, while patients with inherited forms of cancer inherit one mutant allele in the germline while the second allele is somatically mutated in the cancer cells. Three tumor-suppressor genes, *p16INK4A/CDKN2A*, *TP53*, and *DPC4/SMAD4/MADH4*, are inactivated in a significant proportion of PanINs, mirroring their relative frequencies of loss of function in invasive adenocarcinomas.

The *p16INK4A/CDKN2A* gene located on the short arm of chromosome 9 (9p), is one of the most frequently inactivated tumor-suppressor genes in pancreatic cancer [37]. Remarkably, virtually all pancreatic carcinomas have loss of *p16INK4A/CDKN2A* function, in 40% of pancreatic cancer through homozygous deletion, in 40% by an intragenic mutation coupled with loss of the second allele, and in 15% by hypermethylation of the *p16INK4A/CDKN2A* gene promoter [38;39]. The *p16INK4A/CDKN2A* gene encodes the cell-cycle checkpoint protein p16, which binds to the cyclin-dependent kinases Cdk4 and Cdk6, thereby inhibiting binding of cyclin D1, resulting in G1-S cell-cycle arrest [40]. Loss of *p16INK4A/CDKN2A* results in inappropriate phosphorylation of Rb-1, thereby facilitating progression

of the cell cycle through the G1/S transition [41]. Thus, the p16/Rb pathway is inactivated in virtually all pancreatic cancers, leading to an inappropriate progression through the G1 phase of the cell-cycle. Loss of p16 expression is also seen in cancer-associated PanINs, with 30% of PanIN-1A and PanIN-1B, 55% of PanIN-2, and 71% of PanIN-3 lesions, demonstrating loss of nuclear p16 protein expression [42]. In contrast, loss of p16 expression is less frequently observed in PanIN lesions arising in the backdrop of chronic pancreatitis (respectively, 0%, 11%, 16%, and 40% for PanIN-1A, -1B, -2, and -3) [43].

The *TP53* tumor-suppressor gene on chromosome 17p encodes for the p53 protein [44;45]. The p53 protein has a number of important functions in the cell including regulation of the G1/S cell-cycle checkpoint, maintenance of G2/M arrest, and the induction of apoptosis. The *TP53* gene is inactivated in 55-75% of pancreatic cancers, almost always by an intragenic mutation in one allele coupled with loss of the second allele [45]. The loss of *TP53* means that two critical controls of cell number (cell division and cell death) are deregulated in the majority of pancreatic cancers. By immunohistochemistry, p53 accumulation is usually seen in the advanced PanIN-3 lesions, which is consistent with *TP53* gene mutations being a late genetic event in pancreatic cancer progression [46;47].

Another commonly inactivated tumor-suppressor gene in pancreatic cancer is *DPC4,* also known as *SMAD4/MADH4. DPC4* is a tumor-suppressor gene on chromosome 18q and is one of the most commonly inactivated genes in pancreatic ductal adenocarcinoma, detected in approximately 55% of the cases. Inactivation occurs either through homozygous deletion, in approximately 30%, or loss of one allele coupled with an intragenic mutation in the second allele in approximately 25% [48-50]. The *DPC4* gene codes for the protein Smad4 and Smad4 plays a critical role in signaling through the transforming growth factor type β (TGF-β) pathway. The TGF-β pathway is activated when the TGF-β proteins binds to specific cell surface receptors. This triggers an intracellular cascade that results in the nuclear localisation of Smad4. Once in the nucleus, Smad4 has growth controlling effects by regulating the expression of specific target genes [29;51]. Therefore, loss of *DPC4* and, thus loss of Smad4 protein, interferes with the intracellular signaling cascades downstream from TGF-β and activin, resulting in decreased growth inhibition via loss of pro-apoptotic signaling or inappropriate G1/S transition [52;53]. Immunohistochemical labelling for Smad4 protein expression mirrors *DPC4/SMAD4/MADH4* gene status with rare exceptions, and like *TP53*, loss of Smad4 expression is a late genetic event in pancreatic carcinoma progression. Smad4 expression is intact in PanIN-1 and PanIN-2 lesions, but loss of Smad4 expression is observed in 31%-41% of PanIN-3 lesions [49].

Genome-maintenance genes mutations in PanIN lesions

Genome-maintenance genes are those that function to identify and repair damage to DNA. While they do not directly influence cell growth and proliferation, but rather prevent the accumulation of DNA damage and maintain genomic fidelity. When a genome-maintenance gene is inactivated, DNA damage is not repaired efficiently and DNA mutations accumulate. If these mutations occur in cancer-associated genes they can contribute to tumorigenesis [54]. Although gross chromosomal abnormalities are frequent in pancreatic ductal adenocarcinomas, genetic instability also occurs through DNA mismatch repair defects [55]. The DNA mismatch repair genes *hMLH1* and *hMSH2* are examples of genome maintenance genes targeted in pancreatic cancer [56]. Their encoded proteins work together to repair small insertions, deletions, and other sequence mismatches in newly replicated DNA. Either by

mutation or promoter hypermethylation, one of these genes can be inactivated. As a result, DNA repair is compromised, and mutations accumulate in repetitive tracts, producing alterations known as 'microsatellite instability' (MSI). Approximately 4% of pancreatic cancers have MSI and these cancers have a specific microscopic appearance called 'medullary histology.' Medullary histology is characterised by pushing borders, syncytial growth pattern, and lymphocytic infiltrate. Furthermore, MSI is associated with poor differentiation, lack of *KRAS* and *TP53* mutations, and germline mutations of this gene are associated with the human non-polyposis colorectal cancer syndrome (HNPCC) [57-59].

Another class of genome-maintenance genes includes the Fanconi anemia family of genes. Fanconi anemia is a hereditary cancer susceptibility disorder, with the occurrence of hematologic abnormalities or acute myelogenous leukemia at an early stage, usually leading to death before the age of 20. Patients who survive into adulthood often develop solid tumors [60]. The genes mutated in pancreatic cancer include the *BRCA2*, the *FANCC* gene and the *FANCG* gene [61;62]. These genes are targeted in a small percentage of pancreatic cancers, namely less than 10%. Of these, *BRCA2* appears to be particularly significant, because germline *BRCA2* mutations, including a founder germline mutation prevalent in the Ashkenazi Jewish population, result in a predisposition to pancreatic cancer in the affected kindred [63]. In ductal pancreatic cancers 7 to 10% harbour an inactivating intragenic inherited mutation of one copy of the *BRCA2* gene, accompanied by loss of heterozygosity [64;65]. Among three cases of pancreatic cancer with germline mutation of *BRCA2*, loss of remaining wild-type allele was present in a single PanIN-3 lesion, but in none of 13 low-grade PanINs, confirming that bi-allelic inactivation of the *BRCA2* gene, like the *TP53* gene, is a late event in pancreatic cancer [66].

Telomere length abnormalities in PanIN lesions

Telomeres are structures present at the ends of linear chromosomes, comprising hexameric DNA repeat sequences (TTAGGG) in association with telomere-binding proteins. These telomeric repeat sequences prevent fusion between ends of chromosomes, and so we can assume that telomeres serve as sort of protective 'caps.' It appears that telomeres become abnormally short very early in the development of pancreatic neoplasia [67]. These shortened telomeres can presumably lead to the abnormal fusion of chromosome ends and in this fashion to chromosome instability, promoting further neoplastic progression in these cells [54]. Such a chromosome fusion leads to so-called anaphase bridges during mitosis [68]. During cellular replication these anaphase bridges frequently break, generating unstable chromosome ends that are subject to abnormal fusion events and subsequent chromosomal rearrangements [69]. Telomere length abnormalities are one of the earliest event in the pancreatic progression model, with more than 90% of even the lowest grade PanIN lesions demonstrating marked shortening of telomeres as compared with normal ductal epithelium [67]. It is believed that this loss of telomere integrity in PanIN lesions is one of the major causes for the loss of tumor-suppressor genes and the gain of oncogenes described earlier.

Epigenetic abnormalities in PanIN lesions

In addition to genetic changes, we now know that epigenetic abnormalities are a common hallmark of cancers. Epigenetic abnormalities in cancer occurs predominantly trough methylation of CG dinucleotides ('CpG islands') in the promoter region of genes, leading to silencing of transcription [70]. In cancers, there is preferential methylation of the gene

promoter in the neoplastic cells, but not in the corresponding normal cells within the tissue of origin. Numerous studies have showed that promoter hypermethylation of several genes, which have a function in tumor-suppression and/ or critical homeostatic pathways, to be an important mechanism for gene inactivation in many types of cancer [71;72]. A recent study of a large number of microdissected PanIN lesions has found that as many as 70% of the earliest PanIN-1A lesions demonstrate evidence of aberrant promoter methylation [73]. In addition to previously documented genes -*p16* and *proenkephalin*-, this study found evidence of progressive hypermethylation in *NPTX2, SARP2, Reprimo*, and *LHX1* [73-76]. These results suggest that aberrant CpG island hypermethylation begins in early stages of PanINs, and its prevalence progressively increases during neoplastic progression. The aberrantly methylated genes in PanIN lesions can be detected with methylation-specific PCR, making them potentially attractive for early detection. For that reason, the detection of aberrantly methylated genes in the pancreatic juice of patients with pancreatic carcinoma, might be a promising diagnostic strategy [77].

Alterations in apomucin expression in PanIN lesions

The apomucins MUC1, MUC2 and MUC5 are frequently overexpressed in epithelial cancers, particularly those arising in the gastrointestinal tract and pancreas [78]. MUC1 is expressed in the normal pancreatic ducts and acini and is responsible for the maintenance of lumen formation. MUC1 expression is also often encountered in invasive pancreatic ductal adenocarcinomas [79;80]. Maitra *et al,* showed that MUC1 expression was present 43% in PanIN-2 and 85% in PanIN-3, but in only 6% and 5% in PanIN-1A/B. Thus, in the multi-step progression of pancreatic adenocarcinomas, MUC1 expression within normal intra-and interlobular ducts appears to be decreased in the low-grade PanINs (PanIN-1A and 1B). However, MUC1 appears to be subsequently re-expressed in the advanced PanIN lesions, and this expression persists into invasive adenocarcinoma. Of interest, unlike MUC1, the expression of the apomucin MUC2 is uncommon in both normal pancreas and in invasive ductal adenocarcinomas [46;79]. In contrast, MUC2 expression is commonly seen in intraductal papillary mucinous neoplasms (IPMNs) and their associated invasive colloid carcinomas [81]. These mucins can be used to distinguish PanINs from IPMNs, because PanINs, in contrast to IPMNs with intestinal differentiation, do not express MUC2. Furthermore, MUC5 is similar to MUC1 in that it is also expressed in the majority of invasive ductal adenocarcinomas. In contrast to MUC1, however, MUC5 is not expressed in normal ducts, but its expression is up-regulated even in the earliest PanIN lesions and persists thereafter in the majority of lesions of all histologic grades [46;82]. These mucins are also potentially detectable by imaging [83] and they may be useful for screening and as therapeutic targets for the treatment of precursor lesions [84;85].

Aberrant expression of proteins in PanIN lesions

The protein cyclin D1 is a co-factor in the phosphorylation and inactivation of the retinoblastoma (Rb) protein, which plays a central role in cell-cycle regulation [40]. Over-expression of the cyclin D1 protein has been documented in 60-85% of invasive pancreatic adenocarcinoma in imunohistochemistry studies [86;87]. Cyclin D1 overexpression in pancreatic cancer has been associated with a poor prognosis and decrease in survival [88]. In the development of pancreatic cancer, cyclin D1 overexpression appears to be an intermediate step with nuclear overexpression in 29% of PanIN-2 lesion and 57% of PanIN-3 lesions, but

no expression in normal pancreatic ducts, PanIN-1A or PanIN-1B lesions [46].

Cyclooxygenase-2 (COX-2) regulates the metabolism of arachidonic acid into prostaglandins and other pro-inflammatory products. COX-2 has been implicated in tumorigenesis in which metabolites of COX-2 activate a range of signaling pathways, leading to cancer cell proliferation, survival, invasion and angiogenesis [89]. These processes may be secondary to activation of the MAP kinase signaling pathway and nuclear factor kappa B (NF κB)-mediated signaling [90]. In pancreatic cancer COX-2 levels are up-regulated and also in PanIN lesions COX-2 is expressed. In general, COX-2 follows the trend of expressions which increases from normal pancreatic ducts to PanIN to adenocarcinoma, with significantly higher expression in PanIN-2/3 compared with PanIN-1A/1B [91]. The appearances of COX-2 in PanIN lesions suggest the possibility of a potential target for chemoprevention using selective COX-2 inhibitors [92].

Certain proteins were first identified as overexpressed in pancreatic cancer based on global expression analyses and subsequent validation in tissue sections. Many of these proteins, not surprisingly, are also overexpressed in precursor lesions. For example, protein prostate stem cell antigen (PSCA) is overexpressed in 30% of PanIN-1 lesions, and respectively 40%, 60%, 60% in PanIN-2, -3 and invasive cancer, mandating the classification of PSCA as an early event in the progression model [46]. The patterns of protein expression in PanIN lesions are important because the proteins expressed in low-grade PanINs may be reasonable chemoprevention targets, while those expressed late (in PanIN-3 lesions) are potential markers for the early detection of pancreatic neoplasia.

Signaling pathways and PanIN lesions

It is known that several embryonic signaling pathways (Notch, Hedgehog, and Wnt pathways) play an important role in multiple tissues during development *in utero*, and these pathways are for the most part turned off in adult somatic cells, including the exocrine pancreas. Recently, abnormal transcriptional activation of these pathways has been reported in both human and mouse models of pancreatic neoplasia [93-96]. The Notch signaling plays a critical role in maintaining the balance among cell proliferation, differentiation, and apoptosis. Over-expression of Notch pathway receptors (Notch 1-4), ligands (Jagged 1-2), and transcriptional targets (Hes 1) are up-regulated in PanIN lesions as well as in invasive adenocarcinoma. Notch activation in PanIN lesions appears to be ligand-dependent, with Jagged-1 identified by micro-array analysis as one of the significantly over-expressed genes in early PanIN lesions [93;97].

Aberrant activation of the Hedgehog signaling pathway has been reported in PanINs and pancreatic cancer, as well as in genetically engineered murine models *(see discussion below)* of PanIN [94;95]. Global transcriptional profiling of human PanINs revealed up-regulation of extra-pancreatic foregut markers, including pepsinogen C, MUC6, Sox-2, KLF4 and TFF1, as a consequence of overexpression of Gli1, a downstream mediator of Hedgehog signaling. Furthermore, activation of the Hedgehog pathway in a human pancreatic ductal epithelial cell line resulted in a similar up-regulation of foregut markers seen in the early PanIN lesions [98]. Interestingly, the aberrantly expressed markers of foregut are not present in normal ductal epithelium.

Activation of the Wnt signaling pathways usually occurs via activating mutations of β-catenin or loss-of-function mutations of the *APC* tumor suppressor-gene; either event leads to stabilization and nuclear translocation of β-catenin and transcription of Wnt target

genes [99]. Several studies demonstrated that Wnt pathway mutations are rare in pancreatic ductal adenocarcinoma, although they are frequently observed in non-ductal tumors (e.g. solid pseudopapillary tumors, pancreatoblastomas and acinar cell carcinomas) [100;101]. In PanIN lesions, nuclear β-catenin expression is a rare event, and this reiterates the existence of two distinct, genetically divergent pathways of neoplasia in the pancreas: one resulting in the more common, conventional ductal adenocarcinoma and the other resulting in the less common non-ductal neoplasms [102].

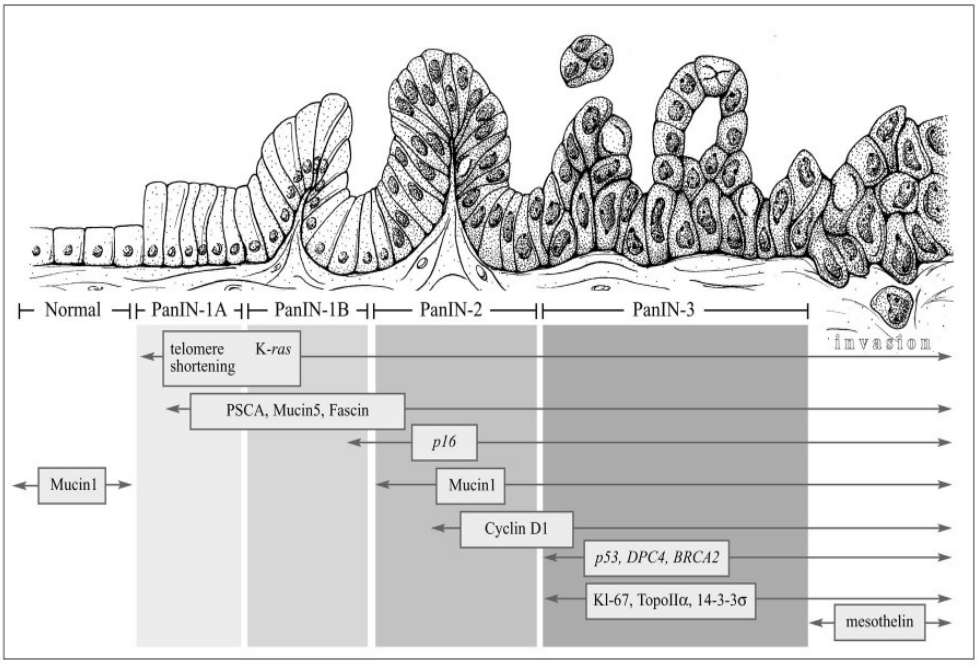

Figure 1: A 'PanINgram' illustrating some of the molecular alterations that occur during the multistep progression of pancreatic adenocarcinomas. The molecular abnormalities listed are not comprehensive, and additional alterations are discussed in the text at the appropriate juncture. Adapted from Maitra *et al*: Multi-component analysis of the pancreatic adenocarcinoma progression model using a pancreatic intraepithelial neoplasia tissue microarray. *Mod Pathol* 2003: 16:902-12 (© Nature Publishing Group).

Mouse models

Since the development of genetically engineered mouse models with pancreatic cancer, our understanding of the genetics of human PanINs and invasive pancreatic cancer has improved a lot. A major breakthrough was achieved in 2003, when Hingorani *and colleagues* developed a mouse model with pancreatic neoplasia that expressed an oncogenic $KRAS^{G12D}$ allele from its endogenous promoter, through Cre-mediated recombinant driven by *Pdx1* regulatory elements [35]. *Pdx1* is involved in early pancreatic cell fate determination. *Pdx1* expression is critical in pancreatic development and homozygous deletion of *Pdx1* causes pancreatic agenesis [103]. The Pdx1-Cre; LSL-$Kras^{G12D}$ mice develop the entire histologic compendium of murine PanIN (mPanIN) lesions observed in the cognate human disease, and in a subset of mice, develop invasive pancreatic carcinomas as well. Although expression of mutant *Kras* itself, is not enough for developing invasive cancer, it is sufficient to initiate PanINs.

The fact that these animals developed PanIN lesions before they developed invasive cancer has helped to validate the hypothesis that PanINs can progress to invasive cancer. However, when engineering mice that mis-express oncogenic *Kras* in the pancreas, where combined with bi-allelic *INK4a*/*Arf* deletion or an oncogenic *Trp53*^{R172H} allele, these mice developed aggressive, metastatic pancreatic cancers, with complete penetrance and shorter latency. On the other hand, abrogation of either *INK4a/Arf* or *TP53* signaling alone in the absence of oncogenic *Kras* does not lead to the development of pancreatic carcinomas or associated precursor lesions, underscoring the crucial importance of *Kras* signaling in initiating the cascade of events, that result in pancreatic carcinogenesis [34;104]. Of interest, the mPanIN lesions in the various LSL-Kras^{G12D} mice not only demonstrate the morphological spectrum of human PanIN lesions, but they also carry many of the alterations described above, such as overexpression of Notch, Hedgehog and COX-2 [35;105]. These mouse models have significantly facilitated defining the role of these genes in the progression of pancreatic neoplasia.

Mouse models can also be used to examine the role of other medical conditions and environmental factors in the development of pancreatic cancer [106;107]. For example, Guerra *et al,* reported that when *Kras* mutations are created in adult mice these genetically engineered mice do not develop lesions or pancreatic cancer. However, if these mice are challenged with a mild form of pancreatitis, they will develop the full spectrum of PanINs and invasive pancreatic carcinoma. This study provides an excellent example of how genetics and environmental factors interplay in the development of pancreatic cancer, especially when we translate these studies into human observations [107;108].

At last, mouse models are potentially useful tools to explore pre-clinical diagnostic and therapeutic strategies for pancreatic neoplasia. As already mentioned, these mouse models not only recapitulate the morphology of the cognate human disease, but also many of the signaling pathways like Notch, Hedgehog and COX-2 [35]. Thus, there is a unique opportunity to explore chemoprevention and treatment strategies in a biologically relevant pre-clinical model.

In conclusion

Putative precursor lesions of pancreatic cancer were documented over a century ago. However, it took many decades to define the various histological types of precursor lesions in the pancreas, and to credential these lesions as true precursors to invasive adenocarcinoma. Nevertheless, the detailed mechanisms involved in the initiation and progression of these precursor lesions remain to be elucidated. An improved understanding of the pathogenesis of PanIN lesions will enable us to develop better tools for primary and secondary prevention of pancreatic cancer, as well as improve existing tools for early diagnosis.

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5

Undifferentiated Carcinoma with Osteoclastic Giant Cells (UCOCGC) of the pancreas associated with the Familial Atypical Multiple Mole Melanoma syndrome (FAMMM)

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Abstract
The familial atypical multiple mole melanoma (FAMMM) syndrome is caused by a germline mutation of *p16*. More than 90% of the sporadic pancreatic carcinomas contain genetic alterations that inactivate *p16*. Patients with the FAMMM syndrome have an increased risk of developing pancreatic cancer. Ductal adenocarcinoma is the most common cancer of the pancreas and the one encountered in patients with FAMMM syndrome. Undifferentiated carcinoma with osteoclastic giant cells, also referred to as UCOCGC of the pancreas, is a rare variant of pancreatic cancer. An UCOCGC of the pancreas associated with FAMMM syndrome is described in this report. Molecular analysis confirmed a germline *p16-Leiden* deletion in the UCOCGC, accompanied by somatic loss of heterozygosity of the second *p16* allele, and absence of p16 protein expression in the neoplastic cells. It is the first case reported and it provides additional evidence that UCOCGC can be considered as a variant of conventional ductal adenocarcinoma of the pancreas.

Introduction

The *p16* gene on chromosome 9p is a tumor-suppressor that inhibits the cell cycle by binding Cdk4 and Cdk6 and interfering with their ability to phosporylate Rb1[1;2]. More than 90% of the sporadic pancreatic adenocarcinomas show complete loss of function of p16 due to a combination of deletion, inactivating mutation and/or hypermethylation of the promoter region [3;4].

The familial atypical multiple mole melanoma (FAMMM) syndrome can be caused by germline mutations of *p16* [5]. Families with the FAMMM syndrome are not only at risk for melanoma but also for the development of pancreatic cancer [6]. FAMMM patients have a 20-fold to 34 fold higher risk of pancreatic cancer compared with the general population, although germline *p16* mutations per se contribute to significantly less than 5% of all familial pancreatic cancer cases. To the best of our knowledge, there are no significant differences in the age of onset, histology, and prognosis of FAMMM-associated ductal adenocarcinomas compared with those occurring in the non-syndromic setting, although the paucity of cases renders a definitive conclusion as unwarranted.

A variety of different types of pancreatic cancer exists, but the most common type is pancreatic ductal adenocarcinoma and this is also the entity that typically is encountered in patients with the FAMMM syndrome [7]. An unusual variant of pancreatic cancer is the anaplastic carcinoma that is accompanied by non-neoplastic osteoclastic like multinucleated giant cells, originally described by Rosai [8]. The giant cells in this neoplasm resemble cells observed in giant cell tumors of bone. A growing body of evidence suggests that undifferentiated carcinoma with osteoclastic giant cells (UCOCGC) of the pancreas is best regarded as a variant of ductal adenocarcinoma with an associated non-neoplastic giant cell reaction [9].

In this report, a case of UCOCGC is described associated with the FAMMM syndrome due to a *p16-Leiden* deletion [7]. It is the first reported case of UCOCGC associated with FAMMM. The case provides indirect support for the notion that UCOCGC can be considered as a variant of ductal adenocarcinoma.

Case Report

A 39 year-old male was referred to us from another hospital. The patient had presented with abdominal discomfort, pain, jaundice and weight loss and had a history of a previous hospitalization for pancreatitis due to alcohol abuse. Computed tomography (CT) and magnetic resonance imaging (MRI) showed heterogeneity and calcification in the pancreas consistent with chronic pancreatitis and no evidence for malignancy although a tumor in the head of the pancreas could not be ruled out with certainty. Endoscopic retrograde cholangiopancreaticography (ERCP) revealed an ampullary lesion (fig. 1A), and the biopsy was initially interpreted as reactive, since epithelial markers were negative on immunohistochemistry.

The patient's family history was remarkable for the occurrence of multiple melanomas and occasionally pancreatic cancer due to the FAMMM syndrome caused by a *p16-Leiden* deletion.

Deeper sections of the ampullary biopsy (fig. 1B) showed osteoclast-like giant cells which expressed CD68 on immunohistochemistry (fig. 1D), whereas immunolabeling for CAM 5.2, pancytokeratin, and CK19 was negative. Immunolabeling for S-100 protein, Melan-A, and HMB-45, performed to rule out melanoma, were also negative. A diagnosis of

UCOCGC was considered and the decision was made to do a mutational analysis of *KRAS2* codon 12, because a previous study by our group had demonstrated that the neoplastic cells of UCOCGC often harbour *KRAS2* gene mutations and that this mutant DNA can be phagocytised by and detected in the non-neoplastic giant cells [10]. A *KRAS2* gene mutation was found (fig. 1C) and, in combination with the family history, it was interpreted as sufficient evidence to justify surgery. A pylorus preserving partial pancreaticoduodenectomy was performed and the surgical specimen grossly revealed a soft hemorrhagic neoplasm in the pancreatic head. The tumor measured 3cm in diameter with involvement of the ampulla (fig. 2A), adjacent to the distal common bile duct. On cut section the tumor was partially cystic and had necrotic areas (fig. 2B). The resection margins were free. Microscopically, the neoplasm had the typical features of an UCOCGC with anaplastic pleomorphic mononucleated cells and scattered osteoclast like multinucleated giant cells without atypia (fig. 2C). Focal ostechondroid differentiation was present. PanIN lesions of various degrees were seen, ranging from PanIN-1A to PanIN-3. Mutation analysis of *p16* performed on the neoplastic cells confirmed the germline *p16-Leiden* deletion and demonstrated loss (loss of heterozygosity, LOH) of the remaining wild-type allele (fig. 2D). Immunolabeling for the p16 protein revealed that the neoplastic anaplastic cells were negative, whereas the non-neoplastic osteoclast-like giant cells were positive (fig. 2C, inset). The patient had an uneventful recovery and was discharged from the hospital after 10 days.

