Molecular biomarkers: their increasing role in the diagnosis, characterization, and therapy guidance in pancreatic cancer

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Abstract
The rapidly expanding knowledge of the pathogenesis of pancreatic cancer at the molecular level is providing new targets for disease characterization, early diagnosis, and drug discovery and development. Gene mutation analysis has provided insight on the pathogenesis and progression from preinvasive lesions to invasive cancer. Gene and protein expression profiling has advanced our understanding of pancreatic ductal adenocarcinoma identifying genes that are highly expressed in pancreatic cancers, providing more insight into the clinicopathologic features of pancreatic cancer, and revealing novel features related to the process of tissue invasion by these tumors. The increasing knowledge of the pathway activation profile in pancreatic cancer is yielding new targets but also new markers to select patients and guide and predict therapy efficacy. The discovery of genetic factors of which the presence predisposes pancreatic cancer to successful targeting, such as the association of BRCA2/Fanconi anemia genes defects and sensitivity to mitomycin C, will eventually lead to a more individualized treatment approach. In summary, several decades of intensive research have originated multiple factors or biomarkers that are likely to be helpful in the diagnosis, characterization, and therapy selection of pancreatic cancer patients. A deep understanding of the relative relevance of each biomarker will be key to efficiently diagnose this disease and direct our patients towards the drugs more likely to be of benefit based on their particular profile. The development of new preclinical models is of paramount importance to achieve these goals. [Mol Cancer Ther 2006;5(4):787–96]

Introduction
Pancreatic cancer remains a devastating disease. In 2004, the estimated incidence of the disease in the United States is 31,860 with an expected 31,270 deaths (1). At the time of diagnosis, 80% of patients have locally advanced or advanced disease for which no curative therapy exists, and 80% of patients treated with curative intent will recur in the first 2 years after surgical resection and will succumb to their disease (2). A desired strategy to improve care is to individualize cancer treatment by directing our patients according to their particular tumor or individual attributes towards those modalities that are more likely to offer a benefit. A cancer biomarker can be defined as a biological feature that provides either diagnostic, prognostic, predictive, or therapeutic information about a particular disease or subject. Some fundamental aspects are worth considering when searching for a biomarker. First, selecting the biological specimens to be used; several types of specimens are available for pancreatic cancer research, including pancreatic tissue, pancreatic juice, and body fluids such as serum or plasma. Second, defining the target for a biomarker; DNA, RNA, or protein can all be used as biomarkers, and the choice of the type of target has relevant implications. Third, it is vital that if a biomarker is to be used in a diagnostic setting, an early alteration with high specificity is also important. This will allow the diagnosis of the cancer before the tumor burden is such that surgical therapy is not applicable, which would be especially relevant in pancreatic cancer. Likewise, if a marker is to be used in a predictive setting, then a correlation of biomarker quantitation with tumor stage is also useful. Finally, reproducibility and ease of interpretation [ideally being dichotomous (present/absent), semiquantitative (−, +, ++, +++), or fully quantitative] are both valuable features.

There is an enormous effort to identify, characterize, and validate meaningful biomarkers because its successful development will represent a step forward in the individualization of cancer diagnosis, therapy, and monitoring. This mini-review will discuss recent advances in the field from a clinically oriented perspective, focusing on those biomarkers that are candidates for being incorporated in the successive steps of diagnosis, therapy selection, and therapy efficacy monitoring.
Source and End Points for Biomarker Development

Sources of Biological Material

Blood

Serum/plasma represents an ideal diagnostic specimen because of its easy and inexpensive accessibility. It has shown to be a particularly useful platform for protein analyses and large population–oriented biomarker assessment. The cellular component of blood is a frequently used source to obtain genomic DNA that can be used to determine gene polymorphism in targeted genes as well as germ-line mutations.

Pancreatic Juice

Pancreatic juice is an attractive source for identifying biomarkers of pancreatic cancer given its specificity. Juice is rich in proteins that are secreted directly from the pancreatic duct, and cancer cells are preferentially shed into the ductal lumen, making pancreatic juice a rich source of both cancer-specific proteins and genetic material. Whereas pancreatic juice may not be as easily accessible as serum, it is a more specific source for searching biomarker candidates associated with pancreatic cancer due to the proximity to the tumor.

Pancreatic Tumor Tissue

Using tumor tissue is the most direct approach to identify biomarkers as they are most likely present in cancer tissue at higher concentrations. An issue with relevant implications is how to prepare the tumor tissue (i.e., either using it as a whole or selecting for analysis only cancer cells). The former approach is logistically more convenient, but also it is rational as it recognizes the fact that in malignancy the tumor-host microenvironment plays a key part in the induction, selection, and expansion of the tumor cells. As an example, a pilot analysis using isotope-coding affinity tag profiling of whole-tumor pancreatic cancer samples showed that many of the differentially regulated proteins in cancer samples, compared with normal pancreatic tissue, were involved in stromal-epithelial interactions and likely originated in nontumor cells (3). This is an example of how the tumor stroma can be used for biomarker discovery as well as a potential therapeutic target. On the other hand, the use of enriched cancer cells may facilitate the discovery of very low abundant biomarkers. In this regard, laser capture microdissection of paraffin-embedded tissues may be useful, particularly for genetic analyses. The protein cross-linkage occurring in fixed tissues and the low amount of protein obtained make this a difficult approach for proteomic studies. Enrichment using procedures based on anti-epithelial antibodies have been proposed to obtain protein and mRNA (4).

