Journal of Molecular Diagnostics, Vol. 10, No. 2, March 2008

DOI: 10.2353/jmoldx.2008.070115

Review

Molecular Genetics of Pancreatic Ductal Adenocarcinomas and Recent Implications for Translational Efforts

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Pancreatic ductal adenocarcinoma (ie, pancreatic cancer) is among the most devastating of human malignancies. It is commonly diagnosed at advanced, already metastatic, and, hence, incurable stages. Despite extensive research efforts in recent decades, pancreatic cancer remains resistant to almost all clinically available therapy regimens. Recent advances in our understanding of the underlying pathophysiology and molecular biology have opened up avenues for the development of novel diagnostic and therapeutic strategies, some of which have shown highly promising preclinical results and are currently being translated into clinical application. Here in we present a review of recent literature on the molecular genetics of pancreatic cancer and emphasize clinical implications for the development of novel diagnostic and therapeutic approaches. ***(J Mol Diagn 2008, 10:111–122; DOI: 10.2353/jmoldx.2008.070115)***

Pancreatic ductal adenocarcinoma (PDAC), which constitutes over 90% of pancreatic cancers in humans, is a devastating and virtually unexceptionally lethal malignancy, afflicting an estimated 213,000 individuals worldwide every year. Accounting for more than 33,000 fatalities annually in the United States, it represents the fourth most common cause of cancer-related deaths.[1] Despite considerable recent research efforts aimed at better understanding the etiology and underlying pathophysiology and at the development of novel diagnostic and therapeutic strategies, this gain in understanding has not yet led to improvement of the overall prognosis of patients suffering from PDAC.[2,3] Herein we present a review of the literature on the molecular genetics of PDAC, with an emphasis on recent data implicating promising novel diagnostic and therapeutic approaches for preclinical evaluation and, possibly, subsequent clinical application for the benefit of patients suffering from this dire malady. In the following text the terms pancreatic cancer and PDAC are used synonymously.

Molecular Abnormalities of Pancreatic Cancer

Genomic (DNA) Alterations

Genomic DNA abnormalities frequently found in pancreatic cancers comprise chromosomal aberrations, copy number changes, activating mutations of oncogenes, as well as specific silencing mutations of tumor suppressor and caretaker genes, epigenetic silencing, telomeric alterations, and mutations in mitochondrial DNA (mtDNA).

Copy Number Aberrations

Due to chromosomal instability, a common feature of most solid tumors, almost every pancreatic cancer harbors several numerical or structural chromosomal alterations revealed by cytogenetic analysis.[4,5] The most common numerical changes observed in pancreatic cancer are losses on chromosomes 6, 12, 13, and 18, as well as gains on chromosomes 7 and 20; chromosomal breaks and rearrangements most frequently occur in regions involving 1p, 1q, 3p, 6q, 7q, 11p, 17p, and 19q.

Another technique that is commonly applied to identify regions of genomic losses at a "higher resolution" by means of polymorphic microsatellite markers is called allelotyping. In a recent study Iacobuzio-Donahue et al

Supported by National Institutes of Health grants R01CA113669 and R21DK072532, The Sol Goldman Pancreatic Cancer Research Center, and an American Association for Cancer Research-PanCAN award (to A.M.). G.F. was supported by a fellowship grant within the Postdoc-Programme of the German Academic Exchange Service.

Accepted for publication October 19, 2007.

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analyzed ~80 pancreatic cancer xenografts by means of 386 microsatellite markers.[6] Allelic losses found most commonly involved chromosomal regions 9p, 17p, and 18q, covering the tumor suppressor genes *CDKN2A*, *TP53*, and *DPC4/SMAD4/MADH4*, respectively. Additional losses were frequently found in 3p, 4q, 5q, 6q, 8p, 12q, 14q, 21q, and 22q. Many of these regions have been linked to candidate tumor suppressor genes, eg, the stress-activated protein kinase *MKK4* (17p), which has been suggested to be involved in metastatic spread,[7] or receptors of the transforming growth factor (TGF)-β signaling pathway *TGFBR1* (9q), *TGFBR2* (3p),[8] and *ACVR1B* (12q).[9] Interestingly, allelotype analysis of microdissected pancreatic intraepithelial neoplasia (PanIN) samples revealed loss of heterozygosity in several of the chromosomal regions also found in pancreatic cancer, including 9p, 17p, and 18q.[10,11]

Comparative genomic hybridization (CGH) can be used to discover genomic deletions as well as amplifications, providing the potential to uncover both potential tumor suppressor genes and oncogenes. For CGH, samples of non-neoplastic and tumor cell DNA are labeled with different dyes and hybridized against each other. Subsequently, the relative ratio of the two dyes indicates regions of cancer-associated gains or losses. Conventional CGH was originally performed using metaphase spreads, with the major drawbacks of relatively low resolution and frequent difficulties to map precisely the regions of genomic amplifications or losses.[12] More recently, several array-based CGH techniques have been developed, using microarrays that are spotted with bacterial artificial chromosomes (BAC arrays),[13] cDNAs (cDNA microarrays),[14] or stretches of oligonucleotides (representational oligonucleotide microarray analysis),[15] providing a significantly higher resolution than conventional CGH (up to 30 kb) and often allowing for precise mapping of deleted or amplified regions and genes included therein. In the setting of pancreatic cancer, array CGH revealed several genomic amplifications, including *C-MYC* (8q), *EGFR* (7p), *KRAS22* (12p), *AKT2* (19q), and *AIB1* (20q), as well as deletions, including *DPC4/SMAD4/MADH4* (18q), *CDKN2A* (9p), *FHIT* (3p), and *MKK4* (17p).[16,17] Using the example of thymidylate synthase, which has previously been linked to responsiveness to 5-fluorouracil treatment, a recent report by Brody et al suggests that results from studies examining copy number aberrations should be interpreted with particular caution, since copy numbers determined in tumor cells are not necessarily identical throughout the whole tumor and might vary over time, in response to chemotherapeutic agents or in metastatic foci as compared to primary tumors.[18]