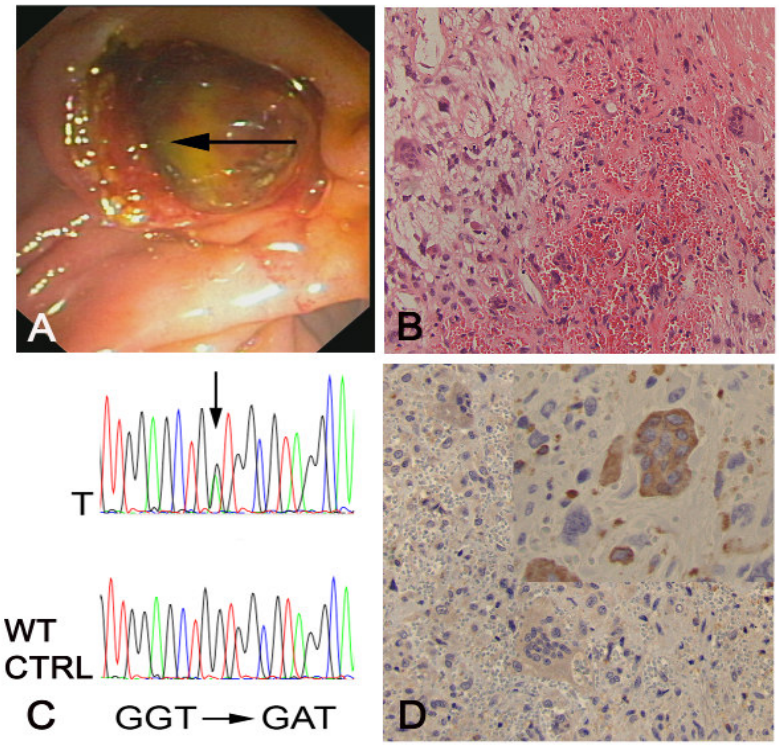

Figure 1:
1A, endoscopic view of ampullary lesion (arrow); **1B**, H&E endoscopic biopsy with osteoclast-like multinucleated giant cells (200X); **1C**, sequence analysis KRAS2 codon 12 mutations; **1D**, immunohistochemistry pankeratin and CD68 (inset). H&E indicates hematoxylin and eosin.

DNA analysis

Genomic DNA was isolated from formalin fixed, paraffin embedded tissue of the initial biopsy, the resected tumor and normal tissue respectively.

The DNA extracted from the biopsy was subjected to PCR amplification of the *KRAS2* gene and subsequent sequence analysis for codon 12 mutations was performed as described elsewhere [11].

The DNA extracted from the surgically resected tumor and normal tissue was PCR amplified for mutational analysis for the *p16-Leiden* deletion, as previously described [7]. This deletion extends 19 bp and removes nucleotides at positions 225-243 of exon 2 [7]. Loss of heterozygosity (LOH) was defined as a decrease of at least 40% in the intensity of one allele in the tumor sample compared to the matched wild type allele in the normal tissue. The quotient of the peak height ratios of normal and tumor tissue was considered as indicative for LOH if <0.6 and >1.6 corresponding with 40% reduction of one allele [12]. It was estimated that from the micro-dissected tumor tissue for LOH analysis 50-70% of the cells were tumor cells.

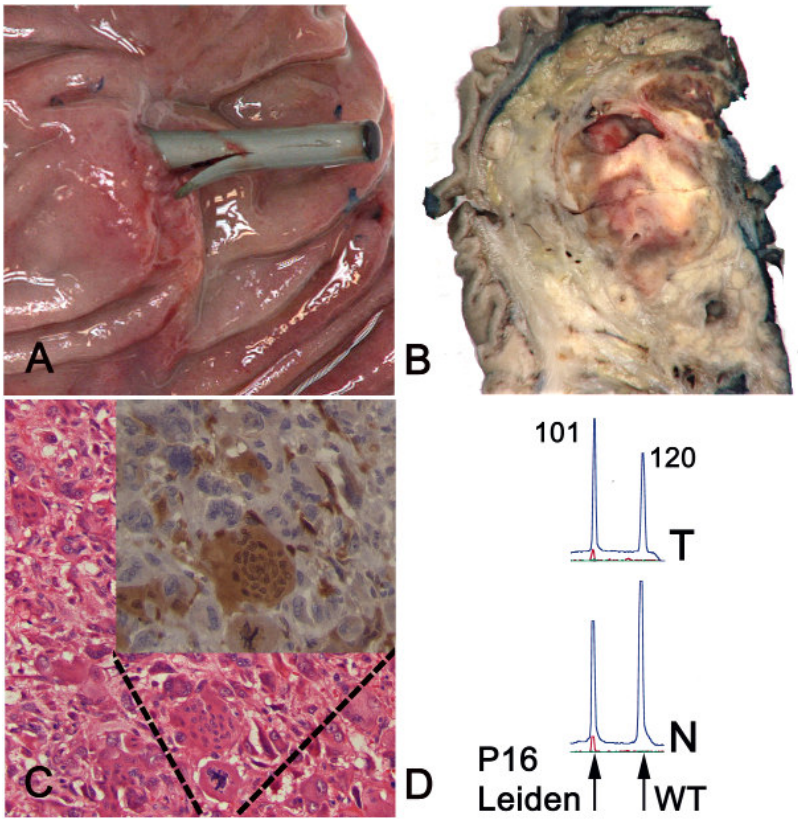

Figure 2:

2A, gross specimen ampulla with stent; **2B**, cross section tumor pancreatic head; **2C**, H&E tumor resection specimen and immunohistochemistry for p16 (tumor cells have no nuclear expression, multinucleated giant cells are positive); **2D**, *p16-Leiden* deletion (arrow, 19 bp shift) and LOH remaining wild-type allele (quotient ratios peak height 1.69). H&E indicates hematoxylin and eosin; LOH, loss of heterozygosity.

Discussion

The FAMMM syndrome is characterized by the familial segregation of multiple melanomas and atypical precursor nevi and can be caused by germline mutations of the *p16* tumor-suppressor gene [5-7]. Carriers of this germline mutation are not only at risk for melanomas and its precursors, but they also have a significantly increased risk of pancreatic carcinoma [5;6]. The 20-fold to 34-fold higher risk of pancreatic cancer in FAMMM patients translates into a 10% to 20% risk of developing pancreatic cancer by the age of 70 years. This is not surprisingly, because the large majority of sporadic (non-syndromic) pancreatic adenocarcinomas harbour acquired *p16* gene mutations, leading to loss of function of this tumor-suppressor [1-4]. FAMMM family members with a specific 19 bp deletion of *p16*, the so called *p16-Leiden* deletion, have an estimated risk of almost 20% of developing pancreatic cancer by the age of 75 years [7].

Pancreatic cancers that are encountered in association with the FAMMM syndrome are typically conventional ductal adenocarcinomas, although unusual cases of adenosquamous and neuroendocrine carcinoma have been reported [7;13]. The UCOCGC of the pancreas is considered a rare variant of ductal adenocarcinomas, characterized by the presence of reactive multinucleated osteoclast like giant cells. Accumulating evidence suggests that UCOCGCs are undifferentiated ductal carcinomas arising from precursor ductal lesions [9]. In addition to morphologic observations, this hypothesis is supported by immunolabeling for the p53 protein which reveals abnormal accumulation of the protein only in the undifferentiated cells. The osteoclast-like giant cells do not accumulate the p53 protein, suggesting that they are *TP53* gene wild-type. Interestingly, we have previously shown that the non-neoplastic multinucleated osteoclast like giant cells can phagocytose degraded neoplastic cells, and, as a result, some of these non-neoplastic cells contain phagocytosed mutant *KRAS2* genes. *KRAS2* gene analysis was used in the current case to arrive at the proper diagnosis [10]. It is at the same time yet another example of the great value of molecular DNA analysis as an adjunct to conventional microscopy.

To our knowledge this is the first case of UCOCGC of the pancreas in a patient from a family with FAMMM syndrome. DNA analysis of the neoplasm and normal tissue in the resection specimen confirmed the expected 19 bp deletion typical for the *p16-Leiden* deletion, accompanied by LOH of the remaining wild-type allele. Accordingly, p16 immunohistochemistry was negative in the neoplastic cells consistent with loss of function of the protein product. By contrast, the osteoclast-like giant cells in this case showed intact labelling for the p16 protein, further supporting the hypothesis that these are non-neoplastic reactive cells. The loss of the wild-type p16 allele in this case supports the contention that the UCOCGC of the pancreas in this patient was causally related to the underlying FAMMM syndrome and not a coincidence. Indirectly, the case therefore also shows that it appears indeed legitimate to consider UCOCGC of the pancreas as a variant of ductal adenocarcinoma.

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6

MicroRNA miR-155 is a biomarker of early pancreatic neoplasia

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Abstract

Background: Intraductal papillary mucinous neoplasms (IPMNs) are non-invasive precursor lesions of pancreatic cancer. Misexpression of microRNAs (miRNAs) is commonly observed in pancreatic adenocarcinoma. In contrast, miRNA abnormalities in pancreatic cancer precursor lesions have not been documented.

Experimental Design: Relative expression levels of a panel of twelve miRNAs upregulated in pancreatic cancers were assessed in 15 non-invasive IPMNs, using quantitative reverse transcription PCR (qRT-PCR). Two significantly overexpressed miRNAs –miR-155 and miR-21– were evaluated by locked nucleic acid *in situ* hybridization (LNA-ISH) in a panel of 64 archival IPMNs. The expression of miR-155 and miR-21 was also evaluated in pancreatic juice samples obtained from 10 patients with surgically resected IPMNs and five patients with non-neoplastic pancreato-biliary disorders ('disease controls').

Results: Significant overexpression by qRT-PCR of ten of the twelve miRNAs was observed in the 15 IPMNs *versus* matched controls ($P<0.05$), with miR-155 (mean 11.6-fold) and miR-21 (mean 12.1-fold) demonstrating highest relative fold-changes in the precursor lesions. LNA-ISH confirmed the expression of miR-155 in 53 of 64 (83%) IPMNs compared to 4 of 54 (7%) normal ducts, and of miR-21 in 52 of 64 (81%) IPMNs compared to 1 of 54 (2%) normal ducts, respectively ($P<0.0001$). Upregulation of miR-155 transcripts by qRT-PCR was observed in 6 of 10 (60%) IPMN-associated pancreatic juice samples compared to 0 of 5 (0%) disease controls.

Conclusions: Aberrant miRNA expression is an early event in the multistage progression of pancreatic cancer, and miR-155 warrants further evaluation as a biomarker for IPMNs in clinical samples.

Introduction

Pancreatic cancer is the fourth most common cause of cancer-related mortality in the United States [1]. The overwhelming majority of patients present with locally advanced or distant metastatic disease, rendering their malignancy surgically inoperable. Despite advances in chemo-radiation therapies over the last few decades, the dire prognosis of pancreatic cancer has remained essentially unchanged. Early diagnosis of this neoplasm at an early, and hence potentially resectable stage, offers one of the best avenues for cure [2].

It is now well established that pancreatic cancers do not arise *de novo*, but rather represent the culmination of a multistep progression involving non-invasive precursor lesions within exocrine pancreatic ducts [3]. The most common precursors are pancreatic intraepithelial neoplasia (PanIN), which are microscopic lesions arising within ducts less than 5mm in diameter [4]. In contrast, IPMNs represent macroscopic precursors of pancreatic adenocarcinoma, typically presenting as cystic lesions within the main pancreatic duct or one of its branches [5]. Since their original description in the 1982 [6], insights into the histological features and genetics of these cystic precursor lesions have expanded considerably. Akin to PanIN lesions, the lining epithelium of IPMNs can demonstrate varying degrees of histologic atypia, ranging from the innocuous IPMN adenoma, through borderline IPMN to IPMN with carcinoma-*in-situ* [4]. In addition to variable risk of progression associated with degrees of histological atypia, biological heterogeneity is also observed within IPMNs based on the nature of the lining epithelium. Thus, IPMNs with intestinal-type or pancreato-biliary epithelium harbour a greater propensity for progression to invasive neoplasia than those with gastric foveolar type epithelium [7;8]. Genetic studies have established that many of the seminal alterations observed in invasive pancreatic cancer, such as mutations of *KRAS2, DPC4/SMAD4*, and *TP53*, are also present in a variable proportion of non-invasive IPMNs, further cementing their status as *bona fide* precursor lesions [3;9]. At the same, a subset of molecular changes identified in IPMNs is not commonly observed in PanINs (for example, mutations of *PI3KCA* and *STK11/LKB1* genes, or expression of the cellular apomucin Muc2) [10-12], suggesting that in addition to histological digression, there are also two distinct molecular pathways to invasive adenocarcinoma in the pancreas [13;14].

MicroRNAs (miRNAs) are a diverse class of 18-24 nucleotide RNA molecules that demonstrate remarkable evolutionary conservation [15]. The principal function of these non-coding RNAs is to regulate the stability and translation of nuclear mRNA transcripts. Physiologic regulation of the cellular transcriptome by miRNAs plays a critical role in development and in homeostasis. Aberrant expression of miRNA is widespread, if not ubiquitous, in human cancers, with the identification of both over- and under-expressed miRNAs in neoplastic cells compared to their normal counterparts [16]. Several recent studies have identified a multitude of misexpressed miRNAs in human pancreatic cancers compared to normal pancreatic tissues [17-20]. In contrast, patterns of miRNA abnormalities in the non-invasive precursor lesions of pancreatic adenocarcinoma remain largely unknown. The profiling of miRNAs in IPMNs has several conceivable advantages. In addition to enhancing our understanding of the pathogenesis of early pancreatic neoplasia, these aberrantly expressed miRNAs potentially represent targets for therapy, as well as candidate biomarkers for the diagnosis of pancreatic cancer precursors in clinical specimens. In this study, we demonstrate that miR-155 and miR-21 are significantly upregulated in the majority of non-invasive IPMNs, and their expression correlates with histological features of progression in these neoplasms. Further, our studies provide promising, albeit preliminary evidence, that

upregulation of miR-155 in clinical samples like pancreatic juice harbors the potential to emerge as a diagnostic adjunct for IPMNs.

Material and Methods

IPMN specimens and other clinical samples

Cryostat embedded sections of 15 non-invasive IPMN specimens were obtained from the surgical pathology archives of Johns Hopkins Hospital. In each case, the patient had undergone surgical resection for removal of a pancreatic cystic neoplasm, and the histology of the lesion was confirmed by one of the authors (AM), who is an expert on pancreatic pathology [4;9]. The IPMN specimens were snap-frozen in liquid nitrogen, embedded in Tissue-Tek OCT compound medium (Sakura FineTek USA, Torrance, CA) and stored at -80°C. The samples were subsequently embedded onto UV-treated PALM membrane slides (Carl Zeiss MicroImaging, Inc., Thornwood, NY) for the purpose of micro-dissection. Locked nucleic acid *in situ* hybridization (LNA-ISH) was performed on a panel of 64 archival IPMNs arrayed on duplicate 1.4 mm cores in tissue microarray (TMA) format, as we have previously described [11;21]. The 64 samples were comprised of 13 IPMN adenomas, 31 borderline IPMNs, and 20 IPMNs with carcinoma-*in-situ*, on the basis of histological atypia in their lining epithelium [4]. Similarly, when classified by the nature of the lining epithelium [7;8], the 64 samples were comprised of 35 pancreato-biliary type, 19 intestinal type, 7 gastric foveolar type, and 1 oncocytic IPMNs; in two cases the epithelium could not be accurately classified and these were labeled as 'unclassified.' In addition, tissue cores from matched non-neoplastic pancreas were available for evaluation in 54 of the 64 IPMNs. We also examined pancreatic juice samples obtained at the time of surgery from 15 patients, including ten patients with histologically documented IPMNs, and five patients with other pancreato-biliary disorders including chronic pancreatitis or bile duct stones ('disease controls'). The pancreatic juice was immediately mixed with Trizol reagent and stored at -80°C for miRNA extraction.

Laser Micro-dissection and miRNA Extraction

The IPMN cryostat sections were fixed in cold methanol and stained with hematoxylin and eosin (H&E) prior to micro-dissection using a PALM MicroBeam (Carl Zeiss MicroImaging, Inc., Thornwood, NY). The neoplastic epithelium was outlined and selectively micro-dissected as described previously [22]. Subsequently, non-neoplastic pancreatic parenchyma from each case was micro-dissected, and 'catapulted' into a different tube. The micro-dissected tissue samples were then subjected to RNA extraction using the mirVana™ miRNA Isolation kit (Ambion/Applied Biosystems, Austin, TX), according to the manufacturer's protocol. For the pancreatic juice samples preserved in TriZol, total RNA isolation was performed by ethanol precipitation, as we have previously described [23].

Quantitative reverse transcription PCR (qRT-PCR) for miRNA

Quantitative analyses of miRNA levels in micro-dissected IPMNs and in pancreatic juice samples were performed using pre-designed TaqMan® miRNA assays (Applied Biosystems, Foster City, CA). The TaqMan® miRNA assays are a two-step protocol, involving reverse transcription with human mature miRNA specific primers, followed by real time PCR with TaqMan® probes. These assays target the mature miRNA sequence only, and the precursors are not detected. For the current study, we selected a panel of 12 miRNAs

previously described as significantly overexpressed in invasive pancreatic cancers (Table 1) ([17-20]): miR-15a, miR-16, miR-17-5p, miR-21, miR-100, miR-107, miR-155, miR-181a, miR-181c, miR-210, miR-221, and miR-223. The non-coding RNU6B (U6 control) was used as housekeeping control. The 15 micro-dissected IPMNs were compared against matched normal pancreata, and each sample was assessed in triplicate for any given miRNA. Relative fold expression was calculated using the $2^{-\Delta\Delta Ct}$ method, as described previously [24]. On the same lines, qRT-PCR was performed on RNA from the 21 pancreatic juice samples for miR-21 and miR-155.

Locked nucleic acid in situ hybridization (LNA-ISH)

LNA-ISH was performed using LNA™ probes against miR-21 and miR-155 (Exiqon, Vedbaek, Denmark), respectively, on archival IPMN tissue microarrays, as per the manufacturer's protocol. The use of LNA™ probes for the successful cataloging of altered miRNAs in archival human cancer tissues has been recently described [25-27]. Briefly, after deparaffinization, the slides were blocked for 2 hours, and then incubated with hybridization buffer containing the digoxigenin (DIG) labeled LNA™ probe in a hybridization oven, overnight. A parallel set of TMAs was hybridized with a 'scrambled' miRNA probe from Exiqon, as previously described [25], as a measure of probe specificity. After several washes for ensuring stringency, the slides were incubated with anti-digoxigenin Fab fragment (1:2000) overnight in a humid chamber at 4°C. The colorimetric detection reaction was performed using NBT/BNI ReadyMix for 48 hours. The slides were then mounted with Cytoseal 60 (Richard-Allan-Scientific). The TMAs were scored on a multi-headed microscope by three of the authors on the panel (J-B.M.K., A.M. and S-M.H.). The LNA-ISH results were scored based on intensity of staining as 0 (negative), 1 (weak), or 2 (strong), and based on the percentage of positive epithelial cells as 0 (<1%), 1 (focal, 1-50%) or 2 (diffuse, >50%), respectively. A 'ISH-score' was generated as the product of intensity times area, similar to what we have previously described with immunohistochemical analyses on TMAs [28;29]. The 'ISH-score' was then binned into a two-tier classification of 'negative' (score 0), and 'positive' (score $\geq$1).

Data processing and statistical analysis

Statistical analyses were performed using SPSS version 11 (SPSS Inc., Chicago, IL). Associations between categorical variables were examined using the Pearson's chi-square and Fisher's exact tests. A *P*-value <0.05 was considered statistically significant. The depiction of miRNA differential expression was plotted using GraphPad Prism.

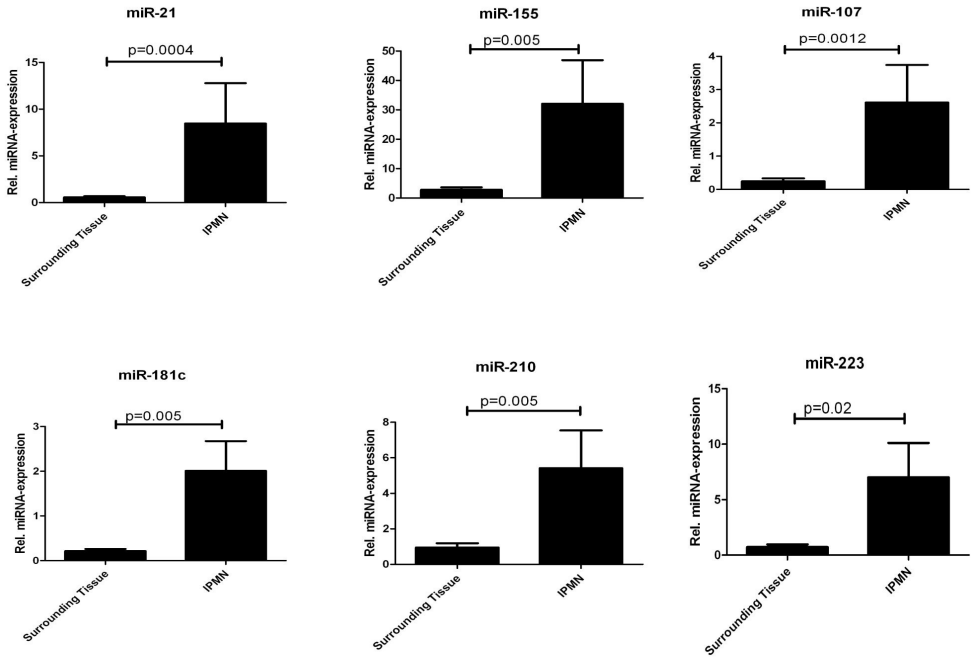

Figure 1: Relative fold expression of miRNAs in micro-dissected IPMNs compared to matched non-neoplastic pancreata. Each panel represents a miRNA assessed by qRT-PCR. The two bars represent either surrounding non-neoplastic tissue, or IPMN epithelium. Each sample was assessed in triplicate, and U6 non-coding RNA was used as housekeeping control. The Y-axis represents the average of the relative fold expression levels for each group (IPMNs *versus* controls). Error bars represent standard errors of the mean. The significance level is designated on the panel. Only six of the 12 miRNAs examined are shown.

Results

Analysis of miRNA profiles in the 15 micro-dissected IPMN lesions confirmed significant overexpression of ten of 12 miRNAs, compared to matched non-neoplastic samples ($P<0.05$) (fig. 1 and table 1); only miR-15a and miR-17-5p did not reach statistically significant differences in expression, although both demonstrated a trend towards upregulation in the neoplastic epithelium. Of the significantly overexpressed miRNAs, miR-21 and miR-155 had the highest relative fold expression levels in IPMNs *versus* non-neoplastic pancreata (12.1-fold and 11.6-fold, respectively). Given the established association of these two seminal 'onco-miRs' with human cancers [25;30], including pancreatic adenocarcinoma [17;20;31], we decided to explore the expression of miR-21 and miR-155 in a larger panel of 64 IPMN lesions by LNA-ISH. Both miRNAs were frequently overexpressed within the neoplastic epithelium of IPMNs, with 53 of 64 (83%) IPMNs expressing miR-155 (fig. 2A) and 52 of 64 (81%) expressing miR-21 (fig. 2B). Expectedly, the scrambled probes failed to demonstrate any evidence of ISH signal (fig. 2C). In contrast, miR-155 expression was observed in only 4 of 54 (7%) matched non-neoplastic pancreata on the TMAs, while miR-21 was expressed in only 1 of 54 (2%) non-neoplastic pancreata ($P<0.001$, Chi-square test; table 2).

Table 1: Relative differential expression of twelve pancreatic cancer associated miRNAs in micro-dissected non-invasive IPMNs

miRNA	Fold-change	*P*-value	Pancreatic cancer reference
miR-21	12.1	0.0004	(17-20)
miR-155	11.6	0.005	(17-20)
miR-107	10.8	0.01	(17, 18, 20)
miR-223	9.7	0.02	(17-19)
miR-181c	9.5	0.005	(18, 20)
miR-181a	7.7	0.01	(18, 20)
miR-221	6.6	0.01	(17-20)
miR-210	5.7	0.005	(18, 19)
miR-16	4.8	0.02	(20)
miR-100	3.8	0.01	(20)
miR-15a	3.5	0.2	(18)
miR-17-5p	3.4	0.1	(17)

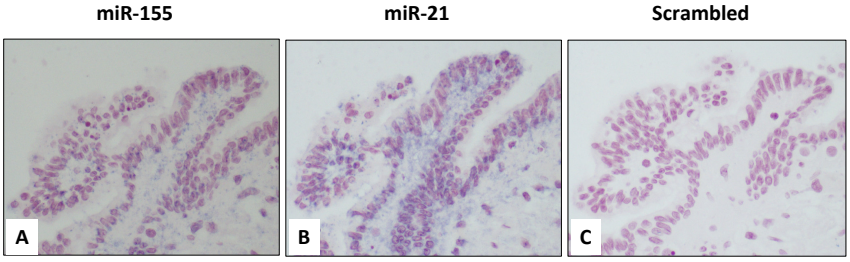

Figure 2: Locked nucleic acid *in-situ* hybridization (LNA-ISH) for miR-155 and miR-21 expression in archival IPMN tissues. High magnification photomicrographs of an archival IPMN with robust expression of DIG-labeled miR-155 probe (**A**) and miR-21 probe (**B**) within the neoplastic epithelium, consistent with expression of these miRNAs within the neoplasm. In contrast, the scrambled miRNA probe demonstrates complete absence of expression (**C**). Magnification 40X.

Upon stratification by the degree of histological atypia in the lining epithelium, a significantly higher proportion of IPMNs with carcinoma-*in-situ* expressed both miRNAs, compared to IPMN adenomas (table 3A). Specifically, 20 of 20 (100%) IPMNs with carcinoma-*in-situ* expressed miR-155 compared to 7 of 13 (54%) IPMN adenomas ($P=0.0002$), while 19 of 20 (95%) IPMNs with carcinoma-*in-situ* expressed miR-21 compared to 7 of 13 (54%) IPMN adenomas ($P=0.008$). However, there were no statistically significant differences between IPMN adenoma and borderline IPMN, or between borderline IPMN and IPMN with carcinoma-*in-situ*, based on either miR-155 or miR-21 expression. When stratified by the histological subtype of the lining epithelium, we did not elicit significant differences in the frequency of miR-155 expression between pancreato-biliary type and intestinal type of IPMNs (table 3B). In contrast, we found a significantly lower proportion of

IPMNs with gastric foveolar type lining expressing miR-155 (4 of 7 cases, 57%), compared to the intestinal type IPMNs (19 of 19 cases 100%; $P = 0.01$). Admittedly the numbers of gastric foveolar type IPMNs in our series are small; nevertheless contingent upon this caveat, it is worth noting that the intestinal types of IPMNs are usually main-duct lesions with a higher propensity for malignancy than the typically branch duct, gastric foveolar type IPMNs [7;8]. Comparable significant differences in expression between the histological variants were, however, not observed in the case of miR-21.

Table 2: Frequency of miR-155 and miR-21 expression in IPMNs and non-neoplastic pancreata by *in-situ* hybridization

miRNA	Expression	ISH-score	Non-neoplastic pancreas	IPMN
miR-155	Negative	0	50 (93%)	11 (17%)
	Positive	1	3 (5%)	16 (25%)
		2	1 (2%)	37 (58%)
miR-21	Negative	0	53 (98%)	12 (19%)
	Positive	1	1 (2%)	39 (61%)
		2	0	13 (20%)
Total			54	64

Note: None of the ISH-scores reached 2x2=4

In light of the promising data with both miRNAs in IPMN tissue sections, we next examined the potential of using these targets as biomarkers of IPMNs in pancreatic juice samples. A total of 15 pancreatic juice samples obtained at the time of surgical resection were examined for relative levels of miR-155 and miR-21 by qRT-PCR. The juice samples were obtained from 10 patients with histologically confirmed IPMNs, and five with other pancreato-biliary disorders ('disease controls'). As seen in figure 3, the mean relative expression level of miR-155 was higher than disease controls in the IPMN juice samples. Specifically, the five disease controls had minimally detectable miR-155 in pancreatic juice, while 6 of 10 (60%) IPMNs had elevated miR-155 in juice samples, with 20-fold or greater relative-fold expression; nevertheless, due likely to the small numbers of samples, the differences did not reach statistical significance (P =0.1). Similarly the five disease controls had minimal miR-21 expression in the juice samples, although the mean level of miR-21 overexpression in the IPMN juice samples was more attenuated than that observed with miR-155, with only two samples having 20-fold or greater relative expression (figure 3).