It is evident, however, that the main limitation of using pancreatic tumor tissue is the very small and finite amounts of material available. A way to overcome this limitation is be to immortalize tumors ex vivo, propagating them on mice after the patient undergoes surgical resection of the primary tumor. Our group has generated a panel of 30 pancreatic cancer xenografts from fresh tumor samples obtained at the time of surgery, which are maintained alive through successive passes in mice. These tumors retain the fundamental features of their original cancer of origin in terms of key gene mutations, as well as genetic and protein expression. In addition, up to 10 successive passages neither induces alterations on these genetic characteristics nor changes their response to antitumor agents. This makes such platform a potentially infinite source of tumor material for correlative studies, as well as a promising platform for drug testing and biomarker discovery.

Genetic Markers

Pancreatic cancer is a genetic disease. Telomere abnormalities and signs of chromosome instability are the earliest alterations, and four genes are mutated in most cases (the KRAS, p16, p53, and MADH4/DPC4 genes). Other genetic abnormalities are seen at a much lower frequency (BRCA2, FANCC, FANCG, FBXW7, BAX, RB1, the transforming growth factor-β receptors TGFBR1 and TGFBR2, the activin receptors ACVR1B and ACVVR2, MKK4, STK11, p300) and will be discussed below if diagnostically or clinically relevant.

Telomere Analysis. Telomere shortening is the earliest and most prevalent genetic change identified in the precursor lesions (5, 6). Telomere attrition is thought to predispose to chromosome fusion and the resulting translocations, followed by their missegregation during mitosis. Later during tumorigenesis, telomerase activity is resumed, compensating the telomere erosive process while permitting continued chromosomal instability. The viability to image and quantitatively assess telomeres makes this alteration a potential diagnostic tool, particularly to characterize early or preinvasive lesions (7).

Genomic Mutation Analysis. The presence of mutations in key genes constitutes an ideal biomarker, as it gives a dichotomous readout and is very specific. However, most of the genetic abnormalities occur in the tumor cells and not in the germ line, and thus the limitation is acquiring tumor source material for analysis. On the other hand, those genes which carry germ-line mutations (such as BRCA2, FANCC, or FANCG) are easily detected and have potential for screening by genetic testing. There are several genes of which the alteration has shown to be relevant in pancreatic cancer, and are summarized in Table 1. The KRAS gene mediates signals from growth factor receptors and other signaling inputs. The mutations convert the normal K-Ras protein (a proto-oncogene) to an oncogene, causing the protein to become overactive in transmitting the growth factor-initiated signals. The gene is mutated in >90% of conventional pancreatic ductal carcinomas (8). Mutant KRAS has been extensively investigated as a marker of pancreatic cancer because mutations are almost entirely limited to one codon, are present in ~90% of pancreatic ductal adenocarcinomas, and can be readily detected using molecular assays. Unfortunately, KRAS mutations are not specific for invasive pancreatic cancer.
and they occur in patients with chronic pancreatitis, in individuals who smoke, and in \textit{in situ} neoplasias from patients without pancreatic cancer (9–11). Mutant \textit{KRAS} can also be detected in the blood of patients with pancreatic cancer, but in this setting, it is more often detectable in patients with advanced-stage cancers, limiting its diagnostic value (12). However, a sensitive assay has been developed that can quantify not just the presence but also the levels of mutant \textit{KRAS} in blood and pancreatic juice, and it remains possible that this quantification of mutant \textit{KRAS} levels could improve the diagnostic utility of \textit{KRAS} as an end point in pancreatic cancer (13). In addition, the addition of \textit{KRAS} mutation detection may improve the diagnostic accuracy of brush cytology done in extrahepatic biliary obstruction (14).

The retinoblastoma protein (Rb1) is a transcriptional regulator and regulates the entry of cells into S phase. Virtually all pancreatic carcinomas suffer a loss of p16 function through homozygous deletions (40%), mutation/loss of heterozygosity (40%), or promoter methylation associated with a lack of gene expression (20%; ref. 15). Biallelic deletions of p16INK4A/CDKN2A are associated with loss of the methylthioadenosine phosphorylase (MTAP), which plays an important role in the salvage pathway for the synthesis of adenosine (16).

In pancreatic ductal adenocarcinoma, \textit{TP53} gene mutations are found in ~70% of invasive cancers and as they generally occur relatively late in the process towards invasive cancer, the detection of \textit{TP53} mutations has been widely investigated as a potentially specific genetic marker in pancreatic cancer (17). Although a few nucleotide hotspots of \textit{TP53} gene mutation are known to exist, mutations occur throughout the gene (18). The presence of \textit{TP53} gene mutations in pancreatic juice samples and in brush cytology specimens has been reported in 40% to 50% of patients with pancreatic cancers (19). The detection of \textit{TP53} mutations by means of immunostaining of the protein product also has the potential to be a useful diagnostic strategy in combination with standard cytology (20).