Nuclear DNA Mutations: Oncogenes

Activating point mutations within the *KRAS* oncogene (12p) are present in 80 to 90% of pancreatic cancers, most commonly affecting codon 12 but also 13 or 61.[19] The activating mutations abolish the intrinsic GTPase activity of KRAS, resulting in constitutive activation of intracellular signal transduction (Figure 1). Of note, activating *KRAS* mutations are not only the most frequently found genetic abnormalities in pancreatic cancer but also seem to be among the earliest changes observed in nonmalignant precursor-lesions, already being present in about 30% of PanIN-1 lesions.[21,22] Rare pancreatic cancers with wild-type KRAS usually harbor mutations of *BRAF*.[23] Since both KRAS and BRAF function in activating the same Ras/Raf/MAP kinase signaling pathway, it explains why mutations of these two genes occur in a virtually exclusive pattern and further underscores the extraordinary importance of this signaling pathway in the genesis of pancreatic cancer. Other oncogenes involved in pancreatic cancer include *CMYC*, *AKT2*, and *EGFR*. *CMYC* amplifications and concomitant overexpression of CMYC can be detected in 50 to 60% of pancreatic cancers,[17,24] providing a potential target for the development of future therapeutic options due to the recent development of compounds specifically inhibiting Myc signaling.[25]

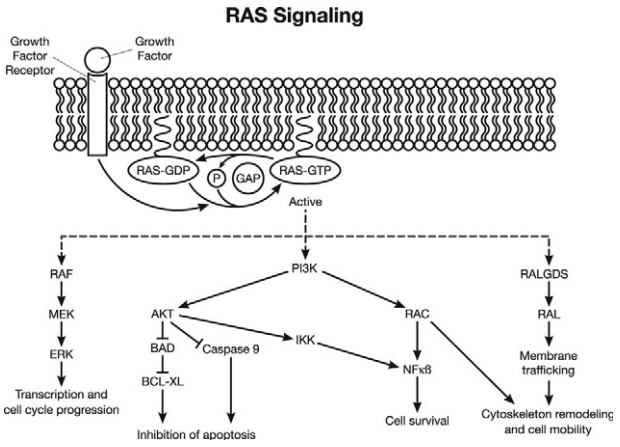

Figure 1. The RAS signaling pathway. RAS proteins are transiently activated by a wide range of extraneous signals, such as binding of growth factor ligands to the cognate growth factor receptor. In its active state, RAS is bound to GTP, and inactivation occurs through guanosine triphosphate-activating proteins (GAPs), which promote GTP hydrolysis. RAS proteins engage a number of downstream effector pathways, including RAF-mitogen activated protein kinase (RAF-MAPK), phosphoinsositide-3-kinase (PI3K), and RalGDS pathways. The proximal proteins in these signaling cascades, in turn, activate downstream intermediaries (eg, MEK/ERK, AKT, RAL) that mediate diverse cellular functions such as proliferation, cell survival, and cell motility. Oncogenic mutations of the *KRAS2* gene, which compromises the intrinsic GTPase activity of the encoded protein, render the pathway constitutively active in pancreatic cancer. (Reproduced with permission from the International Society of Gastrointestinal Oncology).[20]

Although amplifications of *AKT2* are found in less than 5% of cases,[26] the Akt signaling pathway is activated in 30 to 40% of pancreatic cancers,[27] suggesting additional underlying mechanisms of pathway activation, eg, loss of *PTEN*. Specific inhibitors of Akt signaling have been described,[28] which might give rise to novel therapeutic strategies in the near future. Constitutive activation of the EGFR pathway through amplification of the *EGFR* gene or other mechanisms has been described in pancreatic cancer.[16] Recent approaches were aimed to target this signaling pathway using monoclonal antibodies or small-molecule tyrosine kinase inhibitors.[29]

Tumor Suppressor Genes

The cyclin-dependent kinase *CDKN2A/p16* on chromosome 9p inhibits cell cycle progression through the G_1-S checkpoint and constitutes the most frequently inactivated gene in pancreatic cancers.[19,30] More than 90% of pancreatic cancers show loss of CDKN2A/p16 function, which can occur due to homozygous deletions (~40%), mutations with loss of the second allele (~40%), or epigenetic silencing by promoter hypermethylation (10–15%). Interestingly, loss of nuclear p16 protein expression is already observed in 30% of PanIN-1, in 55% of PanIN-2, and in 71% of PanIN-3 lesions.[31] Approximately 30% of homozygous *CDKN2A/p16* mutations also include the *MTAP* gene, which has recently been proposed as potential therapeutic target.[32]

Deleted in pancreatic carcinoma 4 (*DPC4/SMAD4/MADH4*) on chromosome 18q21 is inactivated in about 55% of pancreatic cancers.[33] This is due to mutations in one allele and loss of the second allele in about 25% of cases and due to homozygous mutations in 30% of cases. Of note, *DPC4/SMAD4/MADH4* mutations are very rarely observed in other malignancies.[34] Loss of DPC4/SMAD4/MADH4 function results in reduced growth inhibition and increased proliferation through interference with intracellular signaling cascades downstream of cell surface receptors of the TGF-β family (Figure 2). Abrogation of DPC4 function appears to be a rather late event in the pathogenesis of pancreatic carcinomas, as it is not observed in the majority of PanIN-3 lesions.[35]