Table 3A: Frequency of miR-155 and miR-21 expression in IPMNs stratified by histologic atypia of the lining epithelium

miRNA	Expression	ISH score	IPMN adenoma	IPMN borderline	IPMN carcinoma-in-situ	Total
miR-155	Negative	0	6 (46%)	5 (16%)	0	11 (17%)
	Positive	1	4 (31%)	7 (23%)	5 (25%)	16 (25%)
		2	3 (23%)	19 (61%)	15 (75%)	37 (58%)
miR-21	Negative	0	6 (46%)	5 (16%)	1 (5%)	12 (19%)
	Positive	1	5 (39%)	19 (61%)	15 (75%)	39 (61%)
		2	2 (15%)	7 (23%)	4 (20%)	13 (20%)
Total			13	31	20	64

Note: None of the ISH-scores reached 2x2=4

Table 3B: Frequency of miR-155 and miR-21 expression in IPMNs stratified by histologic subtype of the lining epithelium

miRNA Expression		Gastric	Pancreato-biliary	Intestinal	Oncocytic	Unclassified	Total
miR-155	Negative	3 (43%)	7 (20%)	0	1 (100%)	0	11
	Positive	4 (57%)	28 (80%)	19 (100%)	0	2 (100%)	53
miR-21	Negative	2 (29%)	7 (20%)	3 (16%)	0	0	12
	Positive	5 (71%)	28 (80%)	16 (84%)	1 (100%)	2 (100%)	52
Total		7	35	19	1	2	64

Discussion

It is estimated that as many as half of individuals over the age of sixty harbor PanIN lesions in their pancreata [32]. By contrast, the numbers of IPMNs are likely to be considerably lower in the general population. Nonetheless, due in part to their macroscopic (cystic) nature and the potential for detection upon radiological examination, IPMNs present a unique opportunity for prevention of invasive malignancy in the pancreas [3]. With the enhanced usage of CT and other non-invasive scanning techniques in medical practice, increasing numbers of patients with incidental cysts of the pancreas ('incidentalomas') are being identified [33]. While in many instances these cysts are innocuous in their biological potential (for example, serous cystic neoplasms), others represent *bona fide* precursors of pancreatic adenocarcinomas, such as IPMNs or mucinous cystic neoplasms (MCNs) [34;35]. Identification of molecular aberrations in IPMNs thus attains considerable clinical significance, not only in terms of understanding the biology of early pancreatic neoplasia, but also as a fertile seedbed for generating therapeutic and diagnostic markers, which might stem the progression to a lethal invasive cancer [36].

Several studies have reported miRNA abnormalities in pancreatic cancers, using either array-based platforms or by qRT-PCR [17-20]. For a subset of these miRNAs, the underlying mechanism for misexpression has been elucidated. For example, we have demonstrated that loss of p53 function in pancreatic cancer is associated with decreased expression of its transcriptional target miR-34a in pancreatic cancers [23]; conversely, aberrant Myc-activity in pancreatic and other human cancers results in elevated expression of the miR-17-92 polycistron [37]. In most instances however, the mechanism(s) underlying miRNA misexpression in pancreatic cancer remain unknown. In the current study, we profiled a series of non-invasive IPMNs for miRNA abnormalities using the prior published reports in pancreatic cancer as a guide. Specifically, we selected a panel of 12 miRNAs that have been reported as overexpressed in this malignancy. The miRNAs were selected based on multiple criteria, including their identification in more than one pancreatic cancer miRNA profiling study, the relative fold-elevation compared to normal pancreas, and the putative cancer-associated function of the miRNA. Although this selective approach is less optimal than an unbiased 'forward genetics' strategy, our prior experience with genomic and transcriptomic analyses of IPMNs suggests that these precursors often harbor many of the genetic alterations observed in pancreatic adenocarcinomas [21;38;39]. Indeed, we found that as many as 10 of the 12 miRNAs were significantly overexpressed in the IPMNs compared to non-neoplastic pancreata, establishing that miRNA abnormalities are an early event in the multistep progression of pancreatic cancer.

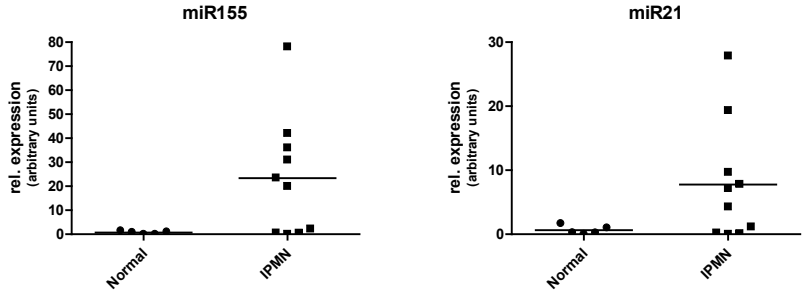

Figure 3: Expression of miR-155 and miR-21 in pancreatic juice samples assessed by qRT-PCR.
Analysis by qRT-PCR of pancreatic juice samples from 10 patients with IPMNs and five disease controls. The X-axis designates the two categories of patients for each miRNA, and the Y-axis designates the relative fold expression in each pancreatic juice sample. The horizontal bar represents the group average. Note that the Y-axis values differ in miR-155 and miR-21, reflecting a greater fold elevation in the former.

We focused our attention on further tissue-based validation of two of the highest differentially expressed miRNAs –miR-21 and miR-155-. Both miRNAs have been reported as significantly overexpressed in invasive pancreatic cancer in all four prior studies [17-20], furthering underscoring their relevance to this malignancy. The primary transcript for miR-21 is expressed from chromosome 17q23.2, and the resulting mature transcript was one of the first oncogenic miRNAs (onco-miR) identified in human cancer [17;30;40]. Upregulation of miR-21 in cancer cells is associated with apoptosis inhibition and acquisition of invasive propertie [41;42]. Several of the coding genes translationally repressed by miR-21 have now been identified, including the tumor-suppressor *phosphatase and tensin homolog* (*PTEN*), downregulation of which results in activation of the Akt signaling pathway [43], and *programmed cell death 4* (*PDCD4*), loss of function of which promotes cellular transformation and metastases [44]. On the same lines, miR-155 is co-expressed in conjunction with the non-coding transcript BIC, from chromosome 21q21.3 [45]. Although initially identified as an overexpressed miRNA in hematological malignancies, miR-155 has now been reported as upregulated in several solid tumors, including pancreatic cancer [40;46]. Of note, a recent study has shown that misexpressed miR-155 in pancreatic cancer appears to repress the function of tumor protein 53 induced nuclear protein 1 (TP53INP1); the latter is a pro-apoptotic, p53-induced protein, and its miR-155-mediated downregulation in pancreatic cancer enhances tumorigenicity *in vivo* [31].

Our LNA-ISH data confirms that abnormal expression of both miR-155 and miR-21 is commonly observed in IPMNs, while these transcripts are absent in the overwhelming majority of normal pancreatic ductal epithelia. As is true for many of the molecular alterations observed in pancreatic cancer precursors [1], there is a gradation in the frequency of miRNA misexpression along the histological continuum of atypia, with a significantly greater proportion of IPMNs with carcinoma-*in-situ* expressing either miRNA compared to IPMN adenomas. Further, when stratified by the histological subtype of lining epithelium, we detected an increased frequency of miR-155 expression in IPMNs with an intestinal type or pancreato-biliary type epithelium *versus* those with gastric foveolar type lining (this difference was statistically significant in the intestinal type IPMNs). Given the greater propensity for the intestinal or pancreato-biliary type IPMNs to progress to invasive adenocarcinomas compared with gastric foveolar IPMNs [7;8], there appears to be a correlation between the proportion of miR-155 expressing cases and intrinsic biological potential of a particular IPMN subtype. Nonetheless, the discrimination was not as unequivocally dichotomous as has been previously reported for certain cellular apomucin expression profiles (specifically, MUC1, MUC2, and MUC5) [7;10].

In addition to profiling IPMN tissues for miRNA abnormalities, we were also interested in determining whether detection of misexpressed miRNAs in pancreatic juice samples might be a feasible biomarker development strategy. To the best of our knowledge, detection of aberrantly expressed miRNAs in pancreatic juice has not been studied to date, although this possibility has been suggested based on the current state of knowledge on miRNA abnormalities in pancreatic cancer. In particular, there is a great need for developing adjunct diagnostic strategies for cystic neoplasms of the pancreas such as IPMNs and MCNs. In contrast to most pancreatic adenocarcinomas that tend to be solid lesions [47], 'conventional' modalities like endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) present considerable challenges in the diagnosis of cystic entities [48]. Typically, the aspirated material tends to be paucicellular, thus impeding accuracy of diagnosis. Of the

available biomarker assays on cyst fluid, only CEA appears to be of modest benefit, with an accuracy of 79% in distinguishing mucinous precursor lesions (IPMNs and MCNs) from other cystic entities in the pancreas [49]. Recent studies have elaborated upon the use of DNA-based molecular assays like microsatellite analysis and somatic mutations in aiding the differential diagnosis of pancreatic cysts [50;51]. Our results provide encouraging, albeit admittedly preliminary, validation that miRNA profiling of pancreatic juice by qRT-PCR is feasible, and further, suggest that such profiling may provide discrimination between pancreato-biliary disease controls and patients harboring IPMNs. Notably, patients with non-neoplastic pancreato-biliary disorders like chronic pancreatitis have minimally detectable miR-155 in their pancreatic juice, while at least 60% of IPMN samples have elevated miR-155 levels (20-fold or greater relative fold expression). It is our intention to build further upon these preliminary studies and examine a larger cohort of patients, factoring in additional standards such as cyst fluid CA19-9 and CEA levels.

In conclusion, we have performed the first miRNA profiling of non-invasive IPMNs, and confirmed that these macroscopic precursors share many of the miRNA abnormalities observed in invasive neoplasia. Two candidate miRNAs -miR-155 and miR-21– are commonly expressed in the neoplastic epithelium of IPMNs, and demonstrate correlation with histological features of progression (carcinoma-*in-situ*) and more ominous biological potential. Finally, elevated miR-155 demonstrates promise as a candidate biomarker for IPMNs in pancreatic juice specimens, warranting further study.

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7

Ligand-dependent Notch signaling is involved in tumor initiation and tumor maintenance in pancreatic cancer

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Abstract

Purpose: Aberrant activation of the Notch signaling pathway is commonly observed in human pancreatic cancer, although the mechanism(s) for this activation has not been elucidated.

Experimental Design: A panel of 20 human pancreatic cancer cell lines was profiled for the expression of Notch pathway related ligands, receptors and target genes. Disruption of intracellular Notch signaling – either genetically by RNA interference targeting *NOTCH1* or pharmacologically by means of the gamma secretase inhibitor GSI-18, was used for assessing requirement of Notch signaling in pancreatic cancer initiation and maintenance.

Results: Striking overexpression of Notch ligand transcripts was detectable in the vast majority of pancreatic cancer cell lines, most prominently, *JAGGED2* (18/20 cases; 90%) and *DLL4* (10/20 cases; 50%). In two cell lines, genomic amplification of the *DLL3* locus was observed, mirrored by overexpression of *DLL3* transcripts. In contrast, coding region mutations of *NOTCH1* or *NOTCH2* were not observed. Genetic and pharmacological inhibition of Notch signaling mitigated anchorage independent growth in pancreatic cancer cells, confirming that sustained Notch activation is a requirement for pancreatic cancer maintenance. Further, transient pre-treatment of pancreatic cancer cells with GSI-18 resulted in depletion in the proportion of tumor-initiating aldehyde dehyrogenase (ALDH)-expressing subpopulation, and was associated with inhibition of colony formation *in vitro* and xenograft engraftment *in vivo*, underscoring a requirement for the Notch-dependent ALDH-expressing cells in pancreatic cancer initiation.

Conclusions: Our studies confirm that Notch activation is almost always ligand-dependent in pancreatic cancer, and inhibition of Notch signaling is a promising therapeutic strategy in this malignancy.

Introduction

Pancreatic cancer is an almost uniformly lethal disease with an overall five-year survival of approximately 5%, and this dire prognosis has not markedly improved over the last few decades [1]. In the United States, approximately 34.000 individuals succumb to this malignancy each year. To date, the only potentially curative therapeutic option is complete surgical resection, but unfortunately, the majority of patients are diagnosed at a locally advanced or distant metastatic stage, thus precluding surgical cure [2]. Currently available treatment options for advanced pancreatic cancer, such as gemcitabine, have had minimal impact in ameliorating survival. Identification of aberrant signaling pathways that can also form the substrate for targeted therapies has thus become an area of foremost priority.

The re-activation of embryonic signal transduction pathways such as Notch and Hedgehog have been reported in a variety of human cancers [3;4]; further, the availability of potent small molecule inhibitors has meant that these pathways can be targeted in these cancers, as we and others have recently shown [5-7]. The Notch signaling pathway is an evolutionarily conserved pathway that plays a major role in cell fate decisions in various tissues during the development of multicellular organisms [8]. In adult tissues, Notch signaling prevents cells from undergoing terminal differentiation, thus maintaining pools of undifferentiated stem/progenitor cells [9;10]. Activation of the Notch signaling pathway has previously been described in several human malignancies, including pancreatic cancer [4;11;12]. For example, our group has shown that expression of Notch gene targets is observed not only in invasive pancreatic cancers, but also in the non-invasive precursor lesions of this malignancy [13]. In a series of elegant studies, Wang *and colleagues* have demonstrated a requirement for active Notch signalling for tumor maintenance in pancreatic cancer, with downregulation of *NOTCH1* contributing to growth inhibition and apoptosis of cancer cells through inhibition of key survival pathways like nuclear factor-kappa B (NF-κB) [14-17]. However, the underlying mechanisms causing aberrant Notch signaling in pancreatic cancer are poorly understood.

In the present study we examine the mechanisms of Notch pathway activation in the setting of pancreatic cancer. We find that endogenous overexpression of Notch ligands, specifically *JAGGED2* and *DLL4,* appears to be the most common mechanism; uncommonly, genomic amplification of the *DLL3* locus on chromosome 19q13 contributes to Notch activation in this malignancy. In contrast to hematological malignancies like T-cell leukemia [18], mutational activation of Notch is rare to absent in pancreatic cancer. Our studies also demonstrate that sustained Notch signaling is required for the viability of a subpopulation of pancreatic cancer cells with tumor initiation properties (i.e., "cancer stem cells"), further supporting the utility of targeting this pathway as a therapeutic strategy in this malignancy.

Materials and methods

Cell lines and culture conditions

Twenty pancreatic cancer cell lines (PANC-1, CAPAN-1, Colo-357, CFPAC, MIAPaCa-2, BxPC-3, AsPc-1, L3.6PL, PL-4, PL-5, PL-8, PL-9, PL-12, PL-13, XPA-1, XPA-3, XPA-4, Panc-8.13, Panc-3.27, and Panc-4.30) were grown as previously described [19]. Immortalized non-malignant human pancreatic epithelial cells (hTERT-HPNE) were cultured as described elsewhere [20]. The hTERT-HPNE cells were used for normalization of expression levels for Notch pathway components amongst the 20 cancer cell lines.

RNA interference-mediated transcript knockdown

For knockdown of *NOTCH1* transcripts, PANC-1 and CAPAN-1 cells were transiently transfected with gene specific or scrambled siRNA using Oligofectamine (Invitrogen) following the standard procedure recommended by the manufacturer. Efficacy of knockdown was confirmed by quantitative reverse transcription-PCR (qRT-PCR). The sequences for the synthetic siRNAs against NOTCH1 (Dharmacon, Lafayette, CO, USA) have been previously described [21]. Similarly, RNAi against *DLL3* was performed in PANC-1 and SU86.86 cell lines using SMARTPool™ siRNA (Dharmacon), followed by qRT-PCR to confirm efficacy of *DLL3* knockdown.

Stable overexpression of Notch1 intracytoplasmic domain in PANC-1 cells

Generation of PANC-1 cells stably overexpressing the Notch-1 intracytoplasmic domain (N1ICD) was accomplished as previously described [21]. Empty vector was used for mock transfection.

Notch pathway inhibitor GSI-18

Synthesis of the gamma-secretase inhibitor [11-endo]-N-(5,6,7,8,9,10-hexahydro-6,9-methanobenzo[a][8]annulen-11-yl)-thiophene-2-sulfonamide (a.k.a. GSI-18) and its ability to block Notch pathway activity in cancer cells have been previously described [21-23].

Notch reporter assays

Assessment of Notch activity following GSI-18 administration was performed using a CBF-1 binding site luciferase reporter (8X-Luc), as previously described, in PANC-1 cells [13]. Renilla luciferase was used as transfection control.

Cell viability assay

Growth inhibition was measured using the CellTiter 96® A_{queous} Cell Proliferation Assay (Promega, Madison, WI, USA), which relies on the conversion of a tetrazolium compound (MTS) to a colored formazan product by the activity of living cells. Briefly, 2000 cells/well were plated in 96 well plates, and were treated with 2, 5 and 10 μM concentrations of GSI-18, for 96 hours, at which point the assay was terminated, and relative growth inhibition compared to vehicle-treated cells measured using the CellTiter 96® reagent, as described in the manufacturer‘s protocol. A panel of six human pancreatic cancer cell lines were examined (PANC-1, CAPAN-1, BxPC3, MIAPaca-2, PANC8.13, PANC3.27) in the MTS assays. Cell viability assays were also performed for PANC-1 and SU86.86 cells following RNAi against *DLL3*, using scrambled siRNA as control. All experiments were set up in triplicates to determine means and standard deviations.

Anchorage independent growth

Anchorage independent growth was assessed in PANC-1 and CAPAN-1 cells following either genetic inhibition (*NOTCH1* siRNA) or with pharmacological inhibition of Notch signaling with GSI-18 (5 μM). Soft agar assays were set up in 6-well plates, each well containing a bottom layer of 1% agarose (Invitrogen), a middle layer of 0.6% agarose including 10.000 cells, and a top layer of medium only. For the pharmacological inhibition experiments, mixtures in each well were supplemented with GSI-18 at the respective concentration or solvent only, and the plates were incubated for three weeks. An independent series of colony assays

was performed in PANC-1 and SU86.86 cells, following genetic knockdown of *DLL3* using siRNA. To assess colony formation, the medium was removed, and 1.5 ml of 0.5% Wright's staining solution was added to each well. After incubation at 4 °C for 12 h, removal of the staining solution and washing twice with PBS, colonies were visualized by trans-UV illumination and counted using the analysis software Quantity One (BioRad, Hercules, California, USA).

Evaluation of aldehyde dehydrogenase (ALDH) activity

ALDH expression was determined at baseline and after pharmacological Notch inhibition in two pancreatic cancer cell lines, E3LZ10.7 and CAPAN-1, where we have previously demonstrated that inhibition of Hedgehog signaling selectively depletes the ALDH "bright" subpopulation [5;6]. After incubation with either vehicle or GSI-18 (5μM) for 24 hours, E3LZ10.7 and CAPAN-1 cells were stained for ALDH expression using the Aldefluor reagent (StemCell Technologies, Vancouver, Canada) according to the manufacturer's instructions and analyzed on a FACSCalibur flow cytometer (Becton Dickinson). ALDH positive cells were quantified by calculating the percentage of total cells that displayed greater fluorescence compared to a control staining reaction containing the ALDH inhibitor diethylamino-benzaldehyde (DEAB), as we have previously described [5;6;24].

Pre-treatment with GSI-18

We have previously shown that transient *ex vivo* pre-treatment with Hedgehog antagonists inhibits both anchorage independent growth and *in vivo* tumorigenicity of pancreatic cancer and glioblastoma cell lines [5;24]. In order to determine the effects of Notch antagonism on tumor initiation, pancreatic cancer cell lines E3LZ10.7 and CAPAN-1 cells were pre-treated with either vehicle or GSI-18 for 24 hours (2 and 5 μM), and allowed to recover in full serum for 24 hours. Thereafter, equal numbers of viable cells from each condition, quantified using trypan-blue dye exclusion assay, were plated in soft agar for colony assays, as described above. Pre-treated and serum-recovered E3LZ10.7 and PANC-1 cells were also injected in athymic (nude) mice for tumor engraftment studies, as described below.

Colony assays with LY294002, an Akt/PI-3-kinase pathway inhibitor

In order to confirm the specificity of Notch inhibition against the tumor initiating component and exclude the potential for artefact, we performed a series of experiments using CAPAN-1 cells treated with LY294002, a small molecule inhibitor of the oncogenic Akt/PI-3-kinase pathway. Two parallel sets of anchorage independent assays were performed: first, a "*pre-treatment*" experiment mirroring the GSI-18 study, with two doses of LY294002 (5 and 10 μM). In this experiment, CAPAN-1 cells were exposed to the drug for 24 hours, followed by full serum recovery and plating in soft agar. The second set of experiments, with the same dosages, utilized a "*continuous*" (conventional) approach, where the cells were incubated in soft agar with continuous exposure to LY294002 for two weeks. Colony counts were performed as described above.

Generation of murine subcutaneous xenografts

All animal experiments conformed to the guidelines of the Animal Care and Use Committee of Johns Hopkins University and animals were maintained in accordance to guidelines of the American Association of Laboratory Animal Care. A total of $5x10^6$ E3LZ10.7 or PANC-1 cells in a volume of 200 μl of 1/1 (v/v) PBS/matrigel, pre-treated

with either vehicle or with GSI-18 at 5μM, and allowed to recover in full serum for 24 hours, were injected subcutaneously into male CD1 nu/nu athymic mice (Charles River). Tumor volumes (V) were determined after measuring the larger (a) and smaller (b) diameters as $V = \frac{a \cdot b^2}{2}$, as previously described [5;6].

Statistical analysis

Kruskal-Wallis analysis was performed using SPSS version 15.0.1 for Microsoft Windows, two-tailed t-test, one way ANOVA and linear regression analysis (Pearson's test) were performed using GraphPad Prism for Windows version 5. *P*-value <0.05 was regarded as statistically significant. Results in bar diagrams are plotted as means and standard deviations if not otherwise indicated.

Results

Endogenous overexpression of Notch ligands in pancreatic cancer

Quantitative real-time qRT-PCR analysis of 20 human pancreatic cancer cell lines compared with hTERT-HPNE cells confirmed variable expression of *NOTCH1* through *NOTCH4* transcripts, with most cell lines not demonstrating any evidence of receptor mRNA overexpression. Thus, compared to hTERT-HPNE cells, only 8 of 20 (40%) pancreatic cancer lines had equal or greater expression of *NOTCH1*, 5 of 20 (25%) had equal or greater expression of *NOTCH4*, and 4 of 20 (20%) had equal or greater expression of *NOTCH2* transcripts, respectively (fig. 1A). Curiously, *NOTCH3* mRNA expression was lower than hTERT-HPNE cells in all 20 cancer cell lines, with several lines not expressing any detectable transcripts. In contrast to receptor mRNA levels, marked upregulation of two of four Notch pathway ligand transcripts (specifically, *JAGGED2* and *DLL4*) were observed in the majority of pancreatic cancer cell lines. This was particularly striking for *JAGGED2* where 18 of 20 (90%) of cell lines had higher transcript levels than observed in hTERT-HPNE (with the majority of cases at >50-fold elevation), and to a lesser extent with *DLL4*, with 10 of 20 lines (50%) demonstrating mRNA overexpression compared to hTERT-HPNE cells (fig. 1B). *JAGGED1* and *DLL1* transcripts were expressed at more attenuated levels (no higher than 10-fold relative overexpression compared to hTERT-HPNE cells), and were upregulated in fewer cell lines within the panel. Consistent with Notch pathway activation, striking overexpression of the Notch target genes *HES1* and *HEY2* (*HERP1*) was seen in 16 of 20 (80%) and 13 of 20 (65%) of PC cell lines, respectively (fig. 1C). In contrast, overexpression of the remaining Notch gene targets, *HEY1* (*HERP2*) and *HEYL* was observed in only a minority of cancer cell lines when compared with corresponding transcript levels in hTERT-HPNE cells. Comparable expression results were obtained when *GUSB* was used as housekeeping control instead of *PGK1* (*data not shown*). Upon correlating Notch ligand levels with that of target genes, *JAGGED2* mRNA expression was most closely and significantly correlated with that of *HES1* transcripts (P =0.045, Pearson correlation), further underscoring the importance of this basic helix-loop-helix (bHLH) transcription factor in the context of pancreatic neoplasia [13;25]. On the contrary, there was highly significant correlation between the patterns of expression of *DLL4* and the Notch target gene *HEYL* in pancreatic cancer cell lines (P =0.003, Pearson correlation), reiterating previous observations that despite the commonalities within the pathway, individual ligands have disparate effects on target genes [21].

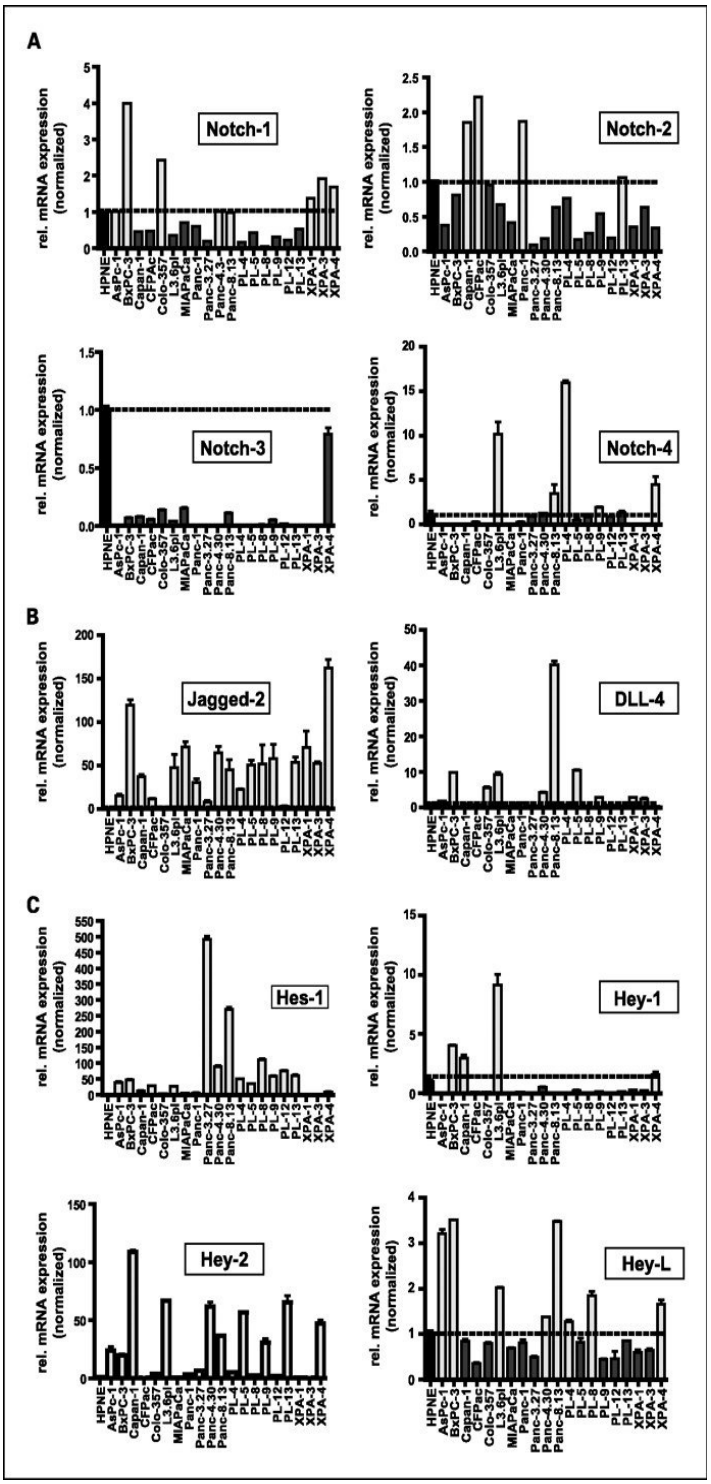

Figure 1: Profiling the Notch pathway in pancreatic cancer cell lines

RNA from a panel of 20 pancreatic cancer cell lines was assessed for expression of Notch receptors *NOTCH1*, *NOTCH2*, *NOTCH3* and *NOTCH4* (**panel A**), Notch ligands *JAGGED2* and *DLL4* (**panel B**) and Notch gene targets *HES1*, *HEY1, HEY2* and *HEYL* (**panel C**), and relative fold levels compared to immortalized hTERT-HPNE cells. Horizontal line indicates normalized ratio of 1 in hTERT-HPNE cells. X-axis corresponds to individual cell line samples, and Y-axis to relative fold level of expression. Light grey bars indicate cancer cell lines with overexpression of corresponding mRNA compared to hTERT-HPNE cells, while dark grey bars indicate cell lines with equal or lesser expression. All assays were performed in triplicate, using *PGK1* as housekeeping control, and independent set of assays was performed using *GUSB* as housekeeping control (data not shown).