Another relevant genetic event present in a significant proportion of pancreatic cancers is alterations in the genes coding the Smad proteins. Signals initiated on the binding of the extracellular proteins transforming growth factor and activin to their receptors are transmitted to the nucleus by the Smad proteins that are the product of the Smad family of genes (\textit{SMAD4} and \textit{DPC4}). Inactivating mutations in \textit{DPC4} are found in 55% of pancreatic carcinomas, and these include both homozygous deletions and intragenic mutations combined with loss of heterozygosity (21). Interestingly, there is a strong correlation between \textit{DPC4} mutation status and \textit{DPC4} protein expression by immunohistochemistry, which may facilitate its use as an adjunct to standard histologic diagnostic procedures (21).

\textbf{Epigenetic Determinants.} Pancreatic cancer has an increasingly relevant epigenetic component in its pathogenesis. Hypermethylation of CpG islands is a common mechanism by which tumor suppressor genes are inactivated. Many (60%) pancreatic carcinomas present hypermethylation of key genes whereas simultaneous methylation sites of at least four loci are seen in a subset (14%) of pancreatic adenocarcinomas (22). Numerous genes undergo aberrant methylation during pancreatic cancer development and methylation of many of these genes is rarely detected in nonneoplastic pancreatic tissues. These genes include \textit{p16} (23), preproenkephalin (\textit{ppENK}; ref. 24), and \textit{SPARC} (25), among others. This may be particularly relevant in the malignant progression of intraductal papillary mucinous neoplasms, where mutational inactivation of those key genes is infrequent but where methylation accounts for \textit{p16} and \textit{ppENK} inactivation or silencing by promoter methylation (23). Many of these genes are aberrantly methylated in a high proportion of pancreatic cancers, making them potentially attractive for early detection. Pilot studies indicate that the detection of aberrantly methylated genes in the pancreatic juice of patients with pancreatic cancer is feasible as a diagnostic tool (26). In the latter study, pancreatic juice samples were collected either intraoperatively or endoscopically from 92 and 13 patients undergoing pancreaticoduodenectomy or investigation for pancreatic disease, respectively. Methylated \textit{ppENK} was detected in the pancreatic juice of 67% of patients with pancreatic ductal adenocarcinoma, in 45% of patients with intraductal papillary mucinous neoplasm, and in 41% of patients with other periampullary carcinomas, using methylation specific PCR. In contrast, methylated \textit{ppENK} was not detected in 20 patients with nonmalignant

\begin{table}[h]
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\begin{tabular}{|l|l|l|}
\hline
\textbf{Gene} & \textbf{Advantage} & \textbf{Disadvantage} & \textbf{References} \\
\hline
\textit{KRAS} & >90% prevalence & Nonspecific (premalignant lesions, smokers) & (9–11) \\
& Early diagnosis tool & & \\
& Potential for use in blood & & \\
& Aid to other diagnostic tools & & \\
\hline
\textit{TP53} & Highly specific & Late event in pancreatic cancer & (18–20) \\
& Good correlation with protein expression by immunohistochemistry & & \\
& Potential use in brush cytology & & \\
& Aid to other diagnostic tools & & \\
\hline
\textit{DPC4} & Strong correlation with protein expression by immunohistochemistry & Relatively low prevalence (55%) & (21) \\
& Aid to other diagnostic tools & & \\
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\end{tabular}
\caption{Somatic mutations with relevance as potential biomarkers in pancreatic cancer}
\end{table}
periampullary disease including 12 patients with chronic pancreatitis. Methylated ppENK was detected in 91% of primary pancreatic. However, one of the difficulties encountered was the high prevalence of methylated ppENK in normal duodenum (91%) and in pancreatic juice obtained by duodenal aspiration rather than by direct cannulation (89%), highlighting the need for an accurate technique to yield meaningful results.

Mitochondrial Mutation Analysis. The emergence of chip technologies has also facilitated investigations into the diagnostic utility of mitochondrial mutations. The mitochondrial genome is particularly susceptible to mutations because of the high level of reactive oxygen species generation, coupled with a low level of DNA repair; in addition, mitochondria can rapidly become homogeneous in colorectal cancer cells using cell fusions (27). Somatic mitochondrial mutations are common in human cancers and have been proposed as a tool for early detection of cancer (28). Potential advantages of mitochondrial DNA as a marker include each cell having more copies of the mitochondrial genome than of nuclear DNA, and the amount of mitochondrial DNA in cancer cells being several times higher than in normal tissues. To efficiently interrogate the mitochondrial genome, a chip-based, high-throughput tool has been developed, and initial studies suggest that it can be used to detect mitochondrial mutations in pancreatic juice samples obtained from patients with pancreatic cancer as well as from the urine of patients with urinary tract cancers (29).