Third, *TP53* on chromosome 17p is inactivated in 50 to 75% of pancreatic cancers, almost exclusively due to intragenic mutations of one allele in combination with loss of the second allele. Wild-type TP53 induces cell cycle arrest and apoptosis in response to DNA damage, and therefore loss of TP53 function allows for accumulation of additional genetic aberrations. Nuclear accumulation of mutated TP53 is only observed in advanced PanIN-3 lesions, and loss of TP53 function is, like DPC4, considered to be a late event in the pathogenic cascade of pancreatic cancer development.[35]

While loss of these three (*CDKN2A*, *DPC4* and *TP53*) genes is found in the majority of pancreatic cancers, other tumor suppressor genes are inactivated in smaller subsets (<10%) of PDAC, eg, *LKB1/STK11* on chromosome 19p,[36] *TGF-βR1* on 9q, *TGF-βR2* on 3p, *RB1* on 13q,[30] and *MKK4* on 17p.[37] Interestingly, MKK4 seems to be inactivated specifically in metastatic lesions, suggesting that wild-type MKK4 might have the ability to inhibit development of metastases through a yet unknown mechanism.

Caretaker Genes

As opposed to alterations of oncogenes and tumor suppressor genes, which both drive the neoplastic process by increasing tumor cell numbers through increased tumor cell growth or inhibition of cell death and cell cycle arrest, a third class of genes is commonly involved in the development of malignant neoplasias, such that when mutated, they act in a fundamentally different way. Such genes are commonly referred to as caretakers or stability genes.[38] Caretaker genes minimize genetic alterations during DNA replication so that loss of their function can lead to accumulation of additional mutations in different other genes.[39]

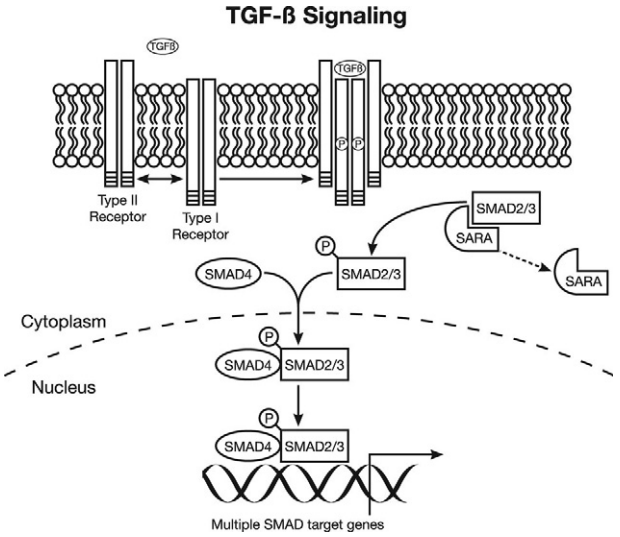

Figure 2. The TGF-β signaling pathway. The TGF-β signaling pathway is activated by binding of the ligand TGF-β to TGF-β type II receptor at the cell surface, which facilitates the recruitment and phosphorylation of type I TGF-β receptor. The latter activates the SMAD transcription factors SMAD 2 or 3 by phosphorylation, which in turn releases them from the cytoplasmic anchoring protein SMAD anchor for receptor activation (SARA). The phosphorylated SMAD 2/3 proteins complex with SMAD4 and translocate to the nucleus where they modulate the expression of multiple SMAD target genes. Note that TGF-β signaling can also occur via SMAD-independent mechanisms (not shown). (Reproduced with permission from the International Society of Gastrointestinal Oncology).[20]

The DNA damage repair genes *hMLH1* and *hMSH2* are inactivated in a small subset of familial pancreatic cancers, but rarely in sporadic cases,[40] mostly medullary carcinomas of the pancreas.[41] These tumors constitute a separate entity that is important to recognize, since on the one hand they tend to carry a more favorable prognosis than ductal adenocarcinomas and, on the other hand, they may indicate hereditary nonpolyposis colorectal cancer syndrome, in which case patients might benefit from genetic counseling. Medullary carcinomas of the pancreas show a typical medullary histology, which is characterized by poor differentiation, pushing borders, and syncytial growth pattern.[41,42]

Moreover, a subgroup of pancreatic cancers carries mutations in genes of the Fanconi's anemia DNA repair pathway. Mutations affecting the *BRCA2* gene have been found in approximately 17% of familial pancreatic cancers,[43] and *FANCC* and *FANCG* mutations have been described as rare findings in sporadic pancreatic cancers,[44] all of which are thought to be part of the Fanconi's anemia DNA repair pathway. (See Kennedy and D'Andrea [45] and Taniguchi and D'Andrea[46] for a recent review and for a schematic overview of the Fanconi's anemia pathway.)

Mutations in Mitochondrial DNA

As they harbor the enzymatic machinery of the respiratory cycle, mitochondria generally contain higher concentrations of free radicals, rendering mtDNA more prone to accumulation of genetic alterations. Moreover, mitochondrial DNA damage repair is overall less effective than in the nucleus, and therefore mutations of mtDNA are quite commonly observed in a variety of malignant neoplasias, including pancreatic cancers.[47,48] Although mutations in mtDNA most likely do not contribute to PDAC pathogenesis but rather represent a bystander effect, they might nevertheless be an interesting diagnostic target. MtDNA is six- to eightfold more abundant in the cell than nuclear DNA, and thus it might be feasible to use the high frequency of mtDNA mutations for the development of screening tests for early detection of pancreatic cancer. A recent study from our own group has demonstrated in principle, that mtDNA mutations can be detected by means of a high-throughput mtDNA microarray of pancreatic juice aspiration samples from patients with pancreatic cancer.[49] It is entirely possible that in the near future less expensive and more sensitive methods will become available, possibly allowing for screening using sample materials that are easier and less invasively to obtain, eg, peripheral blood samples, so that ideally even patients at average or only slightly increased risk could be tested regularly as a tool of secondary prophylaxis.