Amplification of DLL3 is an uncommon "driver" for Notch signaling in pancreatic cancer

Previously published genomic copy number analyses of pancreatic cancer cell lines and xenografts by our group and others have shown that the *DLL3* locus on chromosome 19q13 is included in a recurrent amplicon in this malignancy [19;26;27]. Therefore, we assessed *DLL3* gene dosage in 22 pancreatic cancer cell lines, and found two lines –PANC-1 and SU86.86 that demonstrated 3-fold or greater copy number by genomic Q-PCR, compared to hTERT-HPNE cells (fig. 2A). Transcript profiling confirmed that PANC-1 and SU86.86 had strikingly high expression of DLL3 mRNA, ~200-fold that of hTERT-HPNE cells (fig. 2B). *DLL3* was downregulated by transient RNA interference (RNAi) in both cells lines, and effects on *in vitro* growth and anchorage independence were determined following validation of gene-specific knockdown. No significant effects were observed on either phenotype in PANC-1 cells with *DLL3* RNAi (*data not shown*), suggesting redundant mechanisms for Notch pathway activation in this cell line. In contrast, knockdown of *DLL3* in SU86.86 resulted in significant growth inhibition by MTS assay (fig. 2C, P =0.0005), as well as significant inhibition of anchorage independent growth in soft agar (fig. 2D, P =0.0016). Thus, in a minor subset of pancreatic cancers, Notch pathway activation is likely to be driven by increased *DLL3* copy number and resulting endogenous overexpression of the ligand protein.

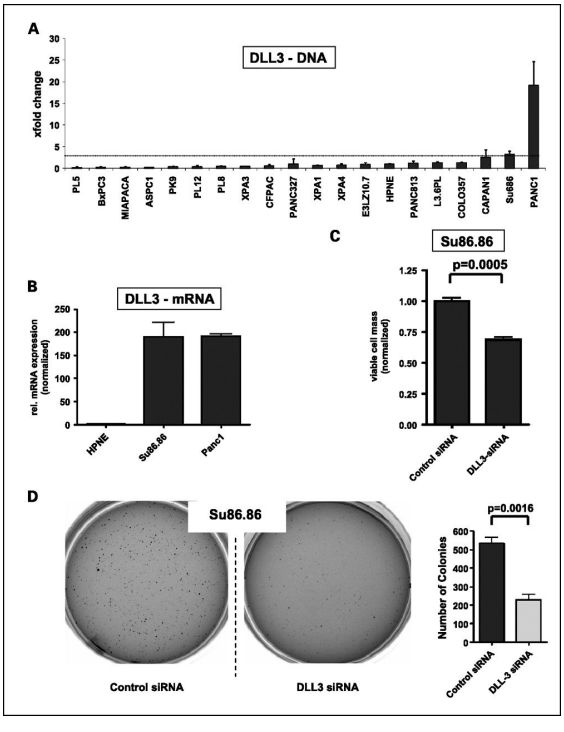

Figure 2: Copy number alteration of DLL3 in a subset of pancreatic cancer cell lines **(A)** Genomic Q-PCR for *DLL3* copy number demonstrates two cell lines (PANC-1 and SU86.86) with an average gene dosage ratio of greater than three. The chromosome 19q13 gene *KCL3* was used as a reference control. Genomic Q-PCR was performed in triplicate, and average ± SD calculated, with the latter expressed as error bars. **(B)** Quantitative reverse transcription PCR (qRT-PCR) for *DLL3* mRNA in PANC-1 and SU86.86 cells demonstrates striking overexpression (~200-fold), relative to levels in hTERT-HPNE cells. **(C)** Knockdown of *DLL3* by synthetic small interfering RNA (siRNA) significantly inhibits in vitro growth of SU86.86 cells, as measured by an MTS cell viability assay at 96 hours, compared to control (scrambled siRNA transfected) cells; (P =0.0005). **(D)** Knockdown of *DLL3* by siRNA significantly inhibits anchorage independent growth in Su86.86 cells, as assessed by colony formation in soft agar, compared to control (scrambled siRNA transfected) cells; (P =0.0016). Colony assays were performed in triplicate, and average ± SD calculated, with the latter expressed as error bars.

NOTCH1 or NOTCH2 mutations are rare to absent in pancreatic cancer

Activating mutations of the *NOTCH* receptors have been suggested to be the underlying driving force of Notch pathway activation in several malignancies, particularly in T-cell leukemias, wherein activating *NOTCH1* mutations are found in as many as 50% of cases [18]. To determine whether such coding sequence mutations of *NOTCH1* or *NOTCH2* exist in the setting of pancreatic cancer, mutational analysis of 20 pancreatic cancer cell lines, as well as 22 patient-derived pancreatic cancer xenografts, was performed by direct Sanger sequencing of the coding regions. All sequence variations from RefSeq (http://www.ncbl.nlm.nih.gov/RefSeq) were first confirmed by replicate PCR, and subsequently cross-matched against the single nucleotide polymorphism database dbSNP (http://www.nlm.nih.gov/projects/SNP). A previously undescribed heterozygous L2458V alteration was identified in the C-terminal PEST domain of *NOTCH1* in the MIAPACA-2 pancreatic cancer cell line (*data not shown*). However, gauged by the low expression levels of Notch pathway target genes in this line (see fig. 1C), the functional significance of this alteration was uncertain. We failed to find any evidence of activating mutations in any of the 42 cancer samples within the *NOTCH1* and *NOTCH2* coding regions.

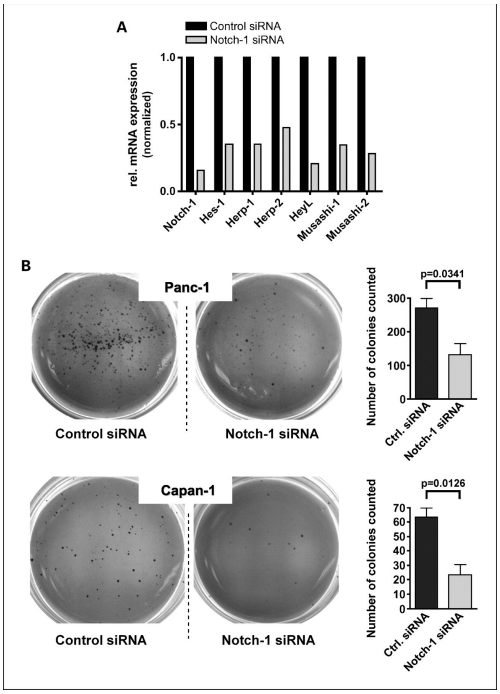

Figure 3: Genetic knockdown of *NOTCH1* function in pancreatic cancer cells inhibits anchorage independent growth

(A) *NOTCH1* RNAi in CAPAN-1 cells leads to >80% downregulation of gene specific transcript levels compared to scrambled siRNA transfected control cells. In addition, efficacy of functional knockdown is confirmed by reduced transcript levels for Notch gene targets, including *HES1, HEY1* [*HERP2*]*, HEY2* [*HERP1*]*, HEYL*, *MUSASHI1* and *MUSASHI2*, compared to scrambled siRNA transfected control cells.

(B) NOTCH1 siRNA significantly inhibits anchorage independent growth in CAPAN-1 and PANC-1 cells, as assessed by colony formation in soft agar, compared to control (scrambled siRNA transfected) cells; (P <0.05). Colony assays were performed in triplicate, and average ± SD calculated, with the latter expressed as error bars.

Sustained Notch signaling is required for pancreatic cancer maintenance

In light of the evidence suggesting ligand-dependent Notch activation in the majority of human pancreatic cancer cell lines, we then evaluated whether sustained Notch signaling is required for the maintenance of pancreatic cancer, and in particular, for anchorage independent growth, a property of transformed cells. We first used RNAi to downregulate *NOTCH1* transcript levels in CAPAN-1 and PANC-1cancer cell lines; efficacy of RNAi was confirmed by real-time PCR demonstrating downregulation of *NOTCH1* transcripts, as well as multiple Notch target genes (fig. 3A). Both cell lines transfected with *NOTCH1* siRNA demonstrated a significant reduction in the number of colonies formed in soft agar compared to scrambled siRNA transfected controls, confirming a requirement of active Notch signaling for anchorage independent growth (fig. 3B).

To complement the RNAi findings, we also studied the effects of pharmacological blockade of Notch signaling in pancreatic cancer cells on *in vitro* growth in monolayers and anchorage independent growth in soft agar. GSI-18 is a previously described gamma secretase inhibitor with potent inhibitory effects on Notch signaling [21-23]. We first established that exposure of PANC-1 cells to GSI-18 leads to significant down-regulation of Notch activity, as observed using CBF1-binding site luciferase reporter assays (fig. 4A). A panel of six pancreatic cancer cell lines was used for *in vitro* growth (MTS) assays. As shown in figure 4B, only modest growth inhibition was observed with GSI-18 at the highest dose (10μM), and cell viability was largely unaffected at 2 and 5μM doses. In contrast, significant reduction in colony formation in soft agar was observed in both CAPAN-1 and PANC-1 cell lines when exposed to 5μM GSI-18 (fig. 4C). Thus, based on the combined results of *NOTCH1* RNAi and GSI-18 treatment, we conclude that continuous blockade of Notch signaling is deleterious for the anchorage independent growth of pancreatic cancer cells.

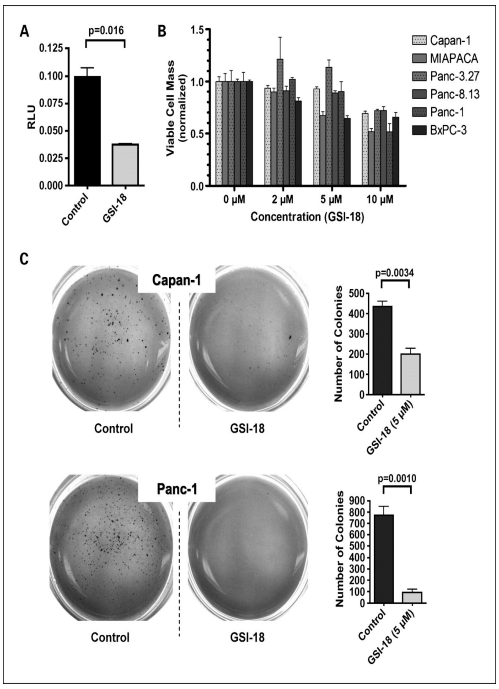

Figure 4: Pharmacological knockdown of Notch function in pancreatic cancer cells inhibits anchorage independent growth

(**A**) Gamma secretase inhibitor GSI-18 (2μM) significantly downregulates CBF-1 binding site luciferase reporter activity (P =0.016) in PANC-1 cells, consistent with inhibition of intracellular Notch function. Control cells are treated with DMSO vehicle. Y-axis depicts relative luciferase activity (RLU). (**B**) Modest dose dependent inhibition of *in vitro* cell growth (assessed by MTS cell viability assay at 96 hours) is observed in a panel of six pancreatic cancer cell lines (CAPAN-1, PANC-1, MIAPACA-2, BxPC3, PANCA8.13, and PANC3.27) upon GSI-18 treatment. Three independent doses (2, 5, and 10μM) are used, and cell viability is normalized to DMSO vehicle treated cells (0μM column). All MTS assays are performed in triplicate, and average ± SD calculated, with the latter expressed as error bars.

(**C**) GSI-18 significantly inhibits anchorage independent growth in CAPAN-1 and PANC-1 cells, as assessed by colony formation in soft agar, compared to control (DMSO-treated) cells; (P <0.005). Colony assays were performed in triplicate, and average ± SD calculated, with the latter expressed as error bars.

Overexpression of Notch 1 Intracellular Domain (N1ICD) rescues GSI-18-mediated inhibition of anchorage independent growth in PANC-1 cells

A mammalian expression vector encoding *N1ICD* was stably transfected in PANC-1 cells ("PANC1-N1ICD"), and overexpression of *N1ICD* as compared to empty vector transfected cells was confirmed by qRT-PCR (fig 5A). Of note, enforced *N1ICD* expression *per se* markedly enhanced anchorage independent growth of PANC-1 cells in soft agar assays (fig. 5B and 5C). Treatment with GSI-18 at a concentration of 5μM led to a more than seven-fold reduction in colony numbers in empty vector-transfected PANC-1 cells, while no significant reduction in colony formation was observed in PANC1-N1ICD cells. Thus, enforced expression of N1ICD is able to rescue PANC-1 cells from the effects of GSI-18, underscoring the relative "on-target" effects of this small molecule inhibitor.

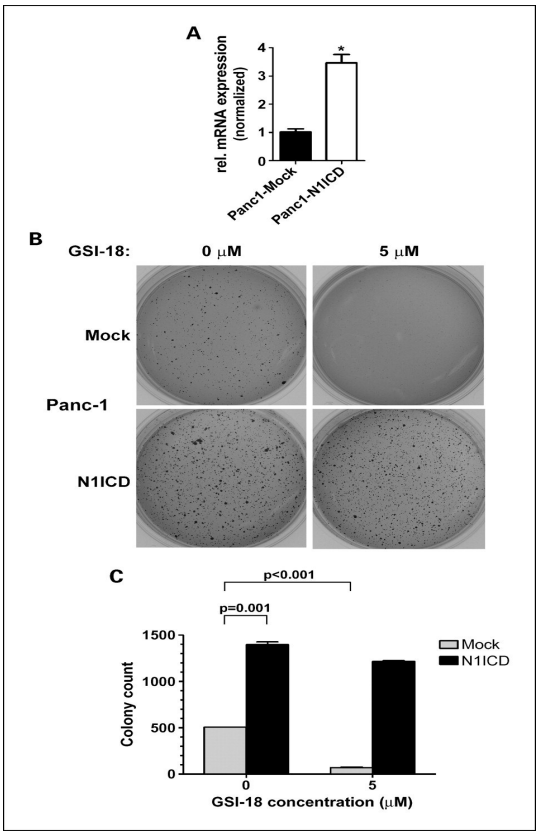

Figure 5: Enforced expression of Notch 1 intracytoplasmic domain (N1ICD) rescues anchorage independent growth phenotype of PANC-1 cells treated with GSI-18

(A) Enforced expression of N1ICD in PANC-1 cells led to increased colony formation and anchorage independent growth in soft agar as compared to empty vector transfected cells.

(B) Treatment with GSI-18 (5 μM) significantly reduced colony formation of empty vector transfected cells, but no significant effects were observed in the N1ICD-overexpressing cells.

(C) Colony counts are provided in diagram.

Transient Notch pathway inhibition eliminates a subpopulation of ALDH "bright" cells with tumor initiating properties in pancreatic cancer

Emerging lines of evidence in solid cancers suggest that a subpopulation of cells with tumor-initiating properties (so-called "cancer stem cells") can be identified by elevated expression of the enzyme aldehyde dehydrogenase (ALDH) [6;24;28]. We have recently identified ALDH "bright" cells in pancreatic cancer that are highly sensitive to Hedgehog pathway blockade with cyclopamine or related small molecule inhibitors [5;6]. We have also shown that selective elimination of these ALDH "bright" cells by transient pre-treatment with Hedgehog inhibitors inhibits subsequent tumor initiation (engraftment) in xenograft models [5]. In order to determine whether this putative tumor initiating population is also Notch pathway dependent, we treated CAPAN-1 and E3LZ10.7 cells with GSI-18 *in vitro* for 24 hours. These two cell lines have been documented to have robust ALDH "bright" cells detectable by the Aldefluor assay [5]. We observed a selective depletion of this subpopulation with transient GSI-18 exposure in both CAPAN-1 and E3LZ10.7 cells (fig. 6A). Upon subsequent plating in soft agar, these transiently pre-treated cells also demonstrated profound inhibition of anchorage independent growth (fig. 6B). Further, when equal numbers of viable E3LZ10.7 or PANC-1 cells, which had been transiently exposed to either GSI-18 or vehicle for 24 hours, respectively, were injected subcutaneously in athymic mice, a significant blockade of xenograft engraftment was observed in both sets of treated cell lines, at 5 weeks of follow up (fig. 6C). These findings underscore the importance of sustained Notch signaling in maintaining the viability of tumor-initiating ALDH "bright" cells in pancreatic cancer, and demonstrate that even transient exposure to Notch antagonists has deleterious effects on tumor engraftment *in vivo*.

One potential pitfall of the "pre-treatment" strategy is the possibility that overall cellular function is sufficiently compromised by the transient exposure to GSI-18 that colony formation and engraftment in nude mice are inhibited, irrespective of any specific impact on the tumorigenic population of cells. To exclude this possibility, we performed a parallel series of colony assays in soft agar, wherein CAPAN-1 cells were either "pre-treated" transiently with LY294002, an antagonist of Akt/PI-3-kinase pathway, prior to plating, or exposed continuously to the drug in a more conventional colony assay format. In contrast to our observations with GSI-18, transient pre-treatment has no effect on anchorage independent growth, while the conventional colony assays demonstrate the expected reduction in colonies at 2 weeks (fig. 6D). This provides additional confirmation that the loss of tumorigenic phenotype observed with transient Notch inhibition is unlikely to be a non-specific deleterious effect on cellular function.

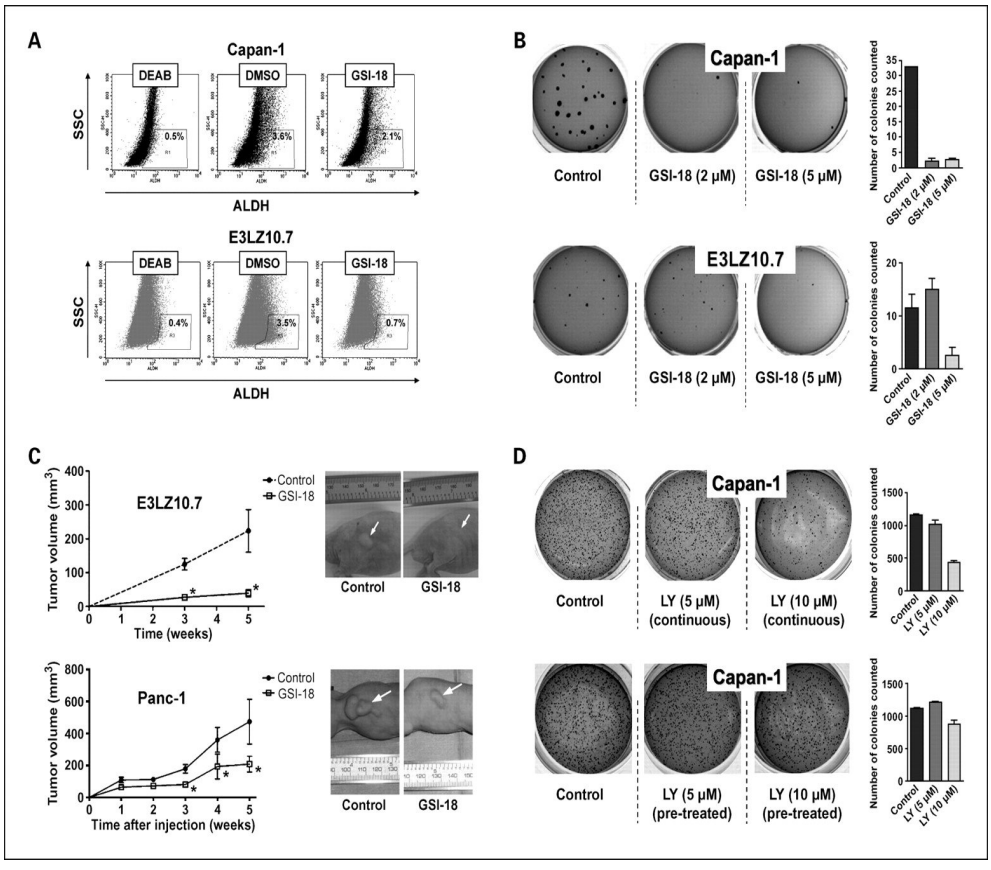

Figure 6: Transient *ex vivo* exposure of pancreatic cancer cells to Notch inhibitors depletes ALDH "bright" cells and impedes tumor initiation *in vivo*

(**A**) Transient incubation of CAPAN-1 and E3LZ10.7 cells with GSI-18 (5 μM) for 24 hours reproducibly diminished the fraction of ALDH "bright" cells as determined by the Aldefluor assay (3.6% to 2.1% for CAPAN-1 and 3.5% to 0.7% for E3LZ10.7). The ALDH inhibitor DEAB was used as negative control in the assay.

(**B**) CAPAN-1 and E3LZ10.7 cells were incubated with 2 and 5μM doses of GSI-18 for 24 hours, followed by full serum recovery for an additional 24 hours, and plating in soft agar for colony assays. No further GSI-18 exposure was administered to the plated cells. Compared to equal numbers of viable plated cells in the DMSO-treated group, reduction in colony formation is observed at 2 and 5μM doses for CAPAN-1 cells, and at 5μM dose for E3LZ10.7 cells.

(**C**) E3LZ10.7 or PANC-1 cells were incubated with GSI-18 at a concentration of 5μM for 24 hours, followed by full serum recovery for an additional 24 hours, and injection of $5x10^6$ cells in the subcutaneous milieu of athymic mice. No further *in vivo* treatment was performed. Compared to equal numbers of viable injected cells in the DMSO-treated group, significant reduction in size of the engrafted tumors is seen with transient GSI-18 exposure in both sets of cell lines, beginning at 3 weeks post-injection and persisting at 5 weeks (*asterisks*).

(**D**) In contrast to the phenotype observed with GSI-18 pre-treatment, no effects of transient exposure are seen with LY294002, a small molecule inhibitor of the Akt signaling pathway, in CAPAN-1 cells. Specifically, CAPAN-1 cells were exposed to two doses (5 and 10μM) of LY294002 in one of two modes: "continuous", wherein cells were incubated with continuous exposure to the drug, as in a conventional colony assay, and "pre-treatment", mimicking the transient pre-treatment exposure for 24 hours performed with GSI-18.

Discussion

The Notch signaling pathway plays a critical role in pancreatic development and in the homeostasis of mature pancreatic tissues [9;29;30]. In the adult pancreas, we and others have shown that Notch activation is predominantly restricted to the centroacinar cells within the exocrine compartment [13;31]. It is believed that these cells represent a persistent pool of progenitor-type cells in the adult pancreas, and that the Notch pathway is a *sine qua non* for maintaining the undifferentiated state of these cells. An abnormal expansion of Notch-expressing cells is observed in states of exocrine injury, while abrogation of Notch signaling impairs subsequent epithelial regeneration, underscoring the importance of this pathway to tissue homeostasis in the pancreas [32-34]. A role for aberrant Notch signaling in pancreatic cancer has emerged from studies conducted in human and animal models of this disease [13-17;35]. For example, the basic helix-loop-helix (bHLH) transcription factor Hes-1 is a prototypal Notch gene target [36], and Hes-1 upregulation is observed at the earliest, non-invasive stages of human and mouse pancreatic cancer [13;35].

In mammalian cells, the canonical Notch pathway includes four distinct Notch receptors, NOTCH1, NOTCH2, NOTCH3 and NOTCH4. Previous reports have elucidated context-dependent and cancer-type specific effects of the Notch receptors on carcinogenesis. Thus, NOTCH1 is oncogenic in T-cell leukemia and in breast cancers [18;37], while loss of NOTCH1 function promotes tumorigenesis in medulloblastoma and in skin cancers [21;38]. In medulloblastoma, by contrast, NOTCH2 appears to be the dominant oncogenic receptor (21). Wang *et al* have demonstrated the primacy of NOTCH1 as the Notch pathway receptor responsible for tumor maintenance in pancreatic cancer [14-17]. Genetic or pharmacological inhibition of NOTCH1 activity in pancreatic cancer has profound deleterious effects on cell growth, cell survival, and invasion, through downregulation of critical signaling moieties like NF-κB, vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMPs) [14-17]. These existing reports provided the seedbed for our current study exploring the mechanisms of Notch activation in pancreatic cancer, and an assessment of the effects of Notch inhibition on the putative tumor-initiating compartment in this malignancy.

The Notch pathway is activated through somatic mutations of *NOTCH1* in approximately 50% of T-cell leukemias [18], and in a minor subset (<5% by conservative estimates) of other solid cancers like breast, lung and colon cancer [39]. The Catalog of Somatic Mutations in Cancer (COSMIC) database (http://www.sanger.ac.uk/genetics/CGP/cosmic/) provides an online compendium of these mutations. Sequencing the complete coding regions of *NOTCH1* and *NOTCH2* genes in 42 pancreatic cancer samples (20 cell lines and 22 xenografts) failed to elicit evidence of activating non-synonymous alterations. A single hemizygous L2458V PEST domain alteration was identified in the MIAPaCa-2 cell line; however, in the absence of functional correlates of pathway activation (as gauged by Notch reporter and target gene analysis), the significance of this change remains uncertain. Of note, a recent large scale sequencing effort of the pancreatic cancer genome also failed to identify somatic point mutations in *NOTCH1* or *NOTCH2*, as well as within any of the genes encoding Notch ligands [40]. These results reiterate that mutational activation of the Notch pathway in pancreatic cancer is rare.