Gene Expression Analysis as a Biomarker Discovery Tool

The analysis of the gene expression of pancreatic cancer cell lines and primary pancreatic cancer tissue, using cultures of normal pancreatic ductal epithelium and normal tissue as control, aims at depicting the functional picture of a cancer system and works on the assumption that those genes differentially expressed in a cancer specimen will be relevant for cancer-related functions. It is not only useful for the discovery of such markers, but as opposed to mutations analysis that is stable, also offers the advantage of allowing the analysis of the differences in expression in a given strain or cell line after exposure to modulating or therapeutic factors. Several studies have been conducted using different methodologies (30–34). Approaches combining the different technologies available for gene classification and identification are likely to render augmented results (34). Among the different genes identified as differentially overexpressed in cancer specimens, only a minority encode known secreted proteins, which are potential candidates to use as serum biomarkers, such as macrophage inhibitory cytokine 1 (MIC-1) or osteopontin (discussed below). Others are not known to be secreted but are specific to pancreatic cancer compared with normal pancreas, and thus can be used as potential markers for improved diagnosis or as therapeutic targets. Two such examples are mesothelin and prostate stem cell antigen (PSCA).

After initial identification, mesothelin mRNA expression was documented in resected primary pancreatic adenocarcinomas and pancreatic cancer cell lines whereas mesothelin protein expression was confirmed by immunohistochemistry in all 60 primary pancreatic adenocarcinomas studied (35). Mesothelin mRNA has also consistently been detected in pancreatic juice of pancreatic cancer subjects (36). In a similar comparison of genetic libraries of cancer and noncancerous pancreatic tissues, three hits were identified: two having already been associated with pancreatic cancer (lipocalin and trefoil factor 2) and the third corresponding to PSCA, a gene previously thought to be largely restricted to prostatic basal cells and prostatic adenocarcinomas (37). The overexpression of PSCA was confirmed in pancreatic cancer cell lines by reverse transcription-PCR and in 36 of 60 (60%) primary pancreatic adenocarcinomas by immunohistochemistry. In 59 of 60 cases, the adjacent nonneoplastic pancreas did not label for PSCA. As diagnostic markers, mesothelin and PSCA have been shown to increase diagnostic accuracy in cytologically borderline cases (38) and were proposed to differentiate primary ovarian mucinous tumors from metastatic pancreatic mucinous carcinomas in the ovary (39). Both mesothelin and PSCA are potential therapeutic targets and are in different stages of preclinical and clinical development in pancreatic cancer, highlighting the effectiveness of gene expression assessment as a discovery tool.

Protein Markers

Serum/Plasma Assessment. A few of the protein markers discovered by gene expression analysis have been evaluated

Table 2. Circulating protein biomarkers undergoing development in pancreatic cancer

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Experimental design</th>
<th>Sensitivity (CA19-9)</th>
<th>Specificity (CA19-9)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIC-1</td>
<td>80 pancreatic cancers</td>
<td>0.71 (0.59)</td>
<td>0.78 (0.88)</td>
<td>(41)</td>
</tr>
<tr>
<td></td>
<td>20 ampullary cancers</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>77 chronic pancreatitis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osteopontin</td>
<td>50 resectable pancreatic cancers</td>
<td>0.80 (0.62)</td>
<td>0.97 (NR)</td>
<td>(42)</td>
</tr>
<tr>
<td></td>
<td>22 healthy controls</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CA242</td>
<td>40 resectable pancreatic cancers</td>
<td>0.75 (0.80)</td>
<td>0.86 (0.68)</td>
<td>(44)</td>
</tr>
<tr>
<td></td>
<td>30 healthy controls</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

NOTE: Sensitivity and specificity for the diagnosis of pancreatic cancer are shown, with the values of CA19-9 used as standard between brackets. Abbreviation: NR, not reported.
in a clinical context, the results of which are shown in Table 2. MIC-1 is a distant member of the transforming growth factor β superfamily originally identified in the setting of macrophage activation (40); MIC-1 was expressed in 14 of 16 primary pancreatic adenocarcinomas (88%) by immunohistochemistry and serum levels were significantly higher in patients with pancreatic ductal, ampullary, and cholangiocellular carcinomas than in those with benign pancreatic neoplasms, chronic pancreatitis, or in healthy controls (41). An elevated serum MIC-1 defined as 2 SD above the mean for healthy controls discriminated equally well as carbohydrate antigen (CA) 19-9, and the combination of MIC-1 and CA19-9 significantly improved diagnostic accuracy, making it a candidate for further testing in pancreatic adenocarcinoma patients. A second protein identified by gene expression analysis was osteopontin (31). Subsequently, it was evidenced that serum osteopontin levels were elevated in 50 patients with resectable pancreatic cancer compared with 22 healthy control individuals, and a cutoff level of 2 SD above the mean rendered a sensitivity of 80% and specificity of 97% for pancreatic cancer diagnosis (42). In contrast, only 62% of these patients with resectable pancreatic cancer had elevated CA19-9.