Telomere Length Abnormalities

Physiologically, telomeres, which consist of repeats of the sequence motif TTAGGG at the ends of each chromosome, confer chromosomal integrity during cell replication and prevent chromosome ends from fusing together.[50] Marked telomere shortening can already be found in more than 90% of even the lowest grade PanIN lesions, so that occurrence of telomere abnormalities are among the earliest known events in the cascade of pancreatic cancer development.[51] In the setting of cancer progression, telomeres play a role similar to that of caretaker genes in maintaining genomic integrity. It is assumed that loss of telomere function might permit subsequent accumulation of additional genomic changes at the chromosomal level that confer progression toward a fully malignant phenotype.[52,53]

Epigenetic Abnormalities

In addition to intragenic mutations and allelic loss, silencing of tumor suppressor genes through epigenetic mechanisms is a frequent finding in many cancers.[54] Transcriptional abrogation by epigenetic silencing is mediated most commonly by hypermethylation of CpG islands in promoter regions of tumor suppressor genes. In pancreatic cancer, epigenetic silencing often affects genes that function as tumor suppressors or are involved in key homeostatic pathways, including *P16/CDKN2A*, *E-cadherin*, *retinoic acid-β*, *osteonectin*, *suppressor of cytokine signaling-1*, and *tumor suppressor in lung cancer 1*.[55,56] More recently, promoter hypermethylation of human *Hedgehog interacting protein* was found in the majority of examined pancreatic cancer cell lines and primary tumor samples,[57] potentially contributing to increased Hedgehog signaling observed in pancreatic cancers. Aberrant DNA methylation occurs already in PanIN-2 and PanIN-3 lesions,[58] and thus diagnostic exploitation of this phenomenon, ie, detection of aberrantly methylated DNA in clinical samples as a strategy for early detection of pancreatic cancer, is currently an area of active research efforts. For example a recent study was successful in detecting methylation of proenkephalin sequences in about 60% of pancreatic juice samples obtained from pancreatic cancer patients but not from controls.[59]

Interestingly, promoter hypermethylation, though most common, is not the only epigenetic modification involved in carcinogenesis. More recently it was found that the reverse means of aberrant gene regulation (hypomethylation) is also exploited by pancreatic cancer cells, ie, some genes, including *maspin*, *S100P*, *mesothelin*, *prostate stem cell antigen*, and *claudin-4*, can be overexpressed due to promoter hypomethylation.[60]

Transcriptomic Changes

Development and better availability of new global gene expression analysis tools such as serial analysis of gene expression and oligonucleotide and cDNA microarrays in recent years have catalyzed an enormous increase in the generation of data characterizing abnormalities at the transcriptional (RNA) level in a variety of human cancers. In particular, several studies were performed to identify genes that are differentially expressed in pancreatic cancers as compared to normal, non-neoplastic pancreatic tissues.[61–68]

Interestingly, six genes—*keratin 19*, *retinoic acid-induced 3*, *secretory leukocyte protease inhibitor*, *stratifin*, *tetraspan 1*, and *transglutaminase 2*—were found to be overexpressed by all three techniques (serial analysis of gene expression and oligonucleotide and cDNA microarrays). It has yet to be elucidated whether up-regulation of these genes is only a bystander phenomenon or is mechanistically involved in malignant transformation and might pose a potential target for the development of novel therapeutic strategies. Moreover, differential gene expression might be used to establish new diagnostic tools such as tumor imaging or early detection of pancreatic cancer.

The tight junction protein claudin 4 was found to be overexpressed in PanIN lesions and in fully invasive pancreatic cancer tissue by microarray analysis, and overexpression was confirmed by immunohistochemistry.[69] Radiolabeled anti-claudin 4 antibodies have recently been tested successfully in a preclinical setting as imaging tools as well as for therapeutic purposes in murine xenograft models of human pancreatic cancer.[70,71]

Mesothelin was originally identified by serial analysis of gene expression as being overexpressed in pancreatic

cancer, and validation by immunohistochemistry revealed it is almost exclusively expressed in neoplastic cells but not in neighboring nonmalignant tissue.[72] As a result of this initial finding, both an experimental tumor vaccine against mesothelin and a conjugated immunotoxin directed against mesothelin are currently undergoing initial evaluation in clinical trials.[73]

Prostate stem cell antigen, originally thought to be restricted to prostatic basal cells and prostate carcinomas, was found by serial analysis of gene expression to be expressed in about 60% of pancreatic cancers, but not in normal, non-neoplastic pancreas tissues.[61] Following up on these findings, prostate stem cell antigen has thereafter been successfully tested as a potential target for immunotherapy[74] as well as for diagnostic imaging in murine xenograft models of human pancreatic cancer.[71]

Yes-associated protein, the mammalian homologue of Yorkie, the main effector of the Hippo pathway, has recently found to be overexpressed at the RNA and protein level in pancreatic cancer cells, suggesting a possible role of this pathway in pancreatic carcinogenesis,[75] likely through interaction with TGF-β signaling.[76] Survivin, a major suppressor of apoptosis, is expressed at the RNA and protein level in low- to high-grade PanIN lesions and fully invasive pancreatic ductal adenocarcinomas in increasing levels, but not in neighboring non-neoplastic tissue, and it is thought to be involved in carcinogenesis as well as drug resistance.[77,78] It might therefore be a promising target both as diagnostic marker for early detection as well as for therapeutic intervention.