In mammalian cells, at least five distinct ligands (JAGGED-1, JAGGED-2, DLL-1, DLL-3, and DLL-4) initiate Notch signaling upon binding to the cognate receptors. We found evidence for striking overexpression of Notch ligand transcripts, especially *JAGGED-2* and *DLL-4*, in the majority of pancreatic cancer cell lines. Thus, as many

as 18 of 20 (90%) of the cell lines examined in our panel demonstrated *JAGGED-2* upregulation compared to hTERT-HPNE cells, with the majority having >50-fold relative expression levels. *JAGGED-2* expression was mirrored by, and correlated with, mRNA expression of the *Hairy enhance of split* family of bHLH transcription factors recognized as Notch gene targets, underscoring the functional relevance of ligand-dependent activation. Ligand-dependent activation of embryonic signaling pathways is not unique to Notch, as we and others have described the existence of an analogous mechanism for both Hedgehog and *wnt* pathways, respectively, in pancreatic cancer [41;42]. In both instances, somatic mutations in downstream components (for example, *GLI1* or *CTNNB1*) are rare to absent, accompanied by endogenous overexpression of stimulatory ligand. An enigmatic question pertains to the upstream genetic influence(s) promoting such profound Notch ligand expression in pancreatic cancer cells. In the context of Hedgehog signaling, we and others have shown that mutant Kras up-regulates the transcription of endogenous Hedgehog ligand in pancreatic cancer cells [43;44]. Whether parallel mechanisms are in place for the Notch pathway remains a matter of investigation. In light of the prior observations by Miele *and colleagues* pertaining to the absolute requirement of Notch signaling for maintaining the neoplastic phenotype of human *Ras*-transformed cells [45], and the demonstration of Notch activation in pancreatic ductal lesions arising in Kras-driven genetically engineered mouse models of pancreatic cancer [35], the existence of such an axis is not beyond the realm of speculation. In passing, we should add that in a minority of instances, Notch activation appears to be a consequence of genomic copy number alterations at chromosome 19q13, the *DLL3* gene locus [19;26;27]. We have confirmed the existence of increased gene dosage, and associated *DLL3* transcript overexpression, in two cell lines, and shown that knockdown of *DLL3* by RNAi can inhibit anchorage independent growth in the SU86.86 cell line. Curiously, *DLL3* RNAi in PANC-1 cells did not exhibit a discernible growth phenotype, suggesting that redundant ligand-driven activation can bypass the blockade of any one single Notch ligand, and underscores the need for targeting downstream elements of this pathway in cancer therapy.

In addition to exploring the mechanisms of Notch activation in pancreatic cancer, we also assessed the potential of Notch as a therapeutic target in pancreatic cancer, and in particular, whether Notch inhibition has a preferential deleterious effect on the tumor-initiating ("cancer stem cell") compartment. In light of the prior series of studies by Wang *and colleagues* [14-17], our findings on Notch inhibition and pancreatic cancer maintenance are mainly confirmatory in nature. Nevertheless, these studies expand the repertoire of pancreatic cancer cell line models in which the anti-cancer effects of Notch inhibition, either by genetic or pharmacological means, are evident. Further, our findings confirm that NOTCH1 is possibly the dominant oncogenic Notch receptor in this malignancy, and that, gamma secretase inhibitors like GSI-18, or other comparable small molecules [7;46], warrant further preclinical evaluation in pancreatic cancer. In contrast to genistein and curcumin, two previously reported Notch inhibitors that are natural plant polyphenols [14-17], the synthetic gamma secretase inhibitors are likely to have a more limited repertoire of targeted intracellular effects. Gamma secretase inhibitors are currently in advanced phase clinical trials for Alzheimer disease, having demonstrated favorable toxicity profiles in healthy volunteers [47], and therefore, the transition to being utilized as an anti-cancer agent may be an option in not too distant a future. Besides pancreatic cancer maintenance, however, a novel finding of our study has been the demonstration that even transient *ex vivo* pharmacological Notch inhibition depletes the putative tumor-initiating population in pancreatic cancer. We

and others have recently identified ALDH "bright" cells detectable by Aldefluor assay as an enriched cancer stem cell compartment in a variety of solid cancers, including pancreatic cancer [5;6;24;28]. The ALDH "bright" cells are eliminated upon systemic Hedgehog inhibitor therapy, and correlate with abrogation of metastases in orthotopic xenograft models of pancreatic cancer [5;6]. Here we have demonstrated that transient *ex vivo* treatment with GSI-18 depletes the ALDH "bright" population in pancreatic cancer cell lines, and this is paralleled by a significant reduction in anchorage independent growth and xenograft engraftment in athymic mice. Due to limited drug availability, we were unable to perform systemic trials in orthotopic xenograft models, but our results lay the groundwork for such analyses in the future. The observation that the ALDH "bright" cells are both Hedgehog and Notch dependent for their viability suggests that combinatorial therapy with small molecule inhibitors against both pathways might have even more potent effects *in vivo* than single agent treatment. Further, our findings reiterate our previously stated postulate that effective therapy of pancreatic cancer will likely require targeting both the "bulk" tumor cells with a conventional anti-metabolite like gemcitabine, as well as a stem cell directed therapy like Notch or Hedgehog inhibitor to eliminate the cells responsible for metastases and disease recurrence.

In conclusion, we demonstrate that ligand-dependent activation of the Notch signaling pathway is common in pancreatic cancer. Pharmacological inhibition of Notch signaling is a valid therapeutic strategy in this malignancy, based on the requirement of sustained Notch activation for tumor initiation as well as for tumor maintenance of pancreatic cancer.

Acknowledgements
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8

The Axl receptor tyrosine kinase confers an adverse prognostic influence in pancreatic cancer and represents a new therapeutic target

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Abstract

Background: Pancreatic cancer is a near uniformly lethal disease and a better understanding of the molecular basis of this malignancy may lead to improved therapeutics. The Axl receptor tyrosine kinase is implicated in cellular transformation and tumor progression, although its role in pancreatic cancer has not been previously documented.

Materials: The immunohistochemical expression of Axl protein was assessed in a panel of 99 archival pancreatic cancers. Endogenous Axl expression was stably downregulated by lentiviral short hairpin shRNA directed against *AXL* mRNA in MIAPaCa-2 cells, and the effects on cell viability, anchorage independent growth, invasion, migration and intracellular effector pathways was assessed, by comparing to lentiviral vector-transfected cells.

Results: Axl labeling was present in 54 of 99 (55%), and was absent in 45 of 99 (45%) cases, respectively. Axl expression in pancreatic cancer was significantly associated with lymph node metastases (P <0.01), and a shorter median survival (12 *versus* 18 months, P <0.01), than in tumors with negative labeling. Stable knockdown of Axl resulted in significant reduction in cell viability (P <0.001), anchorage independent growth (P =0.0031), as well as attenuation of migratory (P <0.001) and invasive properties (P <0.005), compared to vector-transfected cells. Profound inhibition of p42/p44 MAP kinase and PI-3kinase/Akt effector pathways was observed in MIAPaCa-2 cells with loss of Axl function. The reduction in invasion and migration upon Axl knockdown was mirrored by a decrease in the amounts of activated (GTP-bound) GTPase proteins Rho and Rac, significant downregulation in transcript levels of the epithelial-mesenchymal-transition (EMT)-associated transcription factors *snail, slug* and *twist*, and significant decrease in matrix metalloproteinase *MMP-9* mRNA levels.

Conclusion: Expression of Axl tyrosine kinase in pancreatic cancers confers an adverse prognostic influence, and represents a new therapeutic target in this malignancy.

Introduction

Ductal adenocarcinoma of the pancreas (a.k.a. pancreatic cancer) is a near uniformly lethal malignancy that accounts for approximately 34.000 deaths in the United States every year [1]. Worldwide pancreatic cancer causes an estimated 213.000 deaths each year and is the fourth most common cause of cancer-related mortality. For all stages combined, the 1-year survival is around 20%, and the overall 5-year survival rate is less than 5%, despite even the most aggressive therapies currently available [2]. The overwhelming majority of patients present with locally advanced or distant metastatic disease, rendering their disease surgically unresectable. Currently available conventional chemo-radiation therapies are minimally effective in prolonging the median survival of patients with advanced pancreatic cancer [3]. Thus, there is an urgent need for developing more potent therapies, particularly those targeted at aberrantly expressed molecules or pathways in this malignancy [4].

Receptor tyrosine kinases (RTKs) play an important role in signal transduction in both normal and malignant cells [5;6]. There are different families of RTKs which are mainly characterized by differences in the ligand-binding extracellular domains. The mammalian TAM RTK family includes three closely related members: *T*yro-3, *A*xl, *M*er [7]. The extracellular domain of these three RTKs is comprised of two immunoglobulin-like domains followed by two fibronectin type 3-like domains. Axl, also known as Ark, UFO or Tyro7, was originally isolated as a transforming gene from human leukemia cells [8;9]. Since then, aberrant expression of Axl has been reported in a variety of solid cancers, including gliomas, melanomas, lung and breast cancers, amongst others [10-16]. In a subset of these studies, overexpression of Axl in the cancer cells was associated with adverse clinical prognosis, such as advanced stage of disease at presentation and shorter median survival compared to corresponding non-expressing cancers [11;14;17]. Not surprisingly, in addition to promoting transformation and survival, the Axl RTK has been shown to play an important role in mediating cellular migration and invasion, properties that are critical to tumor progression and metastases [10;14;18].

We undertook the current study in light of the surprising paucity of data on the putative role of Axl in pancreatic cancer, particularly given the pervasive importance of metastatic disease as a determinant of survival in this malignancy. Our studies demonstrate that the Axl protein is commonly overexpressed in pancreatic cancer tissues, and that this aberrant expression is associated with a higher propensity for lymphatic metastases and significantly shorter median survival compared to Axl-negative cases within the same cohort. Further, our studies identify an unequivocal role for Axl in enhancing pancreatic cancer growth, migration, and invasion, and provide evidence that this receptor tyrosine kinase is a new therapeutic target in this malignancy.

Material and methods

Immunohistochemistry for Axl protein expression

Immunohistochemistry for Axl was performed on formalin fixed paraffin embedded specimens of 99 surgically resected pancreatic adenocarcinomas, with the samples embedded in a tissue microarray (TMA) format, as previously described [19-21]. Each case was represented by two 1.4mm cores of neoplastic tissue, and two of the corresponding non-neoplastic pancreatic parenchyma. Four-micron sections were cut from the TMAs, and deparaffinized by routine techniques. Thereafter, the sections were quenched with 3% H_2O_2 for 10 minutes. The slides were steamed in 10mM citrate buffer (ph 6.0) to unmask the

epitopes for 20 minutes at 95°C, and then allowed to cool down for 20 minutes to room temperature. Prior to incubating with the primary antibody, the slides were blocked for 30 minutes with a 10% fetal bovine serum solution (Invitrogen, Carlsbad, CA). As primary antibody, we used a 1:100 dilution of hAxl (catalog # AF154, R&D systems, Minneapolis, MN) for 2 hours at room temperature. Labeling was detected with the Dako Envision system (Dako, Envision Plus Detection Kit, Carpinteria, CA) as per the manufacturer's protocol. Sections were counterstained with Harris hematoxylin, a cover slip was applied. Immunohistochemical labeling was evaluated by two of the authors (J-B.M.K. and A.M.) at a multi-headed microscope with consensus reached in all cases. A simple two-tier classification of 'positive' and 'negative' was used, as we have previously described for TMA-based evaluation of immunohistochemical labeling [22-24].

Cell lines and culture conditions

Five pancreatic cancer cell lines (PANC-1, CF-PAC, MIAPaCa-2, PK-9, SU.86.86) were grown in either RPMI 1640 medium (Invitrogen) or in DMEM medium, supplemented with both 10% fetal bovine serum (Invitrogen) and 1% penicillin/ streptomycin (Biofluids, Camarillo, CA). The sources of the pancreatic cancer cell lines used in this study have been described elsewhere [22]. Human pancreatic epithelial cells (HPNE), immortalized by human telomerase reverse transcriptase (hTERT), were used as a control for non-neoplastic pancreas. The derivation and culture conditions for HPNE cells have been previously described [23].

Lentiviral shRNA knockdown of AXL in MIAPaCa-2 cells

Axl-expressing MIAPaCa-2 cells were seeded into 6-well plates at $5x10^5$ cells per well, and infected with either empty pLKO.1 lentiviral vector or with lentivirus expressing *AXL* shRNA (Open Biosystems, Huntsville, AL). Stably infected cells were selected by adding 3 µg/ml of puromycin to the cell culture media. Quantitative reverse transcription PCR (qRT-PCR) and Western blot analysis to confirm *AXL* mRNA and Axl protein knockdown, respectively, was performed as described below.

Western Blot analysis for Axl and downstream effector pathways

Western Blot analysis was performed for detecting Axl expression in the five cancer cell lines, and compared to baseline levels in HPNE cells. Briefly, protein lysates were made from cell pellets, using the following lysis buffer [20 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1% NP40, 0.5% sodium deoxycholate, 1 mmol/L EDTA, 0.1% SDS]; protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and phenylmethylsulfonyl fluoride (PMSF) were added before lysing the cells. Cells were lysed on ice for 30 min with occasional gentle agitation of the tubes. Cell debris was separated by centrifugation at 14.000 rpm for 30 min at 4°C. Protein lysates were resolved by electrophoresis on 12% Tris-glycine gel (Invitrogen) and transferred on to nitrocellulose membranes (LC 2000, Invitrogen). Standard immunoblotting procedures were followed with slight modification: nitrocellulose membranes were blocked for one hour at room temperature and incubated with primary anti-hAxl antibody (dilution 1:1000, catalog #4939S, Cell Signaling, Danvers, MA,) overnight at 4°C. Anti-actin antibody (catalog #sc-1615, Santa Cruz Biotechnology, Santa Cruz, CA) was used as a loading control. The identical protocol was used to confirm Axl protein knockdown in MIAPaCa-2 cells stably infected with lentiviral shRNA. Downstream effector pathways (p42/p44 MAP kinase and PI-3 kinase/Akt) were evaluated in empty vector infected *versus*

AXL shRNA expressing cells, using the following primary antibodies and conditions (all antibodies were purchased from Cell Signaling and used at a 1:100 dilution with incubation overnight at 4^0C): total Akt (catalog #9272), phospho-Akt Ser473 (catalog #9271), phospho-ERK (catalog #9102), and phospho-PDK1 Ser241 (catalog #3061).

RNA extraction and quantitative reverse transcription PCR (qRT-PCR)

MIAPaca-2 cells with empty or *AXL* shRNA-expressing vectors were lysed and RNA was extracted using RNeasy Mini kit (Qiagen, Valencia, CA). RNA was reverse transcribed with oligo-d$(T)_{12-18}$ primers at 42°C for 50 minutes using the SuperScript™ First Strand System (Invitrogen), according to manufacturer's protocol. Primer sequences were designed using Primer3 online primer design software product, and are available upon request. qRT-PCR for AXL transcripts and candidate effector targets of Axl (*Snail, Slug, Twist*, and matrix metalloproteinase *MMP-9*) was performed using the Quantitect™ SYBRGreen PCR kit on a 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA). Relative fold levels were determined using the $2^{-\Delta\Delta Ct}$ method, with *SDHA* used as housekeeping control [25].

Rho/Rac Immunoprecipitation assay

Rho and Rac activation was determined using the Rho/Rac Activation Assay Combo Kit (Cell Biolabs, Inc., San Diego, CA). Briefly, vector-transfected and shRNA-expressing MIAPaCa-2 clones were cultured in T-75 flask in RPMI with 10% FBS. When cells reached 80% confluent, cells were serum starved in RPMI with 0.5% FBS overnight, followed by stimulation with epidermal growth factor (EGF 10ng/mL) for 30 minutes. Cells were washed in cold PBS twice, collected with a cell scraper and lysed on ice using the lysis buffer enclosed in the kit. After pre-cleaning of lysates with glutathione agarose, and shearing of DNA by passing 3 times through a 27-gauge needle, protein concentrations were determined by means of Bradford assays and equal amounts of total protein used for the pull-down step. Beads were loaded on a SDS polyacrylamide gel in laemmli buffer containing 5% beta-mercapto-ethanol (Sigma-Aldrich) and membranes developed with anti-Rho or anti-Rac antibodies at a dilution of 1:1000, as recommended.

In vitro cell viability assays

Cell growth assays using the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide reagent (CellTiter96™, Promega, Madison, WI) were performed on vector-transfected and shRNA-expressing MIAPaCa-2 cells, as described previously [26]. Upon completion of the assay, 20µl/well of the CellTiter96™ solution was added for 1 hour and plates were read on a Wallac-1420 Plate reader at OD=490 nm (PerkinElmer, Boston, MA). All experiments were set up in triplicate to determine means and standard deviations.

Anchorage-independent growth in soft agar

Anchorage-independent growth was assessed by colony formation assays in soft agar, as previously described [26]. Briefly, the soft agar assays were set up in 6-well plates, each well containing a bottom layer of 1% agarose (Invitrogen), a middle layer of 0.6% agarose including $10x10^4$ cells, and a top layer of medium only. Subsequently, the plates were kept in a tissue culture incubator maintained at 37°C and 5% CO_2 for 21 days to allow for colony growth, with top medium being changed on a weekly basis.

The assay was terminated at day 21, when plates were stained with 0.5 ml of 0.005% crystal violet (Sigma-Aldrich) solution at 37°C for 2 hours, and colonies were counted in three independent wells for each condition, using an automated ChemiDoc XRS instrument (Bio-Rad, Hercules, CA).

Modified Boyden chamber invasion and migration assays

Modified Boyden chamber assays to assess the *in vitro* invasion and migration of MIAPaCa-2 cells, with or without endogenous Axl expression, were performed as previously described [26;27]. In Brief, 20 µg/well of Matrigel (BD Biosciences, San Jose, CA) was applied to 24-well trans-well plates with 8-µm pore size (BD Biosciences) and allowed to solidify overnight. Then, culture medium was added and $5x10^4$ cells were seeded into each well. The plates were incubated at 37°C for 72 hours, and thereafter, cells on top of the membrane were removed using a cotton swab, and cells at the bottom were fixed in ethanol and stained with Harris hematoxylin solution. Cells in ten randomly selected microscopic fields were counted in four independent wells for each condition, and means and standard deviations calculated. Transmigrated cells were normalized for viable cell counts in each case, as previously described [26;28]. For assessment of migration, the Boyden chamber assay was performed without addition of the Matrigel plug.

Fluorescence microscopy

The following antibodies were used for immunofluorescence studies: β-tubulin (catalog # 2116, AlexaFluor 555 conjugate, Cell Signaling) and actin (catalog# MAB1501x, Alexa fluor 488 conjugate, Millipore, Temecula, CA.). Briefly, $1.5x10^4$ cells of both MIAPaca-2 vector-transfected and Axl shRNA-expressing clones were each cultured in RPMI with 10% FBS in tissue culture-slides (BD Falcon, Bedford, MA) overnight. Cells were briefly washed in PBS, and were fixed in 2ml of 4% paraformaldehyde in PBS for 15 minutes at ambient temperature. Cells were then washed 3 times for 5 minutes each, and covered with ice-cold 100% methanol and incubated at -20°C for 10 minutes. Following that, cells were rinsed in PBS for 5 minutes. Cells were then blocked in 5% normal rabbit serum in PBS with 0.3% Triton X-100 for 1hr, and incubated in a cocktail of β-tubulin and actin antibodies at a dilution to 1:200 in PBS/0.3% Triton X-100 overnight at 4°C. Cells were then rinsed 3 times for 5 minutes each with PBS. Slides were covered using coverslip with Prolong® Gold Antifade reagent with DAPI (Invitrogen). The edges of the slides were sealed with nail polish. Slides were examined and pictures taken using the appropriate filters.

Statistical analysis

Statistical analyses were performed using SPSS version 11 (SPSS Inc., Chicago, IL). Associations between categorical variables were examined using the Pearson's chi-square tests, and using the two-tailed t-tests for continuous variables, with a *P*-value <0.05 being considered statistically significant. Kaplan-Meier survival analysis was performed in GraphPad Prism for Windows version 4.0 (GraphPad Software Inc., San Diego, CA).

Results

Axl expression in pancreatic adenocarcinoma correlates with an adverse prognosis

Immunohistochemical analysis for Axl expression was performed on a panel of 99 pancreatic cancers using an anti-Axl-specific antibody. The clinicopathologic features of the 99 patients stratified by their Axl expression are summarized in table 1. Axl labeling was observed in 54 of 99 (55%) pancreatic cancers (fig. 1B), and was absent in 45 of 99 (45%) cases (fig. 1A), respectively. In the neoplastic cells, Axl protein was distributed in the cytoplasm with a pronounced membranous accentuation, as would be expected of a RTK (fig. 1C). Normal pancreatic ductal epithelium, by contrast, had minimal to no expression of Axl protein (fig. 1B, arrow). In Axl-negative cancers, expression of the protein was restricted to the tumor-associated vasculature (fig. 1A), a not unexpected finding given the previously reported association between Axl and endothelial function in both normal and tumor tissues [18;28]. In terms of clinicopathological correlation, Axl protein expression in pancreatic cancers was significantly associated with lymph node metastases (P <0.01). Further, Kaplan-Meier survival analysis demonstrated that patients harboring Axl-positive tumors had a significantly shorter median survival than patients with Axl-negative cancers (12 months *versus* 18 months, respectively; P <0.01), underscoring the adverse prognostic influence conferred by Axl expression (fig. 1D).

Table 1: Clinicopathological correlates of Axl expression in archival pancreatic cancers

Clinicopathologic Features		AXL expression		
	Total	**Negative (n=45)**	**Positive (n=54)**	***P-Value***
Gender				
Male	43	19	24	
Female	56	26	30	0.84
Tumorsize (cm)				
≤ 3,0	64	28	36	
> 3,0	35	17	18	0.65
Lymph Node Status				
< 15% positive	52	31	21	
> 15% positive	47	14	33	< 0.01*
Margins				
Positive	65	31	34	
Negative	34	14	20	0.54
Differentiation				
Well or Moderated	53	28	25	
Poorly	46	17	29	0.12
Staging				
Localized	16	5	11	
Advanced	83	40	43	0.22

* Statistically Significant

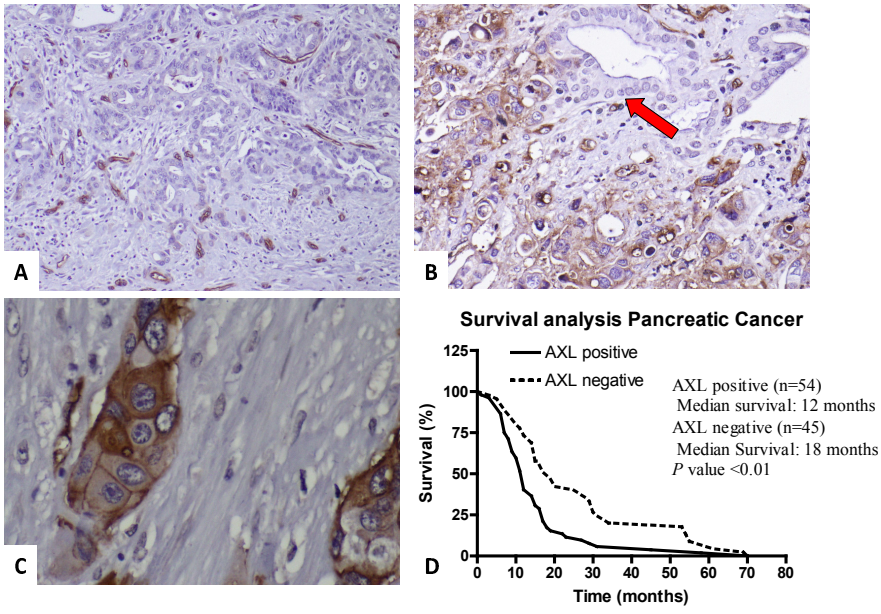

Figure 1: **The Axl receptor tyrosine kinase is overexpressed in pancreatic cancer**
A) A pancreatic cancer with absence of Axl labeling. Only the intra-tumoral blood vessels are positive for Axl expression. **B**) A pancreatic cancer with diffuse overexpression of Axl in the neoplastic cells. Note absence of labelling in adjacent normal ductal epithelium (arrow). **C**) Higher magnification of a pancreatic cancer with Axl expression. The infiltrating adenocarcinoma shows cytoplasmic staining with membranous accentuation for Axl, consistent with the pattern of expression for a receptor tyrosine kinase. **D**) Axl expression is associated with adverse prognosis in pancreatic cancer. Kaplan-Meier survival analysis confirms that patients with Axl-expressing tumors demonstrate a significantly shorter median survival than those with Axl-negative cancers (12 months *versus* 18 months, P <0.01).

Axl protein is overexpressed in pancreatic cancer cell lines compared to HPNE cells

Axl expression was assessed in a panel of five pancreatic cancer cell lines (PANC-1, CF-PAC, MIAPaCa-2, PK-9, and SU.86.86), and in the hTERT-immortalized HPNE cells. Minimal Axl expression was observed in HPNE, while three cancer cell lines (MIAPaCa-2, CF-PAC and PANC-1) had variable Axl overexpression, when compared to the 'baseline' level in HPNE cells (fig. 2A). The MIAPaCa-2 cells were selected for subsequent functional studies comparing Axl-expressing cells to those with knockdown of endogenous protein.

Knockdown of endogenous Axl inhibits cell viability, anchorage independent growth, invasion and migration of MIAPaCa-2 cancer cells

Parental MIAPaCa-2 cells were stably infected with either empty lentiviral vector or virus expressing *AXL* shRNA. Both Western blot analysis (fig. 2B) and qRT-PCR (fig. 2C) confirmed significant knockdown of the endogenous protein in *AXL* shRNA-expressing cells compared to the empty vector infected MIAPaCa-2 cells. Endogenous AXL knockdown led to significant reduction in viability of MIAPaCa-2 cells, compared to vector-transfected cell line, as assessed by *in vitro* MTS assay (fig. 3A) (P <0.001).

Moreover, Axl knockdown inhibited the phenotype of anchorage-independent growth, with a significant reduction in colony formation in soft agar (fig. 3B and fig. 3C) (P =0.0031). Multiple studies have reported that Axl plays an important role in promoting the migration of cancer cells, facilitating tumor progression [10;14;18]. Therefore, we utilized modified Boyden chamber assays to assess the effects of Axl knockdown on *in vitro* invasion and migration, and found a significant reduction in both phenomena compared to MIAPaCa-2 cells with retained Axl function (fig. 4A and fig. 4B, P <0.0005 and P <0.0001, respectively). We also examined the morphology of MIAPaCa-2 cells following Axl knockdown, and these cells demonstrated a striking loss of polarity and absence of filopodia, compared to cells with retained Axl function, which displayed an organized polarity and well-formed filopodia formation (fig. 5).

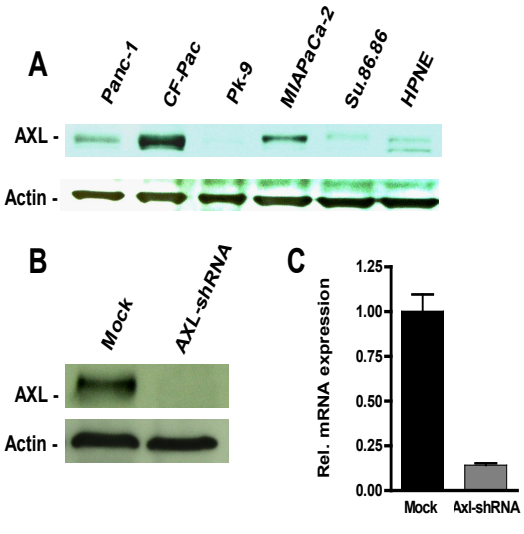

Figure 2: **Axl expression in pancreatic cancer cell lines and stable knockdown of Axl in MIAPaCa-2 cells**

A) Axl protein is overexpressed in three of five pancreatic cancer cell lines (PANC-1, CF-PAC, and MIAPaCa-2), compared to the hTERT immortalized human pancreatic epithelial line HPNE.

B) Endogenous Axl was down-regulated in the Axl-expressing MIAPaCa-2 cells using a lentiviral shRNA vector. Compared to vector-transfected ('mock') clones, the shRNA-expressing cells demonstrate essentially complete loss of Axl protein.

C) Quantitative real-time PCR (qRT-PCR) confirms the downregulation of *AXL* transcripts in shRNA expressing MIAPaCa-2 clones. The assays were performed in triplicate and *SDHA* was used as housekeeping control.