Another marker that has been proposed as a diagnostic tool is CA242. CA242 is a secreted protein that has been assessed as a putative marker of pancreas. In a study comparing preoperative serum carcinoembryonic antigen (CEA), CA19-9, and CA242 in 105 pancreatic cancers, 70 nonpancreatic malignancies, and 30 benign pancreatic diseases, the sensitivity of CA242 was inferior to that of CA19-9, but the specificity was significantly higher (P < 0.01; ref. 43). In CA242-positive patients, the survival time was significantly shorter than that of patients with negative result. In a small series comparing CA242, CA19-9, and CEA in 40 pancreatic cancers and 30 healthy subjects, the authors conclude that although with equivalent sensitivity, CA242 showed a higher specificity than CA19-9 and CEA to diagnose pancreatic cancer (86% versus 68% and 73%, respectively; ref. 44). In both studies, a combination of markers yielded the best diagnostic capacity. However, CA242 has been criticized for its low sensitivity in early pancreatic cancer subjects and also for its potential cross-reactivity with CA19-9 antibody (45).

A different approach is provided by global protein assessment or proteomic analysis. Proteomic technologies provide an excellent means for analysis of body fluids for cataloging protein constituents and identifying biomarkers for early detection of cancers. The traditional approach for quantitative protein profiling is based on two-dimensional electrophoresis for protein separation, followed by mass spectrometric identification of selected proteins or all proteins detected (46). One advantage of the two-dimensional electrophoresis approach is its ability to separate intact isomeric forms of protein that are useful in studies detailing posttranslational modifications, but in addition to being inadequate for high-throughput screening, two-dimensional electrophoresis is suboptimal for small, hydrophobic, very basic, or very acidic proteins. Another approach for disease biomarker discovery and development is using proteomic pattern analysis. This approach primarily compares the pattern of signals observed within a mass spectrum to identify differentially abundant peaks within normal and disease samples for distinguishing of two groups (target and control). Several groups have identified protein fragments in serum by surface-enhanced laser desorption ionization, a technology representative of the latter approach (47, 48). In one study, serum samples from 60 patients with resectable pancreatic adenocarcinoma were compared with samples from 60 age- and sex-matched patients with nonmalignant pancreatic diseases, as well as 60 age- and sex-matched healthy controls, assessing the performance of the individual marker panels alone or in conjunction with CA19-9 (47). Among the peaks identified by surface-enhanced laser desorption ionization profiling that had the ability to distinguish between patient groups, the two most discriminating protein peaks could differentiate patients with pancreatic cancer from healthy controls with a sensitivity of 78% and specificity of 97%. These two markers did significantly better than the current standard serum marker CA19-9. A second study using surface-enhanced laser desorption ionization recognized a set of four mass peaks as most accurately discriminating cancer patients from healthy controls with sensitivity/specificity values of 97.2%/94.4% and 90.9%/91.1% in the training and validation cohorts, respectively (48). When combined with CA19-9, 100% (29 of 29 patients) of pancreatic cancers, including early-stage (stages I and II) tumors, were detected. The robustness of surface-enhanced laser desorption ionization has been evaluated in the context on multi-center designs by showing that the same proteins are amenable for detection in the same samples sets in different laboratories (49).

**Pancreatic Juice Assessment.** Comprehensive characterization of the “pancreatic juice proteome” in patients with pancreatic adenocarcinoma have been carried out (50). Pancreatic juice was first fractionated by gel electrophoresis and subsequently analyzed by liquid chromatography tandem mass spectrometry. A total of 170 unique proteins were identified including known pancreatic cancer tumor markers (e.g., CEA and MUC-1) and proteins overexpressed in pancreatic cancers (e.g., hepatocarcinoma-intestine-pancreas/pancreatitis-associated protein and lipocalin 2). A number of proteins that have not been previously described in pancreatic juice were identified [e.g., tumor rejection antigen (pg96) and azurocidin].

**Novel Ways of Analysis: Interspecies Comparative Proteomics.** The sera resulting from an orthotopic nude mouse model of human pancreatic cancer were screened by surface-enhanced laser desorption ionization for candidate serum biomarkers, followed by an examination of candidate proteins in the plasma of 135 pancreatic cancer patients, 7 pancreatitis patients, and 113 healthy volunteers (51). A 11.7-kDa protein peak correlating with tumor weight was purified by gel filtration, separated by SDS-PAGE, and identified as mouse serum amyloid A by amino acid sequencing and public database searches. The level of serum
Markers for Early Detection and Disease Characterization

Early pancreatic cancer detection is the mainstay for a successful outcome. This is especially relevant in the increasingly characterized subgroup of patients with familial pancreatic cancer. Because the basis for the increase in risk is genetic, the identification of familial pancreatic cancer susceptibility genes should improve our ability to effectively screen high-risk populations. Carriers of mutations in the BRCA2, p16, STK11, cationic trypsinogen, FANCC, and mismatch repair genes are at increased risk of developing inherited pancreatic cancer (53–56). Current screening strategies rely primarily on family history to identify individuals with a significant lifetime risk of developing pancreatic cancer (57). Data arising from screening studies in selected academic centers have shown that many such individuals have asymptomatic preinvasive pancreatic neoplasms that are amenable to cure (58, 59). These studies have used a combination of endoscopic ultrasound and computed tomography scanning of the pancreas and genetic counseling for the study subjects. Less invasive, more specific complementary diagnostic techniques based on molecular abnormalities will likely increase the odds of detecting tumors at early stages and also facilitate the access to screening tools to larger target populations (60).