Moreover, recently aberrant reactivation of the Hedgehog and Notch signaling pathways, and concomitant overexpression of their respective target genes, has been described in the majority of pancreatic cancers.[79–82] Inhibition of Notch-1 lead to growth inhibition and increased apoptosis in pancreatic cancer cell lines *in vitro*,[83,84] and ligand overexpression has been linked to neovascularization *in vivo*.[85] To our knowledge, studies examining *in vivo* effects of Notch inhibition, eg, using xenograft model systems of pancreatic cancer, are still lacking at the time of this manuscript's preparation.

Hedgehog inhibition with the small molecule smoothened inhibitor cyclopamine has been found to increase cytotoxic effects of paclitaxel treatment and radiation on pancreatic cancer cells *in vitro*[86] and to inhibit growth of pancreatic cancer xenografts and metastases *in vivo*.[79,80,87] A simplified, schematic overview of the Hedgehog signaling pathway in mammals is given in Figure 3.

It has been suggested that reactivation of embryonic signaling pathways might be involved in maintaining a subset of cancer cells with stem cell-like properties, ie, putative cancer stem cells. A recent study has described a subpopulation of $CD24^+$, $CD44^+$, and ESA^+ cells with cancer stem cell-like properties, including self-renewal and strikingly enhanced tumorigenic potential in soft agar as well as in athymic mice. Interestingly, this subpopulation was also characterized by marked overexpression of Sonic Hedgehog ligand (SHH) by more than 50-fold as compared to normal tissue, whereas overexpression in "bulk" tumor cells was found to be only about fivefold.[88]

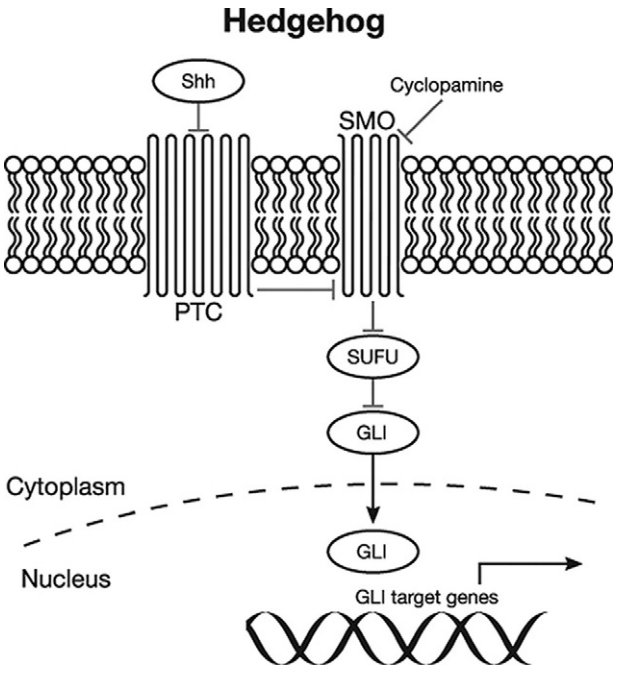

Figure 3. The Hedgehog signaling pathway. The Hedgehog (Hh) signaling pathway involves a series of inhibitory interactions between secreted ligands, cell surface receptors, and cytoplasmic proteins, culminating in the activation of transcription and up-regulation of Hh target genes by the DNA-binding transcription factor GLI. The Hh signaling pathway is activated by binding of Hh ligands—Sonic, Desert, or Indian hedgehog (SHH, DHH, IHH)—to the 12-transmembrane receptor Patched (PTCH). This interaction releases the inhibitory effects of PTC on the 7-transmembrane smoothened (SMO) receptor, and the conformational change with activation that SMO undergoes, in turn, facilitates its inhibition of the cytoplasmic protein Suppressor of Fused (SUFU). SUFU inhibition allows GLI to escape cytoplasmic sequestration, on which it undergoes nuclear translocation and activates the transcription of Hh target genes.

Another study by our own group identified a subpopulation of aldehyde dehydrogenase-"bright" cells with enhanced tumorigenic potential that showed increased expression of the Hedgehog target gene *Gli1* and were diminished by Hedgehog inhibition with cyclopamine.[87] Presently, development of new, clinically applicable Hedgehog inhibitors is being pursued by several pharmaceutical companies and will hopefully soon be available for trials in a clinical setting.

Proteomic Abnormalities

The word proteome denotes the ensemble of proteins synthesized by a cell or, *mutatis mutandis*, a certain type of cells, eg, pancreatic cancer cells, making up the bulk neoplastic tissue. Studying gene expression differences at the protein level can in a way be considered as the most straightforward approach, as it bypasses the need to identify possibly different underlying mechanisms, eg, occurrence of intragenic mutations, hetero- or homozygous deletions, or epigenetic alterations, as discussed above.

The past decade has seen great progress in the development of high-throughput techniques addressing protein expression changes, including chip-based arrays that allow determination of a plethora of proteins within a

given liquid sample in parallel, or tissue microarrays, enabling examination of expression of selected proteins in a great number of tissue specimens with antibody-based protocols like immunohistochemistry or immunofluorescence in one assay.