Knockdown of endogenous Axl is associated with inhibition of multiple effector pathways in pancreatic cancer

We then explored the status of multiple effector pathways that are implicated in pancreatic cancer growth and progression. Two major intracellular effectors downstream of RTKs in both normal and cancer cells are the p42/p44 MAP kinase and the PI-3-kinase/Akt signaling pathways [29]. In MIAPaCa-2 shRNA expressing cells, we found inhibition of both effector arms, as evidenced by reduced phosphorylation of ERK1/ERK2, PDK1^{ser241}, and Akt^{ser473}, compared to vector transfected cells (fig. 6A). In contrast, total Akt levels were unaffected. In light of the profound inhibition of migration and invasion of MIAPaCa-2 cells with loss of endogenous Axl function, we looked at the activation status of the Rho family of GTPases, which control a number of cytoskeletal dynamics including cell migration [30;31], using immunoprecipitation for the active (GTP-bound) complement of Rho and Rac. In contrast to vector-transfected cells, loss of Axl function was associated with marked reduction in the levels of intracellular activated Rho/Rac GTPases (fig. 6B). Finally, we examined the level of expression of the epithelial-mesenchymal-transition (EMT)-associated transcriptional repressors, *snail, slug*, and *twist* [32], and mRNA levels of all three genes were significantly downregulated upon Axl knockdown (P =0.0004, P =0.005 and P = 0.0254), for differential expression of *snail, slug* and *twist,* respectively (fig. 6C). There was also significant downregulation in mRNA levels of the matrix metalloproteinase *MMP-9* in MIAPaCa-2 shRNA-expressing cells compared to vector transfected controls.

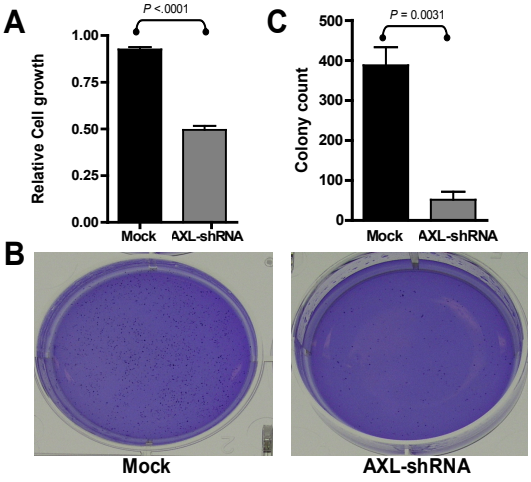

Figure 3: Knockdown of endogenous Axl in MIAPaCa-2 inhibits *in vitro* cell viability and anchorage independent growth

A) *In vitro* cell viability of Axl shRNA-expressing MIAPaCa-2 cells was significantly reduced compared to vector-transfected cells (P <0.0001), as measured using MTS assay. The MTS assays were performed in triplicate, and mean and standard deviations are plotted. **B**) Anchorage independent growth of Axl shRNA-expressing MIAPaCa-2 cells, as assessed by colony formation in soft agar, was significantly reduced compared to vector-transfected cells (P =0.0031). Colony assays were performed in triplicate, and the mean and standard deviations of colony counts were calculated for each condition. **C**) Representative soft agar assay of Axl shRNA-expressing MIAPaCa-2 compared to vector-transfected cells.

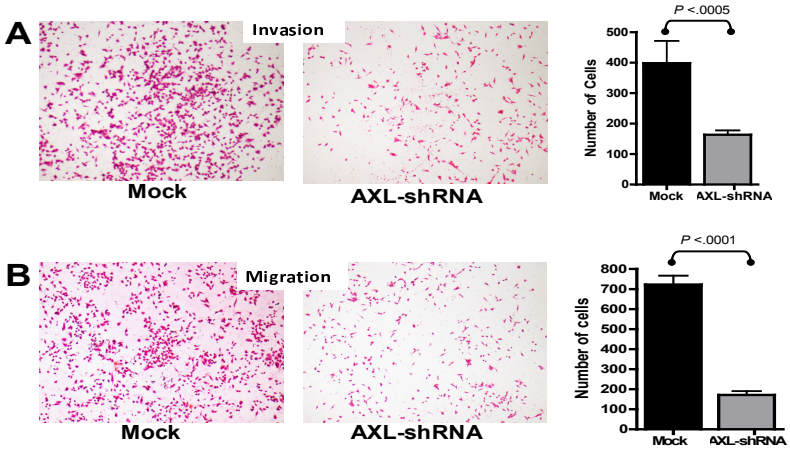

Figure 4: Knockdown of endogenous Axl in MIAPaCa-2 cells inhibits *in vitro* invasion and migration
A) Modified Boyden chamber assay (with Matrigel plug) was performed to assess *in vitro* invasion in MIAPaCa-2 cells. At 72 hours, loss of endogenous Axl function was associated with significant reduction in invasive capacity compared to vector-transfected cells (P <0.0005), when normalized for cell viability. The histogram represents mean and standard deviation of invasion assay performed in triplicate. **B**) Modified Boyden chamber assay (without Matrigel plug) was performed to assess *in vitro* migration in MIAPaCa-2 cells. At 72 hours, loss of endogenous Axl function was associated with significant reduction in migratory capacity compared to vector-transfected cells (P <0.0001), when normalized for cell viability. The histogram represents mean and standard deviation of migration assay performed in triplicate.

Discussion

The Axl RTK belongs to the TAM family of protein kinases, which is comprised of Tyro-3, Axl, and Mer proteins, and which has been implicated in a diverse array of cellular functions such as cell adhesion, migration, proliferation and survival, and regulation of inflammation, amongst others [7]. Following its isolation as a transforming gene in hematological malignancies [8;9], an increasing array of human cancers have been identified with aberrant Axl experession [10-16]. The ligand for the Axl receptor is the vitamin K-dependent protein growth arrest specific-6 (Gas6) [32;33], and this molecule is also overexpressed concurrently with Axl in numerous cancers, thereby sustaining an aberrant Gas6-Axl axis [11;34-38]. To the best of our knowledge, this is the first study to document aberrant expression of Axl in pancreatic adenocarcinomas. By immunohistochemical analysis, 55% of surgically resected pancreatic cancers demonstrated Axl overexpression, and this was correlated with significantly higher prevalence of lymph node metastases, as well as a significantly shorter median survival compared to Axl-negative tumors. The association between Axl overexpression in cancers and an adverse prognosis is not unprecedented, as similar findings have been reported in lung cancer and gliomas, amongst others [11;14]. Since the cohort of cases included for immunohistochemistry was, by definition, restricted to surgically resectable pancreatic cancers without evidence of concurrent metastatic disease, we are unable to document whether Axl upregulation also correlates with distant metastases.

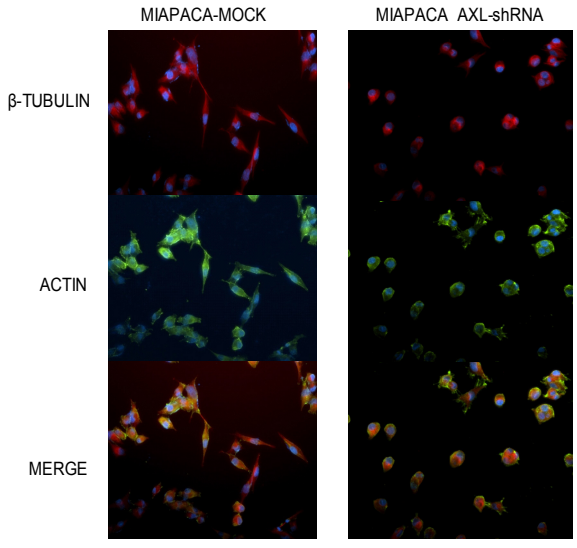

Figure 5: Knockdown of endogenous Axl is associated with reduction in filopdial extensions and loss of polarity in MIAPaCa-2 cells

Immunofluorescence studies demonstrate that vector-transfected MIAPaCa-2 cells have a spindled morphology, with well formed filopdial extensions as seen by β-tubulin / actin compound immunostaining. In contrast, loss of Axl is associated with loss of polarity and cell rounding, and reduction in the filopodial extensions. DAPI is used as a nuclear counterstain

Nevertheless, the higher prevalence of nodal positivity and shortened median survival of patients with Axl-expressing pancreatic cancers suggests that aberrant expression of this protein likely facilitates disease dissemination *in vivo*. This is reaffirmed by the *in vitro* phenotype observed in MIAPaCa-2 pancreatic cancer cells with genetic knockdown of endogenous Axl protein, which results in profound loss of invasive and migratory capabilities, accompanied by a near-complete loss of filopodial extensions. The decrease in invasive/migratory capacity occurs independent of the deleterious effects of Axl knockdown on cell viability, and the substance of these findings is essentially identical to those reported in glioma cell lines with blockade of endogenous Axl function using a dominant negative mutant protein [10]. Conversely, ectopic expression of Axl in lung adenocarcinoma cell lines results in increased filopodia formation and enhanced migratory ability [39]. Not unexpectedly, Axl has a well documented role in *physiological* migration of neuronal and vascular smooth muscle cells [40;41], underscoring the association between *pathological* Axl expression and dissemination of cancer cells from their primary site.

In addition to the observed deleterious phenotype on cell viability, anchorage independent growth, migration and invasion of MIAPaCa-2 cells upon Axl knockdown, we were also able to demonstrate blockade of multiple intracellular effectors in the Axl shRNA expressing cells. Axl promotes the growth and survival of neoplastic as well as non-neoplastic cell types through activation of the p42/44 MAP kinase and the PI-3-kinase/Akt signaling pathways [7;37;42-44], and both effector arms are inhibited in MIAPaCa-2 cells upon Axl knockdown, as evidenced by the decreased phosphorylation of Erk1/2, and PDK1^{ser241} and Akt^{ser473}, respectively. The p42/44 MAP kinase and the PI-3-kinase/Akt pathways are constitutively activated in the majority of pancreatic cancers as a result of upstream somatic *KRAS2* gene mutations [45], and the blockade of these pathways by downregulation of Axl in MIAPaCa-2 cells suggests a role for this RTK in ras-dependent signaling. In light of the inhibitory effects of Axl knockdown on invasion/migration, we also examined the

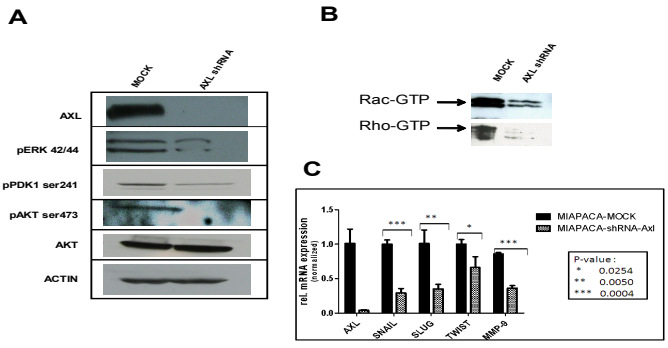

Figure 6: Multiple intracellular effector pathways are disrupted upon inhibition of endogenous Axl function in MIAPaCa-2 cells

A) Lentiviral shRNA-mediated stable knockdown of Axl in MIAPaCa-2 cells blocks the activation of critical intracellular effector pathways known to be activated in pancreatic cancer, including the MAP kinase and PI-3-kinase/Akt signaling pathways. Western blot assay was performed for phospho-ERK1/2, phospho-Akt^{ser473}, and phospho-PDK1^{ser241}, all of which demonstrated reduction in levels of specific phosphorylated protein, compared to vector-transfected cells. In contrast, no changes were seen in the levels of total Akt protein. Actin was used as loading control. **B**) Knockdown of endogenous Axl is associated with reduction in GTP-bound (active) Rho and Rac in MIAPaCa-2 cells compared to vector-transfected cells. The immunoprecipitation assay specifically 'pulls down' GTP-bound forms of both proteins. **C**) qRT-PCR demonstrates that knockdown of endogenous Axl results in reduction in transcripts for *snail* (P= 0.0004), *slug* (P= 0.005), and *twist* (P= 0.0254), whose protein products are transcription factors implicated in epithelial-mesenchymal-transition (EMT), compared to vector-transfected cells. Loss of Axl function was also associated with reduction in transcripts for the matrix metalloproteinase *MMP-9* (P= 0.0004). All qRT-PCR assays were performed in triplicate, and *GUSB* was used as housekeeping control. The Y-axis represents relative fold level in Axl shRNA-expressing cells normalized to vector-transfected cells, and mean and standard deviations are plotted.

status of the Rho family of GTPases in *AXL* shRNA-expressing cells. The Rho GTPases are multi-functional proteins regulating a plethora of cellular activities like endocytosis, vesicle trafficking and gene transcription, but one of their most prominent effects is on cell migration through modulation of the actin cytoskeleton. The active state of Rho proteins is characterized by bound GTP, rather than GDP, which forms the basis for the immunoprecipitation assay described here. We demonstrate a marked reduction in GTP-bound Rho and Rac proteins, consistent with inactivation of these signaling molecules upon Axl knockdown. Previous studies in neuronal cells and NIH3T3 cells with ectopic Axl expression have reported a PI-3-kinase-dependent activation of the Rho GTPases by Axl [40;43], and our results suggest that such as axis is maintained in pancreatic cancer as well. We also demonstrate downregulation of numerous transcripts associated with the phenomenon of cancer cell invasion, including reduction in levels of *snail, slug* and *twist* mRNA, whose products regulate the process of 'epithelial-mesenchymal-transition' or EMT [46]. Acquisition of EMT is required for vascular intravasation and metastatic seeding by tumor cells, and the snail/ slug/ twist family of transcriptional repressors plays an essential role in this phenotypic switch. To the best of our knowledge, this is the first description of an association between transcription factors facilitating EMT and Axl, and provides further mechanistic bases for the observed blockade in invasion/ migration upon knockdown of this RTK in MIAPaCa-2 cells. Finally, we show

reduction in transcripts for the matrix metalloproteinase *MMP-9* in *AXL* shRNA-expressing MIAPaCa-2 cells. MMP-9 (also known as gelatinase-B) is a type IV collagenase involved in basement membrane proteolysis and in tumor angiogenesis, and its expression within the tumor micro-environment promotes invasion and metastases [47;48]. MMP-9 is reported as overexpressed in pancreatic cancer across multiple prior studies [49-51]. Further, a recent study demonstrates that ectopic Axl in cancer cells can upregulate MMP-9, and render the cells more invasive [52]. Our results are in concert with these prior findings, and underscore the importance of sustained Axl expression towards promoting multiple facets of the invasion and metastatic cascade in pancreatic cancer.

Our study does not address the molecular basis for Axl overexpression in pancreatic cancer, although some conclusions can be drawn from existing literature. One common mechanism of RTK activation, with or without associated protein overexpression, is via somatic mutations, and this is best exemplified by the EGFR family of oncogenic receptors in solid tumors [53;54]. Although rare somatic mutations of the *AXL* gene have been reported in lung, ovarian, and gastric cancers (see the Catalog of Somatic Mutations in Cancer, or COSMIC database at http://www.sanger.ac.uk/genetics/CGP/cosmic) [55], the recently completed pancreatic cancer genome sequencing project has failed to detect somatic mutations of this gene in a panel of 24 pancreatic cancers, upon analysis of all coding exons [56]. Thus, mutational events are likely to be a rare to absent basis for Axl activation in pancreatic cancer. Another common mechanism for oncogene overexpression is amplification of the corresponding genomic region in cancer cells [57]. The *AXL* gene is located at chromosome 19q13.1, a site of frequent amplification in solid tumors [58], including in pancreatic cancer [59;60]. While Axl upregulation in a subset of cancers may be the result of copy number alteration, the frequency of 19q13.1 genomic amplification in pancreatic cancer is significantly lower than the prevalence of Axl overexpression in this malignancy (55%), as observed in the current study. This is a trend observed with other recurrent amplicons in pancreatic cancer such as *MYC* and *GATA-6* as well, wherein the frequency of protein overexpression typically exceeds that of genomic copy number alterations [61-63]. Thus, *prima facie* evidence points to transcriptional deregulation of *AXL* as the most frequent cause for overexpression of its protein product in pancreatic cancer, although the proximate transcriptional factor(s) driving this phenomenon remain to be elucidated. Most recently, Mudduluru *and colleagues* have elucidated the promoter sequence of the *AXL* gene, and have identified two potential sources of regulation: first, the existence of multiple specificity protein (Sp)-binding sites to which can bind the Sp1 family of transcription factors, and second, the presence of CpG islands within these Sp-binding motifs that can be reversibly methylated and thereby epigenetically silence *AXL* transcription [64]. Multiple studies have documented overexpression of the Sp1 transcription factor in pancreatic cancer [65-67], and it is tempting to speculate that cancer-specific hypomethylation of the Sp-binding motifs permits unrestricted *AXL* transcription to occur in pancreatic cancers with Axl overexpression. We emphasize the speculative nature of this chain of events, and future studies to better elucidate the mechanism of Axl overexpression in pancreatic cancer are certainly warranted.

An encouraging aspect of our results is the possibility that Axl might become a relevant therapeutic target in pancreatic cancer, a disease where conventional chemo-radiation therapies have had minimal impact on ameliorating prognosis [1]. RTKs have proven to be facile targets for the development of small molecule kinase inhibitors and monoclonal antibodies. Of note, potent small molecule Axl antagonists have recently been identified

and are in active preclinical development [68]. In addition to inhibiting tumor growth, these antagonists have also been shown to block angiogenesis in the tumor microenvironment, which is not an unexpected finding given that the Axl RTK plays a role in the survival of normal endothelial cells [69], as well as that of tumor-associated endothelium [10;18]. In our own immunohistochemical analysis (see figure 1), robust Axl expression was seen within the endothelial cells within the tumor milieu, even in pancreatic cancers where the neoplastic cells *per se* did not express Axl. The *in vivo* application of these, or related, small molecule antagonists in preclinical models of pancreatic cancer will undoubtedly provide additional insights as to the validity of Axl as a therapeutic target in this malignancy.

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9

Widespread activation of the DNA Damage Response in human Pancreatic Intraepithelial Neoplasia

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Abstract

Background: Pancreatic intraepithelial neoplasia (PanIN) lesions are the most common non-invasive precursors of pancreatic adenocarcinoma. Despite the presence of clonal genetic alterations in even the lowest grade of PanINs, only a minor fraction of these lesions ever progress to invasive cancer. We postulated that accumulating DNA damage within the PanIN epithelium activates checkpoint mechanisms.

Experimental Design: Tissue microarrays (TMAs) were constructed from 81 surgically resected primary pancreatic adenocarcinomas, and an independent set of 58 PanIN lesions (31 PanIN-1, 14 PanIN-2, and 13 PanIN-3). Immunohistochemical labeling was performed using antibodies to phospho-γH2AX^{Ser139}, phosphoATM^{Ser1981}, phosphoChk2^{Thr68} and p53. A 'HistoScore' combining area and intensity of labeling in the nuclear compartment was determined for each lesion.

Results: A progressive increase in pH2AX^{Ser139} labeling, consistent with escalating DNA damage, was observed in the non-invasive precursor lesions (HistoScores of 4.34, 6.21, and 7.50, respectively for PanIN-1, -2, and -3), compared to non-neoplastic pancreatic ductal epithelium (HistoScore 2.36) (ANOVA, P <0.0001). In conjunction, activation of the ATM-Chk2 checkpoint pathway was observed in all histological grades of PanIN lesions. Specifically, pATM^{Ser1981} HistoScores for PanIN-1, PanIN-2, and PanIN-3 were 4.83, 5.14, and 7.17, respectively, *versus* 2.33 for non-neoplastic ductal epithelium (ANOVA, P <0.0001); the corresponding HistoScores for pChk2^{Thr68} were 5.43, 7.64, and 5.44 in PanINs-1, -2, and -3, respectively, *versus* 2.75 in non-neoplastic ductal epithelium (ANOVA, P <0.0001). In contrast, absent to minimal nuclear p53 was observed in ductal epithelium, and in PanINs-1 and 2 (HistoScore of 0-1.86), with a significant upregulation (corresponding to mutational inactivation) seen only at the grade of PanIN-3 and invasive carcinoma (HistoScore of 4.00 and 4.22). Nuclear p53 accumulation in invasive cancers was associated with attenuation of the ATM-Chk2 checkpoint, and a restitution to 'baseline' levels.

Conclusions: Activation of the ATM-Chk2 checkpoint pathway is commonly observed in PanINs, including the lowest grade lesions, likely in response to the accumulating DNA damage from events such as oncogene mutations and telomere dysfunction. Loss of p53 function appears to be a critical determinant for bypassing this checkpoint and the subsequent progression to invasive adenocarcinoma.

Introduction

Adenocarcinoma of the pancreas strikes approximately 38.000 individuals each year in the United States, and nearly all die within months of diagnosis [1]. A multistep model has recently been proposed for the development of pancreatic adenocarcinoma, in which non-invasive precursor lesions in the pancreatic ducts undergo histologic and genetic progression towards invasive cancer [2;3]. These morphologically distinct non-invasive lesions have been classified under a uniform nomenclature scheme termed *Pancreatic Intraepithelial Neoplasia* or PanIN [2]. We and others have demonstrated that PanINs share many of the genetic aberrations characteristic of invasive adenocarcinomas, underscoring their classification as neoplastic precursors to invasive carcinoma rather than a reactive/ hyperplastic process [4-10]. Some of the genetic alterations are nearly ubiquitous (e.g., oncogenic KRAS2 mutations and telomere dysfunction) [4;5], suggesting that these are early events in the ductal epithelium, while others, such as loss-of-function of the tumor-suppressor gene *SMAD4* or upregulation of the GPI-anchored protein mesothelin, occur only in the most advanced PanIN lesions that precede invasive cancer [4;10;11].

Autopsy studies have confirmed that PanIN lesions are surprisingly common in the general population, with more than 50% of the general population over 60 years harboring one or more low-grade lesions in their pancreata [12]. Nevertheless, despite this remarkably high prevalence, and the presence of clonal genetic alterations [13], the overwhelming majority of histologically low-grade PanINs do not progress to invasive adenocarcinoma, as obviously gauged by annual incidence rates for this disease. One can speculate, therefore, that these low-grade PanINs either undergo apoptosis and are 'shed' from the body, or that intracellular checkpoint mechanisms come into effect, forestalling or entirely preventing their progression to higher grade PanIN lesions (carcinoma-*in-situ*) and invasive cancer. Recent seminal studies have identified the DNA damage repair protein ataxia telangiectasia mutated (ATM), and its downstream target, the human homolog of the bacterial checkpoint, Chk2, as a pervasive checkpoint in human epithelial pre-cancerous lesions [14-16]. A variety of inciting factors such as telomere dysfunction and oncogene-induced 'replication stress' can cause DNA damage in pre-cancerous lesions, activating the ATM-Chk2 checkpoint and rendering these cells senescent, thereby impeding their progression to invasive malignancy [17;18].

We hypothesized that activation of the DNA Damage Response (DDR) checkpoint in the most common non-invasive precursor lesions of pancreatic adenocarcinoma could provide a putative explanation for the disconnect between PanIN prevalence in the general population and the incidence of invasive adenocarcinoma. Herein, we confirm that activation of the ATM-Chk2 checkpoint is widespread in human PanIN lesions, including in the lowest grade (PanIN-1) lesions. This phenomenon appears to be a consequence of DNA replication stress and the occurrence of double-strand breaks (DSBs), as measured by the progressive accumulation of phospho-histone pH2AX, which forms a scaffold at double-strand breaks [19]. We also provide evidence that loss of p53 function is a critical threshold event in the multistep progression of pancreatic cancer, occurring mostly at or beyond the stage of PanIN-3, allowing the neoplastic epithelium to bypass DDR-induced checkpoints, and progress unimpeded into invasive adenocarcinoma.

Material and Methods

Tissue microarrays (TMAs) were prepared from archival formalin-fixed paraffin embedded sections of 81 surgically resected primary pancreatic adenocarcinomas, as previously described [20;21]; this 'cancer TMA' also included 73 cores of non-neoplastic pancreatic ductal epithelium. An independent set of 58 PanIN lesions (31 PanIN-1, 14 PanIN-2, and 13 PanIN-3) were also arrayed in a 'PanIN TMA,' as previously described [4;7]. For TMA construction, representative areas containing morphologically defined cancers or PanINs were circled on the glass slides and used as a template. The TMAs constructed using a manual Tissue Puncher/Arrayer (Beecher Instruments, Silver Spring, MD), and a 1.4mm core was punched from the donor block to ensure that adequate lesional tissue could be incorporated into the spot.

Immunohistochemistry was performed as previously described [7]. Briefly, unstained 5-µm sections were cut from the paraffin block selected and deparaffinized by routine techniques. Thereafter, the sections were quenched with 3% H_2O_2 for 10 minutes. The slides were steamed in 10mM citrate buffer (ph 6.0) to unmask the epitopes for 20 minutes at 95°C, and then allowed to cool down for 20 minutes to room temperature. Prior to incubating with the primary antibody, the slides were blocked for 30 minutes with a 10% fetal bovine serum solution (Invitrogen, Carlsbad, CA). The following primary antibodies were used for this study: anti-phospho γH2AX^{Ser139} (Upstate/Millipore, Millerica, MA, dilution 1:200), anti-phosphoATM^{Ser1981} (Rockland Immunochemicals, Boyertown, PA, dilution 1:100), anti-phosphoChk2^{Thr68} (Cell Signaling Technology, Beverly, MA, dilution 1:100) and anti-p53 (Santa Cruz Biotechnology, Santa Cruz, CA, dilution 1:200). The specific phospho-antibodies for γH2AX, ATM, and Chk2 were selected based on the published association of phosphorylation at these sites with functional status of the respective protein [14;15;18;22]. Labeling was detected with the PowerVision+ Poly-HRP IHC kit. (Immunovision Technologies, Norwell, MA) following the standard protocol. Slides were counterstained with Harris-hematoxyline solution. Negative controls (primary antibody replaced by serum from appropriate species) were used for each antibody in each run.

Immunohistochemical labeling was scored using a previously described HistoScore scheme [6;23;24], which takes into consideration both the area and intensity of labeling in the appropriate (nuclear) compartment. Specifically, intensity of labeling was designated as 0-3 for absent, weak, moderate and strong, and area of labeling was designated as 0-3 for <5%, 5-25%, 26-50%, and >50%, respectively. The lesional HistoScore was calculated by the product of area and intensity, and subsequently, the average HistoScores for the individual histological grades of PanIN lesions, adenocarcinomas, and normal ductal epithelium were determined.

Statistical analyses were performed using SPSS v17.0 (SPSS Inc., Chicago, IL). Differential expression of phospho-γH2AX^{Ser139}, phospho-ATM^{Ser1981}, phospho-Chk2^{Thr68} and p53 proteins in normal pancreatic ductal epithelium, various grades of PanINs, and invasive pancreatic ductal adenocarcinomas were compared by ANOVA and Duncan's multiple range tests. A P-value < 0.05 was considered statistically significant.

Results

The mean histologic scores of phospho-γH2AX^{Ser139}, phospho-ATM^{Ser1981}, phospho-Chk2^{Thr68} and p53 are summarized in table 1A, while a graphical representation is provided in figure 1. Statistically significant differences between HistoScores for PanINs-1, -2, -3, or invasive adenocarcinoma and that observed in non-neoplastic ductal epithelium for each of the four proteins are indicated in table 1B (calculated using Duncan's multiple range test, level of significance at *P*-value <0.05).