Another relevant aspect is the characterization of precursor lesions of the pancreas, particularly intraductal papillary mucinous neoplasms that share some radiologically features with ductal adenocarcinomas. These are intestinal-type neoplasias that are characterized as having grossly identifiable proliferations of mucin-producing neoplastic epithelium within dilated pancreatic ducts and ductules. The intraductal components of intraductal papillary mucinous neoplasms display a broad spectrum of dysplasia ranging from adenoma to carcinoma in situ, and 30% of intraductal papillary mucinous neoplasms are associated with an infiltrating adenocarcinoma. Although the prognosis of patients with intraductal papillary mucinous neoplasm is better than it is for patients with infiltrating pancreas ductal adenocarcinoma, a subset of intraductal papillary mucinous neoplasms does recur. Developing molecular tools to identify and characterize those cases at higher risk is a need for these patients, particularly to categorize the odds of recurrence. As discussed above, the methylation profile of these tumors can be helpful to differentiate them from other neoplasms (23). Following another strategy, gene expression profile analysis in a pilot cohort followed by immunohistochemistry validation in a larger set of cases has identified a group of transcripts and proteins present in invasive intraductal papillary mucinous neoplasms compared with noninvasive cases, including claudin 4, CXC4, S100A4, and mesothelin (61). A combined analysis of these four factors by immunohistochemistry revealed that 16 of 22 invasive cases expressed at least two factors, as opposed to no expression of any factor in 16 noninvasive invasive cases.

Markers for Prediction of Therapeutic Efficacy

Markers Related to Pathway Activation

Ras/Phosphoinositol 3-Kinase Pathway

Ras is a key member of the signaling pathways that regulate critical cellular functions. Ras proteins transduce growth and differentiation signals from receptor tyrosine kinases to the cell nucleus, thereby initiating gene transcription (62). KRAS mutation has the ability to transform normal cells into a neoplastic phenotype and occurs frequently in human cancers. In pancreatic cancer, activating KRAS mutations are very prevalent (63) and occur early, indicating its role in the initial phases of development of pancreatic cancer. In contrast, their role in established pancreatic cancer is not clear. Because KRAS must be farnesylated to be active (a posttranslational modification mediated by the enzyme farnesyl-transferase), inhibitors of this enzyme have been developed as potential Ras inhibitors (64). However, the successive failure of several inhibitors in pancreatic cancer phase III trials (65) has questioned the relevance of this mechanism, and therefore the value of KRAS mutation as a biomarker predicting efficacy in that setting is also questionable.

One of the key downstream targets of the Ras family is phosphoinositol 3-kinase (PI3K), a heterodimer consisting of a p85 regulatory subunit and a p110 catalytic subunit. Activation of PI3K can occur by binding of the p85 subunit to activated receptor tyrosine kinases or by binding of the p110 subunit to constitutively active KRAS. Activated PI3K catalyzes the conversion of phosphatidylinositol-4,5-biphosphate to phosphatidylinositol-3,4,5-trisphosphate. The best-characterized phosphorylation target of PI3K is...
the pleckstrin homology of Akt. This phosphorylation stimulates the catalytic activity of Akt, resulting in the phosphorylation of a host of other proteins that affect cell growth, cell cycle entry, and cell survival. Preclinical studies have shown that inhibitors of PI3K, such as wortmannin and LY294002, induce dose-dependent apoptosis of pancreatic cancer cells that display constitutive Akt activity in vitro, and inhibit tumor growth of pancreatic cancer xenografts (66). Activation of PI3K is implicated in pancreatic cancer resistance to apoptosis induced by chemotherapeutic or molecular targeting agents, as several studies have shown that treatment with the PI3K inhibitors substantially enhances apoptosis induced by gemcitabine in a concentration-dependent manner (67), although other reports challenge this finding (68). Furthermore, the reduction of phosphorylated Akt levels correlates with the enhancement of gemcitabine-induced apoptosis, suggesting that the PI3K/Akt pathway plays a significant role in mediating drug resistance in human pancreatic cancer cells. It remains to be determined the clinical relevance of these observation for the prediction or guidance of therapy in a clinical setting.