It has become evident from proteomic studies that during the multi-stepwise development of pancreatic cancer, changes in the protein expression pattern do not occur in a random manner but can be grouped into early, intermediate, and late changes, which mirror the stepwise accumulation of genomic alterations as discussed previously.[35] (See Feldmann et al[89] and Singh and Maitra[90] for more detailed reviews of molecular abnormalities observed in precursor lesions of PDAC.) This observation might have direct clinical implications, eg, in the quest to identify novel tumor markers for early detection. While a protein like prostate stem cell antigen, which is already secreted by the earliest PanIN-1 lesions, might be an extremely sensitive marker for early detection, it might nevertheless be of limited clinical value due to the common occurrence of low-grade PanINs in pancreata of older individuals. Detection of mesothelin, which is expressed only in late PanIN-3 lesions and fully invasive cancer tissues, from pancreatic juice could on the other hand have much more severe prognostic implications.

Examination of protein expression patterns from pancreatic juice samples using surface-enhanced laser desorption and ionization mass spectrometry-based protein chips has led to the discovery of hepatocarcinoma-intestine-pancreas/pancreatitis-associated-protein-1 (HIP/PAP-1) overexpression in pancreatic cancers. It has been shown that individuals with high HIP/PAP-1 concentrations >20 μg/ml had a more than 20-fold increased risk of developing pancreatic cancer.[91] Surface-enhanced laser desorption and ionization mass spectrometry has recently successfully been tested to predict pancreatic cancer from patient serum samples with 78% sensitivity and a specificity of 97%.[92]

Another experimental approach to determine altered protein expression from liquid samples exploits liquid chromatography tandem mass spectrometry, which enables identification of proteins based on their individual charge/mass ratios. This technique has recently been used to identify 170 genes expressed in pancreatic juice of patients with pancreatic cancer, including several previously known tumor markers like CEA, MUC1, or HIP/PAP-1.[93]

Mouse Models of Pancreatic Cancer

Xenograft Models

Research aimed at better pathophysiological understanding and development of novel therapeutic strategies against pancreatic cancer has long been hampered by the lack of suitable small animal models that satisfactorily resemble clinical features of human disease. Traditionally, initial screening of potential new drugs for *in vivo* efficacy is most commonly performed on subcutaneous (athymic nude or SCID) mouse xenograft models, derived from either human pancreatic cancer cell lines or primary cancer tissue specimens, which accurately mirror genotypic features of the parental tumors and susceptibility or resistance to anticancer agents even after serial transplantation.[94] While these *in vivo* models are relatively easy to generate and allow for easy screening of putative new drugs on several xenograft tumor lines, and thus are undoubtedly of immense value for initial pharmacological studies, they also suffer from some fundamental drawbacks.[95,96] First, as these xenogenic transplantation models lack T cell or both B and T cell responses, effects of the tumor environment including immunological effects are poorly mirrored. Second, as these models are heterotopic, they do not reflect the physiological microenvironment in the pancreas, including anatomical conditions and effects of direct tumor invasion into neighboring structures, cytokine and chemokine secretion patterns, vascularization and tumor-induced neoangiogenesis. Third, and related to the first two points, subcutaneous xenografts of pancreatic cancers hardly ever metastasize, so that mechanisms involved in metastatic spread cannot be studied using these models, although this is one of the most prominent features of pancreatic cancer in humans that de facto determines the overall survival.

Therefore, recent years have seen increasing use of orthotopic xenograft models, which are more tedious to generate but are able to overcome many of these shortcomings. They are particularly useful to study drug effects on tumor microenvironment including neoangiogenesis and metastatic spread.[87,97] Tumor cells are either injected directly into the mouse pancreas, often in the form of concentrated tumor cell suspensions containing Matrigel, or alternatively are surgically implanted into the murine pancreas as chunks of primary tumor tissues or xenografts. While the first variant is faster and allows generation of xenografts from cell lines without the need to first grow subcutaneous xenografts, the second technique is more reliable in preventing intraperitoneal leakage of tumor cells after injection and also allows for the use of primary tissue samples, thus bypassing the need for *in vitro* established cell lines.

Transgenic Mouse Models

As opposed to subcutaneous or orthotopic xenografts, transgenic mouse models possess the potential to mimic human disease in a syngeneic system. While they can obviously not represent the whole spectrum of genetic aberrations observed in human pancreatic cancers, these models are of tremendous value for translational studies as they also include a syngeneic tumor environment as well as a fully intact immune system. The earliest models, described almost two decades ago, used acinar-specific elastase promoter to target oncogene expression to the murine pancreas, resulting in neoplasms of predominantly acinar histogenesis.[98,99]

The first mouse model most closely resembling histopathological features of human ductal adenocarcinoma was described in 2003.[100] In this model, expression of oncogenic $Kras^{G12D}$ is suppressed by combination with

Lox-STOP-Lox (LSL) constructs. Repression is released on Cre-mediated excision of the LSL cassettes and subsequent recombination. Targeting of transgene expression to the murine pancreas is achieved by expression of Cre recombinase under the control of pancreas-specific promoters Pdx1 or P48. It is commonly assumed that Pdx1-/P48-double positive cells give rise to virtually all mature cells in the pancreas.[100–102] During embryonic development, expression of Pdx1/PF1 starts around E8.5, and P48/PTF1 expression begins slightly later. All of these mice develop ductal lesions resembling human PanIN lesions, which eventually progress into a fully invasive and metastatic adenocarcinoma phenotype in a small percentage (<10%) of animals. The long latency of 6 to 8 months and low frequency suggest the need of additional genetic alterations, likely including the INK4a-Rb or Arf-p53 pathways, whereas the majority of cells expressing oncogenic $Kras^{G12D}$ alone might undergo ras-induced senescence and thus fail to accumulate additional hits required to develop a fully malignant cancer phenotype.[103] Similar to these observations, Grippo et al found multifocal acinar cell hyperplasia in Ela-$Kras^{G12D}$ mice 1 to 2 months of age. Interestingly, by the age of 6 to 18 months, some of these lesions underwent a process the authors referred to as acinar-to-ductal metaplasia and presented with a more duct-like phenotype, including expression of CK-19.[104]