Table 1A: Summary of HistoScores for DDR markers in normal ductal epithelium, PanINs, and pancreatic ductal adenocarcinomas

Antibody	Normal duct (N=73)	PanIN-1 (N=31)	PanIN-2 (N=14)	PanIN-3 (N=13)	Adenocarcinoma (N=81)	*ANOVA P*-Value
pH2AX^{Ser139}	2.36	4.34	6.21	7.50	4.53	< 0.0001*
pATM^{Ser1981}	2.33	4.83	5.14	7.17	4.84	< 0.0001*
pChk2^{Thr68}	2.75	5.43	7.64	5.44	2.43	< 0.0001*
p53	0	1.41	1.86	4.00	4.22	< 0.0001*

DDR: DNA Damage Response; PanIN: Pancreatic Intraepithelial Neoplasia

*Significant at the level of *P*-value < 0.05

Table 1B: Statistically significant differences in HistoScores for DDR markers in PanINs and in adenocarcinomas, compared to HistoScore in non-neoplastic ductal epithelium.

Antibody	PanIN-1 (N=31)	PanIN-2 (N=14)	PanIN-3 (N=13)	Adenocarcinoma (N=81)
pH2AX^{Ser139}	*P* < 0.05*	*P* < 0.05*	*P* < 0.05*	*P* < 0.05*
pATM^{Ser1981}	*P* < 0.05*	*P* < 0.05*	*P* < 0.05*	*P* < 0.05*
pChk2^{Thr68}	*P* < 0.05*	*P* < 0.05*	*P* < 0.05*	NS
P53	NS	NS	*P* < 0.05*	*P* < 0.05*

DDR: DNA Damage Response; NS: not significant; PanIN: Pancreatic Intraepithelial Neoplasia

*Significant at the level of *P*-value < 0.05, *Post-hoc* Duncan's multiple range test.

A progressive increase in phospho-H2AX^{Ser139} labeling, consistent with escalating DNA damage, was observed in PanIN lesions (HistoScores of 4.34, 6.21, and 7.50, respectively for PanIN-1, -2, and -3), compared to non-neoplastic ductal epithelium (HistoScore 2.36) (ANOVA, *P* <0.0001). Interestingly, while invasive adenocarcinomas had a significantly higher phospho-H2AX^{Ser139} HistoScore (4.53) than non-neoplastic ductal epithelium (*P* <0.05, Duncan's test), it was significantly lower than that observed in both PanINs-2 and PanINs-3, respectively. In conjunction with escalating double-strand breaks, a progressive activation of the ATM-Chk2 checkpoint was observed with increasing grade of the PanIN lesions. Specifically, phospho-ATM^{Ser1981} HistoScores for PanIN-1, PanIN-2, and PanIN-3 were 4.83, 5.14, and 7.17, respectively, *versus* 2.33 for non-neoplastic ductal epithelium (ANOVA, *P* <0.0001); the corresponding HistoScores for phospho-Chk2^{Thr68} were 5.43, 7.64, and 5.44 in PanINs-1, -2, and -3, respectively, *versus* 2.75 in non-neoplastic ductal epithelium (ANOVA, *P* <0.0001).

As indicated in table 1B, the HistoScore for each histological grade of PanIN was significantly higher than the corresponding HistoScore in the non-neoplastic ductal epithelium, for both proteins (P <0.05, Duncan's test). In both instances, attenuation of the checkpoint was observed in invasive cancers (HistoScores of 4.84 and 2.43, respectively for phospho-ATM^{Ser1981} and phospho-Chk2^{Thr68}), such that in the case of phospho-Chk2^{Thr68}, no significant difference in HistoScores was observed between cancer and non-neoplastic ductal epithelium. In contrast to all of the aforementioned proteins, absent to minimal nuclear p53 expression was observed in non-neoplastic ductal epithelium, as well as in PanINs-1 and -2 (HistoScore of 0-1.86), with a significant upregulation (corresponding to mutational inactivation) seen only at the grade of PanIN-3 and invasive carcinoma (HistoScore of 4.00 and 4.22). Representative photomicrographs demonstrating expression of these four proteins in various PanIN lesions culminating in invasive carcinoma are illustrated in figure 2.

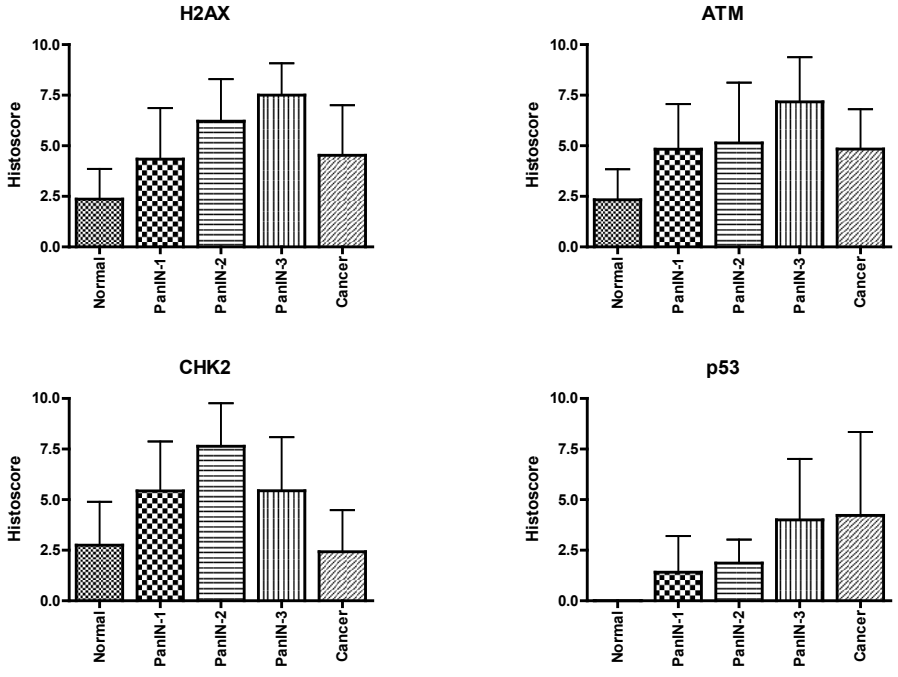

Figure 1: Histograms illustrating the HistoScores for each of the four proteins analyzed in this study, including phospho-γH2AX^{Ser139}, phospho-ataxia telangiectasia mutated^{Ser1981} (ATM^{Ser1981}), phospho-Chk2^{Thr68} and p53. The HistoScores are stratified by the normal ductal epithelium, Pancreatic Intraepithelial Neoplasias (PanINs)-1, -2 and -3, and invasive adenocarcinoma. The mean HistoScore and standard deviations are represented for each grade of lesion. See text for details and statistical analyses.

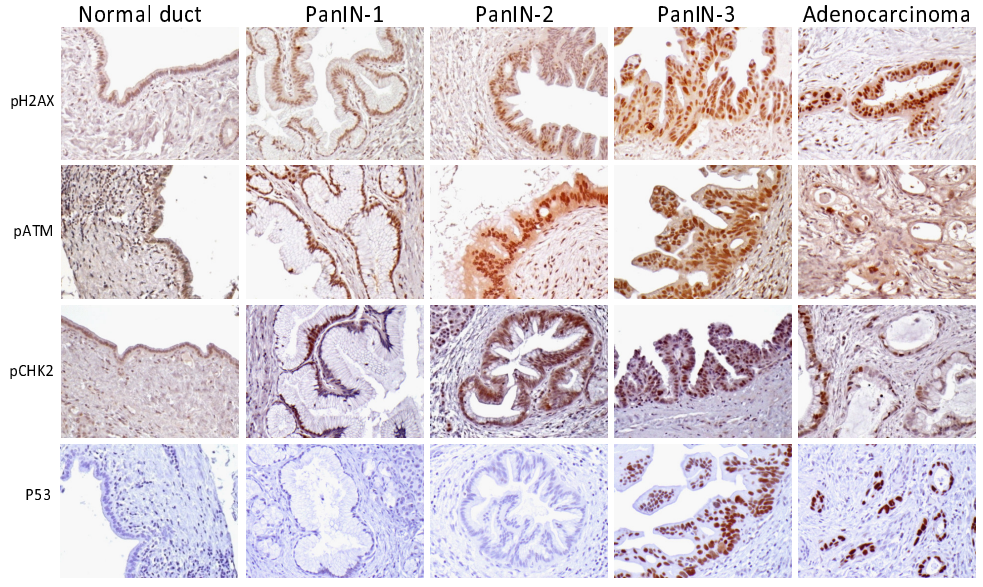

Figure 2: Representative photomicrographs illustrating expression of phospho-γH2AX^{Ser139}, phospho-ataxia telangiectasia mutated^{Ser1981} (ATM^{Ser1981}), phospho-Chk2^{Thr68} and p53 in various histological grades of pancreatic ductal lesions.

Discussion

A diverse array of intracellular signals may activate the so-called DNA Damage Response (DDR) checkpoint in cells, including DNA damage itself, as well as critical telomere shortening, and oncogene activation (*reviewed in* [26-30]). Telomere dysfunction and oncogene activation appear to precipitate so-called 'replicative stress,' leading to DNA damage, and culminating in activation of the DDR checkpoint [16;17;26]. The principle DNA damage phenotypes observed in the setting of the DDR are double-strand breaks (DSBs), and these foci can be recognized by the binding of phosphorylated histone γH2AX to the damaged chromatin [19;31]. Phosphorylated γH2AX forms a scaffold for the DNA repair machinery to engage at the site of double-strand breaks, and phosphorylated γH2AX therefore serves as surrogate readout for DNA damage in cells. In mammalian cells, ATM, and its target, the bacterial checkpoint homolog protein Chk2, are the most important 'sensors' of double-strand breaks [30;32]. Activation of ATM was originally described as an intracellular response to ionizing radiation, which in turn, results in activation of Chk2 protein through phosphorylation of a Thr^{68} moiety [33;34]. As countless examples in experimental animals models and cognate human scenarios have documented, abrogation of the DDR checkpoint itself, or secondary defects in p53 that bypass this checkpoint, enable cells to bypass senescence and restitute proliferation even in the face of genomic damage (*reviewed in* [29;32;35;36]).

In recent years, evidence has emerged to support aberrant activation of the DDR checkpoint in human epithelial pre-cancerous lesions. For example, Bartek *and colleagues* described widespread abnormalities of the ATM-Chk2 axis in non-invasive precursor lesions of human bladder, colon, and breast cancers [15], while Gorgoulis *et al* described comparable findings in the context of lung and epidermal tissues [16]. In all of these instances, DDR checkpoint activation was accompanied by evidence of DNA double-strand breaks, as assessed by phosphorylated γH2AX expression. Of note, p53 function was generally retained in the non-invasive precursor lesions, while progression to invasive cancer was accompanied by p53 inactivation, underscoring a selection pressure for clones with p53 dysfunction [15;16]. Further, DDR in pre-cancerous lesions was observed *prior* to the onset of genomic instability that characterizes invasive cancer, suggesting that widespread allelic imbalances were not the underlying basis for checkpoint activation within the epithelium.

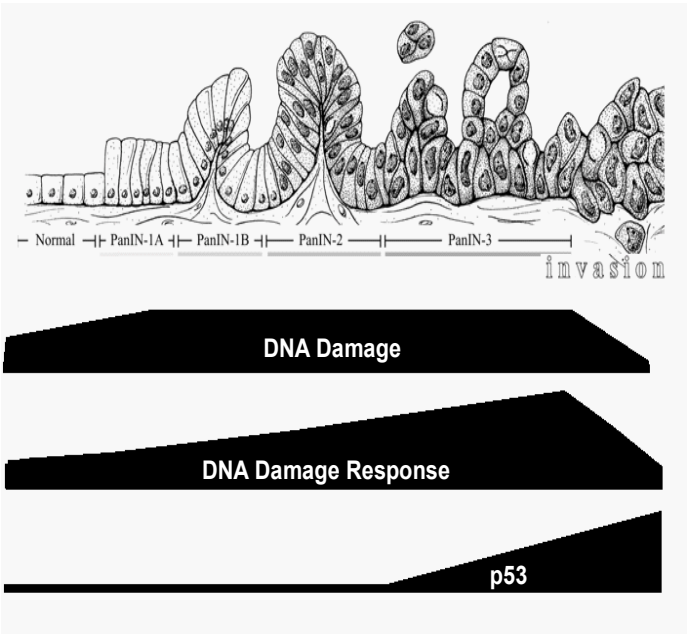

Figure 3: A proposed model of DNA Damage Response (DDR) mediated by the ataxia telangiectasia mutated (ATM)-Chk2 checkpoint in the pancreatic ductal epithelium. In response to double strand breaks, DDR can be observed in the earliest Pancreatic Intraepithelial Neoplasia (PanIN) lesions, and the increase in ATM-Chk2 expression parallels the histological progression to high-grade PanIN-3. Inactivation of p53 function at the stage of PanIN-3 and beyond is associated with bypass of the DDR checkpoint and progression to invasive cancer.

In the current study, we report that histological progression along the PanIN continuum is associated with an escalating degree of DNA damage, as assessed by phosphorylated γH2AX expression, as well as activation of the ATM-Chk2 checkpoint. For three of these proteins (phospho-γH2AX^{Ser139}, phospho-ATM^{Ser1981}, and phospho-Chk2^{Thr68}), we found significant differences in the HistoScores between non-neoplastic ductal epithelium and even PanIN-1, implying that DDR activation is one of the earliest molecular events in the multistep progression of pancreatic cancer. Nuclear accumulation of the p53 protein is a reliable surrogate for mutational inactivation [37], and this was minimally observed up to the stage of PanIN-2, consistent with retained p53 function. In contrast, a significant upregulation of nuclear p53 protein was seen in PanIN-3 and in invasive adenocarcinoma, reinforcing the need for p53 function to be lost so that neoplastic cells can bypass the DDR checkpoint. In our series, abrogation of p53 and progression to invasive adenocarcinoma was associated with a restitution of activated Chk2 expression to 'baseline' levels (i.e., no significant differences

in HistoScores between adenocarcinoma and non-neoplastic epithelium). Thus, the results described herein are comparable to those observed in precursor lesions at other epithelial sites [15;16], and provide a unifying model for containing the unimpeded progression of precursor lesions to invasive cancer. A pictorial representation of these three interdependent processes (DNA damage, DDR and p53 accumulation) along the histological continuum of PanIN lesions is presented in figure 3, and underscores the temporal significance of p53 mutations in bypassing the ATM-Chk2 checkpoint.

One pertinent question that remains unanswered is the inciting event(s) leading to DDR within the pancreatic ducts, as genomic instability alone is unlikely to explain the rather widespread nature of the response. We believe that the reasons are multifactorial, with *KRAS* gene mutations and telomere dysfunction being the most likely culprits, as both are known to induce DNA damage [17;18;22;26;28;38]. In fact, our group has previously shown that telomere attrition is present in >90% of PanIN-1 [4], providing a rational basis for 'replicative stress' and induction of DDR in the earliest precursor lesions. Re-activation of telomerase activity in invasive adenocarcinomas, and the consequent reduction in replicative stress, might underlie the paradoxical attenuation of double-strand breaks (i.e., phospho-γH2AX^{Ser139} labeling) observed in the invasive adenocarcinoma samples, when compared to levels in higher-grade PanIN lesions. The potential role of mutant *KRAS* in DDR has emerged from a recent mouse model of pancreatic cancer mediated by the expression of mutant *Kras* from its endogenous promoter, wherein markers attributable to senescence are uniformly observed in the murine PanIN lesions, but are lost on progression to invasive adenocarcinoma, [22]. Furthering this parallel between human and murine disease is the observation that mice expressing mutant *Kras* alone develop invasive cancers in a minority of cases (<10%) [42], while cooperating hits that allow cells to bypass checkpoints (e.g., loss of *Trp53* or *Ink4a/Arf*) results in complete and accelerated penetrance of the malignant phenotype [38-42].

In summary, we report widespread activation of the DDR checkpoint in the most common non-invasive precursor lesions of pancreatic cancer, including in the lowest grade PanINs. We observe that a DDR-induced checkpoint in PanINs is contingent on retained p53 function, and that the inactivation of this 'gatekeeper' gene is likely one of the most critical events in opening the floodgates to invasive neoplasia. Finally, our results may provide a functional basis to the discordance between the rather common occurrence of PanIN lesions observed in the elderly population, and the relatively uncommon incidence of pancreatic adenocarcinoma.

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10

Summary and Concluding remarks

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Summary and Concluding remarks

Ductal adenocarcinoma of the pancreas is a very aggressive disease with a high mortality rate. Pancreatic carcinoma is the fourth leading cause of cancer-related death in Western countries, despite the fact this cancer accounts for only about 3% of all malignant tumors. The lethal nature of this cancer stems from its propensity to rapidly disseminate to the lymphatic system and distant organs. This aggressive biology and resistance to conventional and targeted therapeutic agents leads to a typical clinical presentation of an incurable disease at the time of diagnosis. The poor prognosis and late presentation of pancreatic cancer patients emphasize the importance of early detection, which seems to be the *sine qua non* in the fight against pancreatic carcinoma.

The growing understanding of the accumulation of genetic changes that accompanies pancreatic carcinogenesis enables the potential use of molecular markers in early detection and management, at a time that the disease is still resectable and potentially curable. The possibility for early detection of pancreatic cancer may be realized by increasing knowledge of the histology and molecular genetics of precursor lesions of pancreatic cancer in conjunction with the development of sensitive and specific screening tests (both invasive and non-invasive) to detect early pancreatic cancer. Better understanding of the embryonic signaling pathways activated in pancreatic cancer may yield new targets but also new markers to select patients and guide and predict therapy efficacy. It is hoped for the future that the understanding of genetic alterations will lead to the rapid discovery of an effective biomarker of pancreatic carcinogenesis.

Chapter 3 of the thesis reviews the molecular insights on pancreatic ductal adenocarcinoma and its precursor lesions, including insights gained trough experimental models of pancreatic carcinogenesis. Intensive research over the last two decades has shown that pancreatic cancer is fundamentally a genetic disease, caused by inherited germline and/ or acquired somatic mutations in cancer-associated genes. Multiple alterations in genes that are important in pancreatic cancer progression have been identified, including tumor-suppressor genes, oncogenes, and genome-maintenance genes. Furthermore, identifying precursor lesions within pancreatic ducts has led to the formulation of a progression model of pancreatic cancer and subsequent identification of early- and late- changes leading to invasive cancer. The recognition that invasive pancreatic ductal adenocarcinomas arise from non-invasive intraductal precursors has highlighted the need for more accurate molecular markers that can not only accurately diagnose pancreatic cancer, but can ultimately also identify these precancerous lesions.

At this moment three distinct precursor lesions have been elucidated in pancreatic cancer. Pancreatic intraepithelial neoplasia (PanIN) is the most common non-invasive precursor lesion of pancreatic cancer. In **chapter 4** we discuss some of the most common seminal alterations that are seen in PanIN lesions and likely contribute to the stepwise genetic progression model of pancreatic cancer. This chapter describes that PanINs share many of the genetic aberrations associated with invasive adenocarcinomas, underscoring their classification as true 'neoplasms' rather than as a reactive/ hyperplastic process. Understanding the molecular mechanisms that facilitate PanIN progression toward invasive adenocarcinomas is critical, because these non-invasive neoplasms represent one of the best targets available for early detection and chemoprevention strategies for pancreatic cancer. This tumor progression

model could potentially be utilized for the identification of patients at-risk, the development of more sensitive molecular-based diagnostic tests, or screening tests for the presymptomatic diagnosis of pancreatic cancer, for more accurate prognostication, and for the improvement of staging procedures. Furthermore, these genetic alterations may be used for the development of novel gene-based therapies.

Chapter 5 is a case-report about a rare variant of pancreatic cancer, namely undifferentiated carcinoma with osteoclastic giant cells, also referred to as UCOCGC of the pancreas. An UCOCGC of the pancreas associated with the familial atypical multiple mole and melanoma (FAMMM) syndrome due to the *p16-Leiden* deletion is described in this report. In addition to nevi and melanomas, patients with the FAMMM syndrome have an increased risk of developing pancreatic cancer. The FAMMM syndrome is caused by a germline mutation of *p16*. Molecular analysis confirmed a germline *p16-Leiden* deletion in the UCOCGC, accompanied by somatic loss of heterozygosity of the second *p16* allele, and absence of p16 protein expression in the neoplastic cells. On the contrary, the osteoclast-like giant cells were positive for the p16 protein, further supporting the hypothesis that these are non-neoplastic cells. Indirectly, the case therefore also shows that it appears to be legitimate to consider UCOCGC of the pancreas as a variant of ductal adenocarcinoma.

As several studies have highlighted, miRNA expression is deregulated in pancreatic cancer. In contrast, patterns of miRNA abnormalities in the non-invasive precursor lesions of pancreatic adenocarcinoma remain largely unknown. The aim of **chapter 6** was to determine whether microRNAs are being misexpressed in one of the precursor lesions of pancreatic cancer. We found significant overexpression by qRT-PCR of ten of the twelve miRNAs observed in 15 IPMNs *versus* matched normal pancreata. Two significantly overexpressed miRNAs –miR-155 and miR-21– were further evaluated by locked nucleic acid *in-situ* hybridization (LNA-ISH) in a panel of 64 archival IPMNs. Both miRNAs were frequently overexpressed within the neoplastic epithelium of IPMNs, with 53 of 64 (83%) IPMNs expressing miR-155 and 52 of 64 (81%) expressing miR-21. In contrast, miR-155 expression was observed in only 4 of 54 (7%) matched non-neoplastic pancreata on the tissue micro arrays, while miR-21 was expressed in only 1 of 54 (2%) non-neoplastic pancreata. When we looked to the histological continuum of atypia, a significantly greater proportion of IPMNs with carcinoma-*in-situ* expressing either miRNA compared to IPMN adenomas. The expression of miR-155 and miR-21 was also evaluated in pancreatic juice samples obtained from 10 patients with surgically resected IPMNs and five patients with non-neoplastic pancreato-biliary disorders (‘disease controls’). Upregulation of miR-155 transcripts by qRT-PCR was observed in 6 of 10 (60%) IPMN-associated pancreatic juice samples compared to 0 of 5 (0%) disease controls. In summary, aberrant miRNA expression is an early event in the multistage progression of pancreatic cancer, and miR-155 warrants further evaluation as a biomarker for IPMNs in clinical samples.

In **chapter 7**, we carried out a study of the mechanisms of the Notch pathway activation in the setting of pancreatic cancer. We found that endogenous overexpression of Notch ligands, specifically *JAGGED2* and *DLL4,* appears to be the most common mechanism. Of interest, we verified genomic amplification of the *DLL3* locus on chromosome 19q13 contributing to Notch activation in this malignancy. However, mutational activation of Notch seems to be

rare to absent in pancreatic cancer, this in contrast to other malignancies like T-cell leukemia. This study also demonstrated that sustained Notch signaling is required for the viability of a subpopulation of pancreatic cancer cells with tumor initiation properties (i.e., 'cancer stems cells'), and reiterate the utility of targeting this pathway as a therapeutic strategy in this malignancy. Thus, we confirmed that Notch activation is almost always ligand-dependent in tumor initiation and tumor maintenance in pancreatic cancer, and inhibition of Notch signaling is a promising therapeutic strategy in this carcinoma.

In **chapter 8** the significance of the receptor tyrosine kinase Axl was evaluated by immunohistochemical analysis of 99 patients with pancreatic cancer. In cancer tissues, positive and negative Axl protein expressions were seen in 54 of 99 (55%) and in 45 of 99 (45%) cases, respectively. Axl protein expression was significantly associated with lymph node status. Moreover, patients with positive Axl staining had a shorter median survival than patients with negative expression; 12 months *versus* 18 months respectively. In pancreatic cancer cell lines, the expression of the protein Axl was confirmed. Stable knockdown of *AXL* transcripts was enabled in Axl-overexpressing MIAPaCa-2 cells using lentiviral short hairpin shRNA, and resulted in significant reduction in cell viability, anchorage independent growth, as well as attenuation of migratory and invasive properties, compared to vector-transfected cells. Further, we explored the status of multiple effector pathways that are implicated in pancreatic cancer growth and progression. Two major intracellular effectors downstream of RTKs in both normal and cancer cells are the p42/p44 MAP kinase and the PI-3-kinase/Akt signaling pathways. In MIAPaCa-2 shRNA expressing cells, we found inhibition of both effector arms. The reduction in invasion and migration upon Axl knockdown was mirrored by a decrease in the amounts of activated (GTP-bound) GTPase proteins Rho and Rac, downregulation in transcript levels of the epithelial mesenchymal transition (EMT)-associated transcription factors *snail*, *slug* and *twist*, and decrease in matrix metalloproteinase *MMP-9* mRNA levels. Taken together, these reports support and emphasize an important role of Axl in pancreatic tumorigenesis. Perhaps Axl is an interesting and novel signaling pathway in pancreatic cancer. Nevertheless, future investigations will be needed to establish the detailed signaling mechanisms by which Axl involves the initiation and progression of cancer.

Finally, in **chapter 9** DNA Damage Response markers were characterized in PanIN lesions to determine whether or not these are involved in early pancreatic neoplasia. As compared to pancreatic ductal epithelium a progressive increase in phospho-γH2AX^{Ser139}, phosphoATM^{Ser1981} and phosphoChk2^{Thr68} labeling consistent with escalating DNA damage, was observed in the non-invasive precursor lesions. Conversely, absent to minimal nuclear p53 was observed in ductal epithelium, and in PanINs-1 and -2, with a significant upregulation (corresponding to mutational inactivation) seen only at the stage of PanIN-3 and invasive neoplasia. To conclude, activation of the ATM-Chk2 checkpoint pathway is commonly observed in PanIN lesions, including the lowest grade lesions, likely in response to the accumulating DNA damage from events such as oncogene mutations and telomere dysfunction. These results suggest that ATM and CHK2 activation at early stage of pancreatic tumorigenesis suppresses tumor progression. Loss of p53 function appears to be a critical determinant for bypassing this checkpoint and the subsequent progression to invasive adenocarcinoma.

Taken together the above studies in this thesis have shown that molecular markers may indeed have a role in identifying patients predisposed to pancreatic cancer, and improving early detection, diagnostic accuracy and prognostication. Our increased knowledge of both the molecular changes in pancreatic cancer as well as its precursor lesions may provide the basis for developing more sensitive screening strategies and the identification of new drug targets enabling directed drug design. Nevertheless, immediate translation of these results into routine daily practice is still limited due to various reasons. The suitability of these markers and new techniques in early detection of pancreatic cancer needs further investigation in future studies. Fundamental issues of sensitivity and specificity remain to be addressed for the newest markers. For instance, each possible marker or panel of markers needs to be assessed in normal pancreatic tissue, precancerous stages, various stages of invasive pancreatic cancer and in benign disease such as chronic pancreatitis. Ideally, a screening test should be easy to perform in a variety of clinical situations to detect pancreatic cancer in a pre-clinical stage, using molecular analysis in sources such as blood, stool, pancreatic juice, fine-needle aspiration of a pancreatic mass, or brush cytology specimens of the pancreatic duct. Moreover, recognizing individuals at high risk of developing pancreatic cancer and applying markers that could identify the precancerous lesions of the pancreas in these individuals could allow such lesions to be resected before the occurrence of pancreatic cancer.

Although a lot of pancreatic cancer research concentrates on the early detection of pancreatic neoplasia, it is also yet important to focus on increasing our knowledge about pancreatic carcinogenesis and the development of the precursor lesions. The past decade has brought tremendous progress in understanding the genetic changes in pancreatic cancer; however the survival rate of pancreatic cancer has still not improved. Therefore, future studies should also explore new pathways to get new and better insights in the tumorigenesis of the pancreas, like our studies of Axl and the ATM-Chk2-p53 pathway. Also, the detailed mechanisms of already known pathways, such as Notch and Hedgehog, should be further examined. All this will enable us to develop better tools for primary and secondary prevention of pancreatic cancer, as well as improve existing tools for early diagnosis.