**Phosphatase and Tensin Homologue/Mammalian Target of Rapamycin Pathway**

The tumor suppressor gene phosphatase and tensin homologue deleted from chromosome 10 (PTEN) is known to play a major role in embryonic development, cell migration and apoptosis. PTEN acts as a lipid phosphatase that regulates major signal transduction pathways and effectively terminates PI3K-mediated signaling. PTEN mutation, which occurs frequently in many solid tumors, is associated with constitutive activation of the PI3K/Akt pathway, resulting in tumors that are generally resistant to apoptosis. In pancreatic cancer, PTEN is not mutated but functionally abrogated through loss of expression. It was found that >60% of pancreatic cancer cell lines and tumor tissues had a decreased or loss of expression of PTEN. In a murine model of conditional PTEN inactivation, this genetic defect that leads to acquisition of a profound metastatic ductal phenotype is accompanied by loss of differentiated acinar units and the occurrence of invasive cancer (69). PTEN status in tumor cells has been implicated as an important predictor of sensitivity to rapamycin analogues that inhibit the mammalian target of rapamycin (mTOR; ref. 70), and inhibitors of mTOR reverse doxorubicin resistance conferred by PTEN mutation in prostate cancer cells (71).

mTOR (also named FRAP or RAP1 and RAPT1; refs. 72, 73) acts as a nutrient or growth factor sensor and likely has similar essential actions in regulating mammalian cellular functions, although some of the biochemical pathways may be distinct from those in yeast. The main function of mTOR, in the context of cell proliferation, is the regulation of translation initiation, presumably mediated by the activation of P70-S6 kinase (P70-S6K1) and the inactivation of 4E-binding protein (4E-BP1). The increase in the translation of a subset of mRNAs brings about protein products that are required for traverse through the G1 phase of the cell cycle. mTOR regulates essential signal transduction pathways and is involved in the coupling of growth stimuli with cell cycle progression. The expression of the PI3K/mTOR signaling pathway in human pancreatic cancer cells and tissues was investigated, documenting that the mTOR and P70-S6K1 from the PI3K/mTOR signaling pathway were activated in all of the pancreatic cancer cell lines examined (74). In addition, temsirolimus (a rapamycin prodrug) was effective in a xenograft model of the pancreas cell line ASPC1. In a novel direct pancreatic cancer xenograft model, temsirolimus showed a high degree of tumor growth inhibition with significant anti-tumor effect in most of the cases studied.1 In a phase II trial in patients with glioblastoma multiforme treated with temsirolimus, high levels of phosphorylated P70-S6K1 in baseline tumor samples seem to predict a patient population more likely to derive benefit from treatment (75). The efficacy of rapamycin in patients with pancreatic cancer and the value of the phosphorylation status of P70-S6K1 as a predictor of mTOR inhibitors and as a pharmacodynamic marker of mTOR inhibition are being further evaluated.

**Genetic-Based Predictors**

**BRCA2/Fanconi Anemia Gene Mutations**

The causative genes of Fanconi anemia (FANCC and FANCG) play a role in human tumorigenesis. The BRCA2 gene represents Fanconi complementation group D1 and is thought to aid DNA strand repair, and BRCA2 has been categorized as a genome maintenance gene rather than a standard tumor suppressor. Seven to ten percent of “sporadic” pancreatic cancers (more in instances of familial aggregation) harbor an inactivating intragenic inherited mutation of one copy of the BRCA2 gene, accompanied by loss of heterozygosity (76). Somatic BRCA2/Fanconi anemia gene mutations are present in a subset of pancreatic cancer patients, particularly in familial cases (53, 77). It has been recently documented that the presence of mutations is predictive of sensitivity to cross-linking (mitomycin C, cisplatin, chlorambucil, and melphalan) chemotherapeutic agents in pancreatic cancer preclinical models (78). Strategies to translate this paradigm to pancreatic cancer patients harboring defects in the BRCA2/Fanconi anemia pathway are being actively investigated.

**MTAP**

The MTAP gene, on chromosome 9p21, is frequently included within homozygous deletions of the p16INK4A/CDKN2A gene. MTAP plays an important role in the salvage pathway for the synthesis of adenosine. Novel chemotherapeutic strategies exploiting the selective loss of MTAP function in cancers have been proposed, using inhibitors of de novo AMP synthesis such as t-alanosine (79). MTAP expression is lost in ~30% of infiltrating pancreatic cancers and in a lower percentage of other periamppullary neoplasms, and this loss is the result of homozygous deletions encompassing both the MTAP and p16INK4A/CDKN2A genes (80). Thus, pancreatic cancer is a promising cancer type in which to explore novel strategies to exploit the selective loss of MTAP function.
Drug Metabolism Markers: Deoxycytidine Kinase and Response to Gemcitabine

Deoxycytidine kinase is essential for the phosphorylation of gemcitabine (2',2'-difluorodeoxycytidine), a deoxycytidine analogue that is the standard cytotoxic agent in the management of pancreatic cancer. Deoxycytidine kinase activity has been related to gemcitabine sensitivity in preclinical cell line–based xenograft models including pancreatic cancer (81). Deoxycytidine kinase protein expression measured by immunohistochemistry correlated with gemcitabine activity in a novel direct xenograft pancreatic cancer model, exemplifying a method of assessment more easily translated to the clinic.\(^1\)