In fact, newer transgenic models have recently been described, in that $Kras^{G12D}$ expression is directed to the pancreas by means of Cre recombinase under the control of a Pdx1 promoter in combination with inhibition of the INK4A/Arf or p53 pathways, respectively: LSL-$Kras^{G12D}$; INK4a/$Arf^{lox/lox}$;Pdx1-Cre,[105] LSL-Kras;$p53^{lox/lox}$;Pdx1-Cre,[106] and LSL-Kras;$Trp53^{R172H}$;Pdx1-Cre.[107] Mice with knockout of the INK4a/Arf locus, resulting in loss of both murine p16 and p19 function, develop poorly differentiated carcinomas very rapidly and start to die before 7 weeks of age,[105] whereas inhibition of p53 function, either by genetic knockout[106] or by introduction of a dominant negative p53 allele,[107] preferentially leads to moderately to well-differentiated adenocarcinomas. Of note, abrogation of either INK4a/Arf or p53 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, eventually culminating in a fully malignant phenotype during pancreatic carcinogenesis.[105]

The described models, and especially the latter,[107] to date also represent those recapitulating most closely the clinicopathological characteristics of human pancreatic adenocarcinomas and thus carry enormous potential for future translational studies in providing an excellent platform for preclinical evaluation of novel drugs and other therapeutic approaches. Several signaling pathways shown to be aberrantly activated in human pancreatic cancers are also found to be turned on in the discussed mouse models, including the Hedgehog and Notch signaling pathways. As these pathways can be targeted by using blocking antibodies or small-molecule inhibitors, for example, it is tempting to speculate whether therapeutic regimens exploiting blockade of these pathways might have an effect on survival in these preclinical models and might moreover eventually be translated into applications in a clinical setting.

Using a mouse model of TGF-α-induced pancreatic cancer previously described by Wagner and colleagues,[108] a recent study from the same group found that pancreatic carcinomas occurring in C57BL/6-EL-TGF-alpha;$Trp53^{-/-}$ mice induced a distinct immune response including secretion of proinflammatory cytokines and occurrence of tumor-specific regulatory T lymphocytes in the host. Surprisingly, tumor-derived cell lines did not form xenograft tumors in immunocompetent mice of the same genetic background, a finding that is yet to be fully understood. From these results the authors conclude that spontaneous tumors arising in this mouse model are recognized by the host immune system and that therefore transgenic models might be more suitable than xenografts to evaluate certain immunotherapeutic regimens in a preclinical setting.[109]

An immunotherapeutic approach using tumor vaccination with tumor/dendritic cell fusion supplemented by injection of superantigen staphylococcal enterotoxin B leads to generation of tumor-specific cytotoxic T lymphocytes and increased survival in a previously described transgenic mouse model of acinar cell-type pancreatic cancer overexpressing MUC1,[110,111] which is based on a model originally described by Tevethia et al, in which pancreatic acinar carcinomas are induced by pancreas-specific expression of a transgene containing the N-terminal amino acids 1–127 of large T-cell antigen under the control of THE elastase-1 promoter.[112]

In the last few years, several other transgenic mouse models targeting different pathways thought to be involved in pancreatic carcinogenesis, which allow deeper insight in underlying etiological and pathogenetic mechanisms, have been described. Three recent mouse models addressed the role of TGF-β signaling in pancreatic cancer (see Figure 2 for an overview of the TGF-β pathway) by pancreas-specific deletion of either SMAD4[113,114] or TGF-β receptor type II (TGFBR2).[115] Interestingly, as observed for interruption of INK4a/Arf and p53 signaling pathways, neither *SMAD4* nor *TGFBR2* deletion alone is sufficient to induce pancreatic neoplasia. However, when combined with oncogenic $Kras^{G12D}$, development of fully malignant carcinomas is enhanced with shorter latencies than observed for $Kras^{G12D}$ alone. While mice lacking pancreatic TGFBR2 expression in the presence of $Kras^{G12D}$ all develop well-differentiated adenocarcinomas and die of their disease within 200 days, with a median survival of 59 days,[115] loss of *SMAD4* in $Kras^{G12D}$-expressing pancreata leads to development of premalignant precursor lesions of intraductal papillary mucinous neoplasm or mucinous cystic neoplasm type, which can progress into fully malignant carcinomas. For the latter, median survival of 8 months[114] and 7 to 12 weeks[113] have been described. TGF-β signaling has previously been proposed to mediate epithelial-to-mesenchymal transition,[116] and in line with this concept, INK4A/Arf-null;$Kras^{G12D}$ mice with wild-type SMAD4 usually present with poorly differentiated carcinomas, whereas mice that also lack SMAD4 expression, develop mostly

well- to moderately differentiated pancreatic adenocarcinomas that express epithelial markers such as E-cadherin or cytokeratin-19.

Studies examining the role of Hedgehog signaling demonstrated that overexpression of the Hedgehog ligand Sonic Hedgehog (SHH) in the pancreas is sufficient to induce formation of PanIN lesions.[79] Introduction of a dominant-active form of the activating Hedgehog transcription factor GLI2 (CLEG2) leads to formation of poorly differentiated pancreatic carcinomas that lack CK-19 expression in about 30% of PDX1-Cre;CLEG2 mice, which seem to develop without evidence of PanINs as precursor lesions.[117] Of note, combined expression of CLEG2 and $Kras^{G12D}$ in the murine pancreas results in formation of PanIN lesions and pancreatic carcinomas in all studied mice, with shorter latency and dramatically decreased overall survival of only 3 to 8 weeks.