It is clear that there is still a long way to go, in finding an absolute cure for this uniformly lethal disease. Future studies will have to focus on all the different issues in order to benefit even more from the recent advances in our understanding of *molecular characteristics of pancreatic carcinogenesis*. In view of the dismal outlook for a patient with pancreatic carcinoma the energy would definitely be well spent.

Inch by inch, we are coming closer to better insights in this disease. As complex as pancreatic cancer is, our increasing knowledge of each of the various facets of the cancer has provided potential new targets for the early detection and treatment of this cancer. *One inch, at a time…*Our goal is to fight for that *inch. "Because we know when we add up all those inches that is going to make the difference between winning and losing between living and dying…"*

(Al Pacino, *Any given Sunday*)

11

Samenvatting in het Nederlands

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Samenvatting in het Nederlands

Inleiding voor de Conclusie

Het menselijk lichaam is opgebouwd uit miljarden cellen. Door middel van celdeling worden voortdurend nieuwe cellen gevormd. Dit is noodzakelijk om te kunnen groeien, maar ook om beschadigde en verouderde cellen te kunnen vervangen. Deze celdeling wordt goed gereguleerd en gecontroleerd. Hierbij spelen genen een belangrijke rol. De genen bevatten het erfelijke materiaal, aangeduid als DNA. Soms gaat er iets mis met de celdeling en dan worden er allerlei regelmechanismen in werking gesteld om de fout te herstellen. Indien ook dat niet lukt, kan het lichaam besluiten om de 'zieke cel' af te voeren. Echter soms weet een zieke cel te ontsnappen aan alle regelmechanismen en wordt het een cel die maar door blijft delen. Er ontstaat dan overmatige celdeling, die tot een gezwel of tumor leidt. Deze tumor is kwaadaardig (maligne) van aard, en dit wordt ook wel kanker genoemd. Een tumor werkt niet meer samen met andere cellen en kan andere delen van het lichaam binnendringen (invasieve groei). Bovendien, kunnen groepjes van deze cellen losraken en via de lymfe- en/of bloedbanen in andere organen terecht komen. Dit proces heet uitzaaien.

Kanker ontstaat niet zomaar vanuit het niets, maar ontwikkelt zich gedurende een stapsgewijs proces, gedurende vele jaren. Dit proces van tumorprogressie wordt onder andere bepaald door het genetische materiaal in het DNA, dat alles structureert in het menselijk lichaam. Zo ook alle regelmechanismen om een tumor te onderdrukken. Nochtans kan het voorkomen dat in de loop der jaren in het DNA fouten (mutaties) ontstaan. Indien deze fouten zich op blijven stapelen, vormt zich DNA wat minder goed in staat is om cellen te ontdekken met eigenschappen van kanker en deze kankercellen hetzij te herstellen of te vernietigen. Hierdoor kan uiteindelijk in de loop der tijd een tumorcel ontstaan die uit kan groeien tot kanker, in de pancreas heet dit adenocarcinoom. Dit gehele proces van *'ontstaan van kanker'* wordt ook wel met een moeilijk woord carcinogenese of tumorigenese genoemd. Ook in de pancreas begint kanker niet zomaar... Intussen is bekend dat het kwaadaardige pancreascarcinoom vaak wordt voorafgegaan door een zogenaamd voorloperstraject. Dit voorloperstraject wordt beschouwd als een soort overgangsgebied tussen normaal pancreas weefsel en het maligne pancreas weefsel (kanker). In dit overgangsgebied van de pancreas zijn inmiddels goed gedefinieerde laesies omschreven. Simpelweg heten deze laesies precursor laesies. Bij pancreaskanker zijn drie verschillende types precursor laesies bekend. Deze zijn: PANcreatic Intraepithelial Neoplasias (PanINs), Mucinous Cystic Neoplasms (MCN) en Intraductal Papillary Mucinous Neoplasms (IPMNs). Deze laesies zijn dus nog geen kanker, maar kunnen het wel worden. Elke laesie kan opgevat worden als een vorm van pre-maligniteit voordat pancreaskanker ontstaat.

Zoals al eerder aangegeven, vinden er in de menselijke cel continu allerlei processen plaats. Deze processen spelen zich af op moleculair en genetisch niveau en zijn onder andere door het DNA zeer strak georganiseerd. Vaak is het zo dat al deze processen als een serie van aan één schakelingen op elkaar volgen. De moleculen *–lees eiwitten-* grijpen als een systeem van radartjes op elkaar aan. Door middel van deze 'kettingreactie' van acties tussen moleculen onderling, worden er in de menselijke cel allerlei processen aangestuurd en functies uitgevoerd. Deze 'kettingreacties' in de cel worden ook wel 'pathways' genoemd. 'Pathways' vervullen een belangrijke rol in de celhuishouding. Vaak is het zo dat 'pathways' onderling weer met elkaar communiceren en kan het gebeuren dat de ene 'pathway' de andere 'pathway' activeert.

In dit proefschrift zijn verschillende studies uitgevoerd naar het pancreascarcinoom. Onder andere zijn er verscheidene precursor laesies bestudeerd om deze beter te doorgronden en daarbij mogelijke aangrijpingspunten te ontdekken voor eventuele biomarkers voor de vroege detectie. Tevens zijn er onderzoeken gedaan naar een aantal 'pathways,' om te zien in hoeverre deze 'pathways' een belangrijke rol spelen in de pancreas carcinogenese.

De Conclusie

Het ductale adenocarcinoom van de pancreas is een zeer agressieve ziekte met een hoge mortaliteit. Het pancreascarcinoom is de vierde oorzaak van kanker-gerelateerde sterfte in de Westerse wereld, ondanks het feit dat deze kanker slechts 3% van alle kwaadaardige maligniteiten representeert. Het dodelijke karakter van deze kanker komt voornamelijk door het gegeven dat deze kanker zeer snel kan uitzaaien naar het lymfatische systeem of op afstand gelegen organen in het lichaam via de bloedbaan. Deze agressieve biologische aard en resistentie tegen de huidige therapeutische middelen, leidt tot een typisch klinische presentatie van een ziekte die ongeneesbaar is ten tijde van diagnose. De slechte prognose en de late presentatie van pancreaskanker benadrukt het belang van vroege detectie, een *sine qua non* voor de strijd tegen pancreascarcinoom.

Het groeiende inzicht in de carcinogenese van de pancreas en de hiervoor verantwoordelijke accumulatie van genetische veranderingen biedt perspectieven voor de ontwikkeling van moleculaire markers voor de vroege detectie en behandeling van het pancreascarcinoom, op een moment dat chirurgische verwijdering van de kanker nog steeds mogelijk is en daardoor nog potentieel geneesbaar. De mogelijkheid tot vroege detectie kan gerealiseerd worden door een beter begrip van de histologie en moleculaire genetische veranderingen in voorloperstadia en tumor laesies van het pancreascarcinoom, in combinatie met de ontwikkeling van sensitieve en specifieke testen om pancreascarcinoom in een vroeg stadium te ontdekken. De toegenomen kennis over de tumorprogressie cascade van pancreaskanker heeft tot gevolg dat er nieuwe genen, maar ook nieuwe markers kunnen worden ontdekt, waarmee de effectiviteit van bepaalde therapieën kan worden voorspeld in geselecteerde patiëntgroepen. Het is te hopen voor de toekomst dat het inzicht in de verschillende genetische veranderingen zal leiden tot de ontdekking van effectieve markers voor de pancreas carcinogenese.

Hoofdstuk 3 van dit proefschrift beschrijft de moleculaire inzichten van het pancreascarcinoom en zijn voorloperlaesies, inclusief inzichten opgedaan in muismodellen van pancreas carcinogenese. Intensief onderzoek van de laatste twee decennia heeft aangetoond dat pancreascarcinoom een genetische ziekte is, veroorzaakt door aangeboren kiemcel en/of verworven somatische mutaties in kankergerelateerde genen. Verscheidene veranderingen in genen die belangrijk zijn in de tumorprogressie zijn beschreven, inclusief de zogenaamde tumor-suppressie genen, oncogenen en 'care-takergenen'. De ontdekking van precursor laesies in de pancreas duct heeft geleid tot de formulering van een progressiemodel van het pancreascarcinoom en tot een verdere identificatie van vroege en late veranderingen in de pancreas leidend tot invasieve kanker. De erkenning dat het invasieve pancreas ductaal adenocarcinoom ontstaat uit niet-invasieve ductale precursor laesies benadrukt de noodzaak van meer accurate moleculaire markers, die niet alleen pancreaskanker goed kunnen diagnosticeren, maar ook juist die specifieke precursor laesies (het zogenaamde pre-maligne voorloper tumorstadia) kunnen identificeren.

Op dit moment zijn er drie goed omschreven precursor laesies bekend in pancreaskanker. De zogenaamde 'Pancreatic Intraepithelial Neoplasia,' ook wel PanIN laesie genoemd, is de meest voorkomende niet-invasieve precursor laesie in pancreaskanker. In **hoofdstuk 4** worden de meest voorkomende veranderingen die bekend zijn in PanIN laesies besproken en waarschijnlijk bijdragen in het tumorprogressie-model van pancreaskanker. Dit hoofdstuk beschrijft dat PanIN laesies veel dezelfde genetische veranderingen delen die geassocieerd worden met het invasieve adenocarcinoom. Dit gegeven onderstreept duidelijk het feit dat PanIN laesies als een echte pre-maligniteit moeten worden gezien, in plaats van de PanIN laesies slechts als een reactief/ hyperplastisch proces te beschouwen. Begrip van het moleculaire mechanisme dat bijdraagt dat een PanIN laesie zich ontwikkelt tot een invasief adenocarcinoom is cruciaal, omdat deze niet-invasieve neoplasie een van de beste beschikbare aangrijpingspunten vertegenwoordigt voor vroege detectie en chemopreventie strategieën voor de behandeling van pancreaskanker. De specifieke moleculaire veranderingen die beschreven zijn in dit model, kunnen worden gebruikt voor diverse klinische doeleinden. Bijvoorbeeld voor de detectie van patiënten met een verhoogd risico op het krijgen van pancreaskanker, de ontwikkeling van nieuwe, meer sensitieve diagnostische methodes, screening voor pancreaskanker voor een betere prognose en voor de verbetering van de stagering. Bovendien zouden deze genetische veranderingen kunnen dienen als basis voor de ontwikkeling van nieuwe therapieën.

Hoofdstuk 5 is een case-report over een zeldzame variant van pancreaskanker, namelijk het ongedifferentieerde carcinoom met osteoclast-achtige meerkernige reuscellen, in het Engels ook wel afgekort als UCOCGC van de pancreas. Een UCOCGC van de pancreas geassocieerd met het familiare atypische multiple moedervlek en melanoom (FAMMM) syndroom veroorzaakt door een zogenaamde *p16-Leiden* deletie is beschreven in dit case-report. Patiënten met het FAMMM syndroom hebben naast moedervlekken en melanomen, een verhoogd risico op het verkrijgen van pancreaskanker. Het FAMMM syndroom wordt veroorzaakt door een kiemcel mutatie van het gen *p16*. De moleculaire analyse van deze patiënt met het UCOCGC bevestigde de *p16-Leiden* deletie vergezelt met een verworven verlies van de heterozygotiteit van het tweede *p16* allel. Tevens toonde de neoplatische cellen een afwezigheid van het p16 eiwit aan. Dit in tegenstelling tot de osteoclast-achtige reuscellen, die wel positief waren voor het p16 eiwit. Dit sterkt de hypothese dat deze cellen als niet neoplastische cellen moeten worden beschouwd. Indirect, lijkt dit case-report een bewijs voor het feit dat het legitiem is, om UCOCGC van de pancreas als een variant van het ductale adenocarcinoom te beschouwen.

Verschillende studies hebben doen uitkomen dat microRNA expressie gedereguleerd is in pancreaskanker. Opvallend genoeg, is het nog grotendeels onbekend hoe deze abnormale patronen van microRNA zich manifesteren in de non-invasieve precursor laesies van pancreas adenocarcinoom. Het doel van **hoofdstuk 6** is om te bepalen of microRNAs tot abnormale expressie komen in een van de precursor laesies van pancreaskanker. Gekeken is naar het verschil in expressie van bepaalde microRNAs tussen normaal pancreas weefsel en IPMN laesies, een precursor laesie van het pancreascarcinoom. Dit is gedaan door middel van een bepaalde techniek genaamd kwantitatieve Real-Time PCR (qRT-PCR). In dit onderzoek waren 10 van de 12 onderzochte microRNAs significant verhoogd aanwezig in de IPMN laesies *versus* het normale pancreas weefsel, waarvan twee microRNAs (miR-155 en miR-21)

duidelijk het hoogst. Deze twee microRNAs zijn vervolgens verder geëvalueerd door middel van 'Locked nucleic acid *in-situ* hybridisatie' (LNA-ISH) op tissue micro arrays van pancreas tumoren met verschillende stadia van IPMN laesies. LNA-ISH bevestigde de expressie van miR-155 in 53 van de 64 (83%) IPMNs vergeleken met 4 uit 54 (7%) in normaal ductaal pancreas weefsel. Voor miR-21 waren deze cijfers 52 van de 64 (81%) IPMNs vergeleken met 1 uit 54 (2%) in normaal ductaal weefsel. Vervolgens is gekeken naar het verschil in expressie van beide microRNAs, gerelateerd aan de histologische graad van de IPMNs. IPMNs met carcinoma-*in-situ* (een hoge graad IPMN, die dicht tegen kanker aan zit), bevatte relatief gezien, significant meer expressie van zowel miR-155 als miR-21, dan IPMNs met adenoma (een lage graad IPMN). Als laatste is ook de expressie van deze twee microRNAs onderzocht in pancreassap van in totaal 10 patiënten die chirurgische verwijdering van hun IPMN hadden ondergaan en 5 patiënten met niet-kwaadaardige pancreato-billiare klachten. Deze laatste 5 patiënten fungeerde als controlegroep. Dit onderzoek is eveneens gedaan door middel van qRT-PCR. Hierbij werd een upregulatie van miR-155 waargenomen in het pancreassap in 6 uit 10 (60%) van het IPMN- geassocieerde pancreassap tegen 0 uit 5 (0%) van de controlegroep. Samenvattend, is in dit onderzoek aangetoond dat abnormale expressie van microRNAs een vroege stap is in het ontstaan van pancreaskanker. Gebaseerd op de resultaten van onze studie is miR-155 een interessante kandidaat als mogelijke biomarker voor IPMNs in klinische relevante toepassingen.

Hoofdstuk 7 beschrijft een studie uitgevoerd naar het mechanisme van de activatie van de 'Notch pathway' in pancreaskanker. Tijdens de embryogenese heeft de 'Notch pathway' een belangrijke rol bij de aanleg van de pancreas. Na de embryogenese schakelt de 'Notch pathway' zichzelf uiteindelijk uit. Recentelijk is ontdekt dat in pancreaskanker de 'Notch pathway' weer geactiveerd wordt. Notch lijkt dus belangrijk in de carcinogenese van de pancreas. In deze studie is gekeken naar het exacte mechanisme van de 'Notch pathway' activatie. Gevonden is dat endogene over-expressie van Notch liganden, vooral *JAGGED2* en *DLL4*, het meest voorkomende mechanisme schijnt te zijn. Tevens is geverifieerd dat het gen *DLL3* op de locus van chromosoom 19q13 geamplificeerd is en zo bijdraagt tot de activatie van Notch. In tegenstelling tot andere maligniteiten zoals T-cel leukemie waar het Notch gen wel gemuteerd is, lijkt het erop dat in pancreaskanker een mutatie van Notch ongewoon is. Deze studie demonstreert ook dat een continu aanhoudend signaal van Notch vereist is voor de levensvatbaarheid van een subpopulatie van pancreascellen met tumor geïnitieerde eigenschappen, zogenaamde 'kanker stamcellen.' Dit gegeven bevestigt het nut om deze 'pathway' als mogelijk aangrijpingspunt te gebruiken voor een eventuele klinische benadering van deze maligniteit. Kortom, geconcludeerd kan worden dat Notch activatie bijna altijd ligand afhankelijk is in het aanzetten tot tumorgroei en vitaal is voor de tumorigenese in pancreaskanker. Inhibitie van het Notch signaal kan allicht een veelbelovende therapeutische strategie zijn voor de behandeling van dit carcinoom.

In verscheidene humane kankersoorten, zoals borst- en longkanker, is aangetoond dat de receptor tyrosine kinase Axl een functie vervuld bij het ontstaan van kanker. Verrassend genoeg, is Axl nog nooit bestudeerd in pancreaskanker. In **hoofdstuk 8** is de betekenis van de receptor tyrosine kinase Axl geëvalueerd door middel van een immunohistochemische analyse in 99 patiënten met pancreaskanker. In kankerweefsel is zowel positieve als negatieve expressie waargenomen van het eiwit Axl. Positieve kleuring werd waargenomen

in 54 van de 99 (55%) gevallen en negatieve kleuring in 45 van de 99 (45%) gevallen. De expressie van het Axl eiwit was tevens significant geassocieerd met de lymfeklier status. Daarbij hadden patiënten met een positieve kleuring van Axl kortere overlevingscijfers dan patiënten met een negatieve kleuring, respectievelijk 12 maanden *versus* 18 maanden. In cellijnen van pancreaskanker is de aanwezigheid van het Axl eiwit bevestigd. Een zogenaamde 'knockdown' van het *Axl* gen (uitschakeling van het *Axl* gen), resulteerde in kankercellijnen met verminderde groei-, migratie- en invasie-eigenschappen. Verder is de status van verschillende 'downstream pathways' bestudeerd welke betrokken zijn bij de groei en vooruitgang van de kanker. In zowel normale cellen als kankercellen worden deze 'downstream pathways' geactiveerd als reactie op de receptor tyrosine kinase Axl. De uitschakeling van het *Axl* gen had tot gevolg dat verscheidene 'downstream pathways' verminderd tot expressie kwamen. Alles bij elkaar genomen, ondersteunen en benadrukken de onderzoeken van dit hoofdstuk een belangrijke rol voor Axl in de tumorigenese van de pancreas. Allicht is Axl een interessante en nieuwe 'signaling pathway' in pancreaskanker. Desalniettemin, is toekomstig onderzoek zeker nodig om de exacte mechanismen bloot te leggen van deze 'pathway,' om zodoende beter te doorgronden waarom Axl betrokken is bij de kankerontwikkeling en verdere progressie van de kanker.

Tot slot, zijn in **hoofdstuk 9** DNA Damage Response markers gekarakteriseerd in PanIN laesies om te bepalen of deze betrokken zijn bij de vroege neoplasie van de pancreas. Zoals eerder beschreven in de inleiding, wordt de celdeling strak gereguleerd door het DNA. Indien er fouten ontstaan, worden er allerlei regelmechanismen in gang gezet om deze fouten te herstellen. Voorbeeld van zo een mechanisme is de 'DNA Damage Response pathway.' Deze 'pathway' kan onderzocht worden door te kijken naar de specifieke uitingen op eiwitniveau: pγH2AX^{Ser139}, pATM^{Ser1981}, pChk2^{Thr68}, en p53. In dit hoofdstuk is gekeken naar verschil in expressie van bovengenoemde eiwitten in normaal pancreas weefsel, in de PanIN laesies en in kankerweefsel. In vergelijking met het normale ductale pancreas epitheel, is een progressieve toename van kleuring en dus activiteit waargenomen van pγH2AX^{Ser139}, pATM^{Ser1981} en pChk2^{Thr68} in de non-invasieve precursor laesies (PanINs). Daarentegen, in normaal ductaal epitheel, PanIN-1 en PanIN-2 laesies werd afwezige tot minimale expressie van het nucleaire eiwit p53 geobserveerd. Een duidelijke upregulatie van p53 (wat correspondeert met inactiviteit ten gevolge van een mutatie) werd alleen gezien in PanIN-3 laesies en invasieve neoplasie. Kortom, de ATM-Chk2-p53 checkpoint pathway is een duidelijk voorkomend verschijnsel in PanIN laesies. Deze resultaten suggereren dat ATM en Chk2 samen proberen de tumorprogressie te onderdrukken in de vroege fase van pancreas tumorigenese. Het lijkt erop dat verlies van het *p53* gen een kritische determinant is in het omzeilen van dit checkpoint en dat dit leidt tot verdere progressie van het invasieve adenocarcinoom.

De bovenstaande studies in dit proefschrift tonen aan dat moleculaire markers een rol zouden kunnen spelen in het identificeren van patiënten met een verhoogd risico op pancreaskanker, het verbeteren van (vroeg)diagnostiek en het schatten van de prognose. De toegenomen kennis van moleculaire veranderingen in zowel het pancreascarcinoom als de voorloperstadia, kan misschien de basis bieden voor het ontwikkelen van meer sensitieve detectiemethoden en de identificatie van nieuwe aangrijpingspunten voor geneesmiddelen. Desalniettemin, de onmiddellijke toepassing van deze technieken in de dagelijkse praktijk zal om meerdere

redenen voorlopig beperkt blijven. De beschikbaarheid van deze markers en nieuwe technieken voor vroege detectie van pancreaskanker zal verder onderzocht moeten worden in toekomstige studies. Fundamentele zaken als sensitiviteit en specificiteit blijven belangrijk voor de nieuwste markers. Bijvoorbeeld, de bruikbaarheid van elke mogelijke marker of panel van markers, moet worden onderzocht in normaal pancreasweefsel, voorloperstadia, verschillende tumorstadia van pancreascarcinoom en in goedaardige aandoeningen, zoals chronische pancreatitis. Een screening test zou idealiter, gemakkelijk uitvoerbaar moeten zijn in verscheidene klinische situaties om pancreaskanker in een vroege fase te detecteren, zoals moleculaire analyse van bronnen als bloed, feces, pancreassap, dunne naald aspiratie van pancreascellen, of brush cytologie van de ductus pancreaticus. Bovendien, zou herkenning van patiënten met een verhoogd risico tot de ontwikkeling van pancreaskanker en daarbij markers die zich richten op het identificeren van de voorloperstadia van de kanker in deze individuen, kunnen betekenen dat deze laesies chirurgisch verwijderd kunnen worden voordat pancreaskanker zich ontwikkeld.

Ondanks het gegeven dat vandaag de dag veel onderzoek van pancreaskanker gericht is op de vroege detectie van pancreaskanker, is het ook nog steeds zeer belangrijk om te focussen op een vergroting van onze kennis over pancreas carcinogenese en de ontwikkeling van zijn precursor laesies. Het afgelopen decennium is veel progressie geboekt in het verkrijgen van kennis over de verschillende genetische veranderingen in de pancreas carcinogenese; echter de overlevingscijfers van pancreaskanker zijn nog steeds niet verbeterd. Toekomstige studies zouden zich daarom moeten richten op de ontdekking van nieuwe 'pathways' om nieuwe en verbeterde inzichten te verkrijgen in de tumorigenese van de pancreas, zoals onze studies over Axl en de ATM-Chk2-p53 pathway. Tevens zouden ook de mechanismen van reeds bekende 'pathways,' zoals Notch en Hedgehog, verder in detail in kaart gebracht moeten worden. Dit alles bij elkaar zal ons in staat stellen om zowel betere methodes te ontwikkelen voor primaire en secundaire preventie van pancreaskanker, als het verbeteren van de huidige methodes voor vroege diagnose.

Het is duidelijk dat er nog steeds een lange weg te gaan is, voor een absolute genezing van deze universeel dodelijke ziekte. Toekomstige studies zullen zich moeten concentreren op de verschillende beperkingen en problemen, zodat in de toekomst nog meer geprofiteerd kan worden van de recent opgedane kennis omtrent de *moleculaire kenmerken van pancreas carcinogenese*. Gegeven de huidige slechte vooruitzichten voor een patiënt met pancreaskanker is iedere inzet op dit terrein zeker goed besteedt.

Inch by inch, beetje bij beetje, komen we tot betere inzichten in deze ziekte. Hoe ingewikkeld pancreaskanker ook is, onze verbeterde kennis op elk verschillend facet van deze vorm van kanker heeft geleid tot potentiële nieuwe aangrijpingspunten voor vroege detectie en behandeling van deze kanker. *One inch, at a time...* Ons doel blijft te vechten voor elke *inch. "Because we know when we add up all those inches that is going to make the difference between winning and losing, between living and dying…"*

(Al Pacino, *Any given Sunday*)

About the author
&
List of Publications

Curriculum Vitae

Jan-Bart Michiel Koorstra was born on the fourth of June 1985 in Naarden, the Netherlands. In the year 1997, Jan-Bart started his secondary pre-university education at the Willem de Zwijger College in Bussum. He graduated in 2003 and moved to Leiden in the same year. From there he started to study Medicine at the Free University (VU) in Amsterdam.

In September 2007, Professor dr. G. Johan A. Offerhaus gave Jan-Bart the unique opportunity to visit the Johns Hopkins University School of Medicine in Baltimore, Maryland, United States. Supported by a grant from the Dutch Cancer Foundation (KWF), Jan-Bart worked for almost twelve months as a student research fellow at the Department of Pathology in the Sol Goldman Pancreatic Cancer Research Center under supervision of Dr. Anirban Maitra and Prof. dr. Ralph H. Hruban. At Johns Hopkins, most of the studies on pancreatic cancer in this thesis were initiated.

Upon his return from Baltimore, Jan-Bart continued his Medical School program and began his clinical rotations. In December 2008, Jan-Bart obtained his Master of Science degree in Medicine. Since his homecoming in the Netherlands, Jan-Bart combined his clinical rotations with finishing his thesis on *Molecular characteristics of pancreatic carcinogenesis* in the lab of Prof. dr. G. Johan A. Offerhaus.

For the research work conducted at Johns Hopkins, Jan-Bart received the VUmc Research Award 2008 and the Hippocrates Award 2009.

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Al Pacino, *Any given Sunday* speech:

"I don't know what to say really. Three minutes till the biggest battle of our professional lives. It all comes down to today. Now either we heal as a team, or we're gonna crumble. *Inch by inch*, play by play, till we're finished. We're in hell right now, gentlemen. Believe me. And we can stay here, get the shit kicked out of us, or we can fight our way back into the light. We can climb out of hell. *One inch at a time...*

Now I can't do it for you. I'm too old. I look around, I see these young faces, and I think... I mean… I've made every wrong choice a middle-aged man can make. I pissed away all my money, believe it or not. I chased off anyone who's ever loved me, and lately, I can't even stand the face I see in the mirror. You know when you get old in life, things get taken from you. That's part of life. But you only learn that when you start losing stuff. You find out *life's this game of inches*. And so is football. Because in either game, life or football, the margin for error is so small. I mean... one half a step too late or too early and you don't quite make it. One half second too slow too fast, you don't quite catch it. *The inches we need are everywhere around us*. They are in every break of the game, every minute, every second. On this team, *we fight for that inch*. On this team, we tear ourselves and everyone else around us to pieces for that *inch*. We claw with our fingernails for that *inch*. *Because we know when we add up all those inches, that's gonna make the difference between winning and losing between living and dying*! I'll tell you this - in any fight, it's the guy whose willing to die who's gonna win that *inch*. And I know if I'm going to have any life anymore, it's because I'm still willing to fight and die for that *inch*. Because that's what living is! The *6 inches* in front of your face...

Now I can't make you do it. You've got to look at the guy next to you, look into his eyes. Now I think you're gonna see a guy who will go that *inch* with you. You're gonna see a guy who will sacrifice himself for this team, because he knows when it comes down to it, you're gonna do the same for him.

That's a team, gentlemen. And either we heal, now, as a team, or we will die, as individuals. That's football, guys. That's all it is. Now, what are you going to do?"