Immunologic Markers

The discovery of pancreas tumor-specific antigens and the subsequent ability to harness this technology have become an area of intense interest for tumor immunologists and clinicians alike. One of such biomarkers, sialylated MUC-1 mucin, which is expressed preferentially in pancreatic cancer cells (82) and was correlated with tumor aggressiveness and patient survival (83), has been proposed and used as a potential antigen in a pilot, feasibility trial of vaccination in patients with pancreas and biliary cancer (84). Interestingly, mesothelin, one of the pancreas-related proteins recently discovered and discussed before, has been shown to be a potentially relevant immunologic target (85). Patients vaccinated with irradiated whole pancreatic cancer cell vaccines presented an induction of CD8\(^+\) T-cell responses to multiple HLA-A2-, HLA-A3-, and HLA-A24-restricted mesothelin epitopes and developed antimesothelin specific antibodies (86).

Markers for Therapeutic Efficacy Monitoring

Sialylated Lewis\(^a\) blood group CA19-9 is widely used to monitor response to therapy in patients undergoing treatment (87, 88). The kinetics of CA19-9 serves as an early indicator of response to treatment (89): an early decrease during the first weeks of chemotherapy with gemcitabine was associated with a better survival (90) and patients with the greatest degree of biomarker decline (>75%) lived significantly longer (12.0 versus 4.3 months, \(P < 0.001\); ref. 91). The authors of the latter study conclude that serial CA19-9 measurements correlate well with clinical outcomes in patients with advanced pancreatic cancer, and that decline in this biomarker should be entertained for possible use as a surrogate end point in clinical trials for the selection of new treatments in this disease.

Conclusions

The rapidly expanding knowledge of the pathogenesis of cancer at the molecular level is providing new targets for disease characterization, early diagnosis, drug discovery and development, the most advanced of which are summarized in Table 3. Gene mutation analysis has provided insight on the pathogenesis and progression from preinvasive lesions to invasive cancer. Gene and protein expression profiling has advanced our understanding of pancreatic ductal adenocarcinoma in several ways. First, in excess of 200 genes have been identified that are highly expressed in pancreatic duct adenocarcinomas but not in normal pancreatic ductal epithelium. Each of these highly expressed genes offers new opportunities for development of diagnostic tests or therapeutic targets. Second, many genes relating to the clinicopathologic features of pancreatic cancer have been identified. Third, gene expression studies have revealed novel features related to the process of tissue invasion by pancreatic cancers, identifying new possibilities for drug delivery focused on tumor-stromal interactions. The increasing knowledge of the pathway activation profile in pancreatic cancer is yielding new targets but also new markers to select patients and guide and predict therapy efficacy, such as the activation status of P70S6K1 and mTOR inhibitors. Strategies aiming at incorporating these evaluations in the clinical are being actively pursued. In addition, there is a mounting number of genetic factors, the presence of which predisposes pancreatic cancer to successful targeting, such as the association of BRDA2/Fanconi anemia genes and sensitivity to mitomycin C and other cross-linking chemotherapeutic agents. Selection strategies based on the presence of this somatic genetic variant are under way to clinically test the relevance of this observation. In summary, several decades of intensive research have originated multiple factors or biomarkers that are likely to be helpful in the diagnosis, characterization, and therapy selection of pancreatic cancer patients. A deep biological understanding of the relative relevance of each biomarker will be key to efficiently diagnose and direct our patients towards the drugs more likely to benefit from its particular modulation profile. The development of new preclinical models is paramount to achieve these goals.

Table 3. Key biomarkers and their potential use in pancreatic cancer

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Potential utilization</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>KRAS</td>
<td>Diagnosis</td>
<td>(13)</td>
</tr>
<tr>
<td>DPC4</td>
<td>Diagnosis</td>
<td>(21)</td>
</tr>
<tr>
<td>Mesothelin</td>
<td>Diagnosis</td>
<td>(35)</td>
</tr>
<tr>
<td>PSMA</td>
<td>Diagnosis</td>
<td>(38)</td>
</tr>
<tr>
<td>MIC-1</td>
<td>Diagnosis</td>
<td>(41)</td>
</tr>
<tr>
<td>Osteopontin</td>
<td>Diagnosis</td>
<td>(42)</td>
</tr>
<tr>
<td>PTEN</td>
<td>Therapeutic guidance</td>
<td>(70)</td>
</tr>
<tr>
<td>Phospho-P70S6K</td>
<td>Therapeutic guidance</td>
<td>(75)</td>
</tr>
<tr>
<td>BRCA2</td>
<td>Therapeutic guidance</td>
<td>(78)</td>
</tr>
<tr>
<td>MTAP</td>
<td>Therapeutic target</td>
<td>(80)</td>
</tr>
<tr>
<td>Deoxycytidine kinase</td>
<td>Therapeutic guidance</td>
<td>(81)</td>
</tr>
</tbody>
</table>

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\(^1\) The development of new preclinical models is paramount to achieve these goals.
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Molecular biomarkers: their increasing role in the diagnosis, characterization, and therapy guidance in pancreatic cancer

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