Overexpression of COX-2 in the pancreas under the control of a keratin 5 promoter leads to formation of IPMN- and PanIN-like lesions, enhanced ras signaling, inflammation, and fibrosis in a subset of mice.[118] Clerc and colleagues described development of pancreatic carcinomas through acinar-to-ductal transition in three of 20 mice overexpressing CCK2/gastrin receptor in pancreatic acinar cells.[119]

Other Promising Translational Studies

Some novel therapeutic strategies that were developed based on recent advances in our understanding of molecular biology and pathophysiology of pancreatic cancer are actually on the verge of being tested in the clinics and might soon be beneficial, if not for all, for at least a subset of patients suffering from PDAC. Targeting aberrantly reactivated developmental signaling pathways, namely the Hedgehog,[79,80] Notch,[84,120] and Wnt[121,122] signaling pathways, by means of blocking antibodies or small-molecule inhibitors are highly promising future treatment options in PDAC, as already mentioned above. These approaches are particularly exciting, as first evidence is emerging that at least some of these strategies might target distinct subpopulations of cancer cells with stem cell-like properties[87,88] and could therefore carry the potential to overcome development of drug resistance and tumor recurrence commonly observed today with standard chemotherapy regimens in several malignancies. However, as evaluation of treatment strategies targeting these pathways has only just begun and, moreover, the concept of cancer stem cells is still highly and controversially discussed in the scientific community, it is not possible yet to give a final judgment as to the full therapeutic potential of these approaches.

Multiple immunotherapeutic trials have been performed in a clinical setting at several institutions, including our own, with variable success.[123] Overall, immunotherapeutic regimens using monoclonal antibodies have so far been the most successful approaches. However, tumor vaccination protocols for pancreatic cancer are also increasingly translated into clinical therapies, some of which showed extremely encouraging initial results. A phase I study conducted at our own institution using a whole tumor cell vaccine coadministered with granulocyte macrophage–colony-stimulating factor was shown to be safe and potentially effective in patients with early stage disease.[124] Follow-up phase II clinical trials are currently ongoing.

Another idea is based on the recent observation that a small subset of PDAC that carry mutations in the Fanconi anemia/BRCA2 pathway is highly sensitive to cross-linking agents such as mitomycin C or cisplatin *in vitro* and *in vivo*.[44,125] Although affecting only 5 to 10% of human PDAC, these findings might soon be translated into effective chemotherapeutic treatment regimens for these cases. A phase II clinical trial is presently underway.

Overexpression of the chemokine receptor CXCR4 is thought to be involved in the development of metastases, possibly through attraction of CXCR4-positive tumor cells by SDF-1alpha/CXCL12.[126] Moreover, tumor neoangiogenesis and growth of subcutaneous murine xenografts could be inhibited by administration of anti-CXCR4 blocking antibodies.[127] Therefore, the SDF-1/CXCR4 signaling axis might be yet another promising therapeutic target for PDAC in the near future.

Using *in vitro* and *in vivo* model systems, treatment with tumor necrosis factor-related apoptosis-inducing ligand has been demonstrated to be a valid novel therapeutic strategy in several solid tumors, including pancreatic cancer, especially when combined with other substances sensitizing cancer cells to tumor necrosis factor-related apoptosis-inducing ligand by overcoming resistance to apoptosis.[128,129] This strategy is also most likely soon to undergo initial evaluation in a clinical setting.

Clinical use of several commonly administered cytotoxic chemotherapeutics is often limited by systemic adverse effects. Utilization of nanotechnology carries the potential to minimize these problems, by encapsulating drugs in nanoparticles and via specific delivery to tumor cells by passive (through enhanced permeation and retention effect) or active targeting (nanoparticles coated with antibodies or receptor analogs directed against tumor-specific surface antigens), thereby drastically reducing the total amount of drug needed to achieve a therapeutic response.[130] Moreover, encapsulation into nanoparticles can overcome poor water solubility, a common shortcoming of multiple substances being screened as potential drugs. An example is curcumin, a plant extract from *Curcuma longa*, which has long been known in traditional Indian medicine and has well-described anti-inflammatory and antineoplastic properties *in vitro*. While promising *in vivo* results have been achieved in animal models of PDAC in that comparably huge doses of curcumin could be administered,[131] as well as in clinical trials in preventing progression of preneoplastic colon adenomas,[132] broader application in different tumor types has been hampered by poor water solubility and almost zero resorption from the gastrointestinal tract and systemic bioavailability.[133,134] Nanoencapsulation of curcumin has recently been shown to render the drug readily water soluble while retaining its biological properties

in vitro.[135,136] However, the *in vivo* efficacy of these new formulations has yet to be evaluated.

Conclusion

Pancreatic cancer continues to prove resistant against most clinically available treatment options to date, and it remains to be one of the worst killers among human malignancies. It seems unlikely that potent therapeutic options, which would enable us to definitely cure or, more desirably, prevent all pancreatic cancers, will be available in the near future. However, based on recent advances, as described in this article, there is increasing hope that tenacious research efforts and better understanding of underlying biological characteristics will finally yield enhanced survival and better quality of life for patients suffering from this disease. Multimodal strategies combining different therapeutic strategies are likely to have the greatest chance for improvement in the coming years.

Acknowledgments

We thank Jennifer Parsons-Brumbaugh and Joseph Dieter for preparation of the illustrations. We apologize to colleagues whose work could not be cited due to lack of space.

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