# Pancreatic Cancer: The Role of Molecular Markers in Diagnosis and Management

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## Keywords

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Abstract: Despite an annual incidence of just 40,000 new cases per year, pancreatic adenocarcinoma (PAC) remains the fourth most common cause of cancer-related mortality in the United States, a fact indicative of the considerable diagnostic and therapeutic challenges posed by this malignancy. The availability of increasingly sophisticated molecular techniques over the last decade has intensified the search for biomarkers not only to predict outcome and response to therapy in established pancreatic malignancy but also to identify premalignant pancreatic lesions in at-risk individuals. A wealth of information regarding the complex sequence of genetic abnormalities in PAC has been gained from recent in-depth molecular analyses, and lately the role of epigenetic alterations in the development and maintenance of pancreatic carcinogenesis has been more clearly described. In addition, advances in serum proteomic methods and the collection of circulating tumor cells offer hope for the development of noninvasive techniques for biomarker discovery. At present, we are awaiting the development and validation of robust biomarkers suitable for clinical application in this disease. Herein, we discuss the current status of molecular markers in the diagnosis and management of PAC and review potential clinical applications thereof.

# Introduction

Pancreatic adenocarcinoma (PAC) is the fourth most common cause of cancer-related mortality in the United States.<sup>1</sup> It is characterized by frequent presentation of advanced disease, a high rate of local and distant recurrence following surgical resection, and relative resistance to local and systemic therapies. There is a pressing need to identify new molecular markers to predict outcome and response to therapy in PAC, and to assist in the detection of premalignant pancreatic lesions. The elucidation of the sequence of acquired genetic abnormalities leading to the development of invasive PAC has aided the development of tissue biomarkers of preinvasive malignancy. Furthermore, improved awareness and understanding of hereditary genetic abnormalities predisposing to PAC offer the potential for both screening of at-risk individuals and development of a molecularly targeted therapeutic approach. Several newly identified molecular markers of invasive PAC offer potential therapeutic targets, and pharmacogenomic studies have yielded putative biomarkers of response to established cytotoxic therapy for PAC. However, prospective validation of all putative predictive and prognostic biomarkers must be achieved before their incorporation into clinical decisionmaking, and the temptation to select therapy based on nonvalidated data, no matter how promising, should be resisted. Here we review the diagnostic and therapeutic implications of molecular markers in premalignant pancreatic lesions and invasive PAC.

# Molecular Markers of Early and Pre-Invasive PAC

#### The Pancreatic Progression Model

The most commonly seen genetic alterations in PAC have been well described; these include near ubiquitous activating KRAS mutation in up to 90% of cases, with frequent inactivation of tumor suppressor genes p53, p16, and SMAD4, either by intragenic mutation with loss of the remaining allele or by homozygous deletion (Table 1). Three precursor lesions to PAC have been identified: microscopic pancreatic intraepithelial neoplasia (PanIn) 1-3, intraductal papillary mucinous neoplasm (IPMN), and mucinous cystic neoplasm (MCN). IPMN and MCN may be identified on cross sectional imaging, and PanIn is a histologic diagnosis, graded 1-3 with progressive histologic and molecular changes.<sup>2</sup> Although PanIn 3 is almost always seen in association with invasive PAC, PanIn 1 may be seen in specimens without evidence of invasive malignancy and has unclear significance with regard to future development of invasive PAC. Study of the type and frequency of molecular alterations found in these precursor lesions has provided valuable insight into the sequence of genetic events driving pancreatic tumorigenesis. Telomere shortening occurs in 90% of PanIn 1 specimens, whereas an activating KRAS mutation is seen in 45%.<sup>3</sup> Inactivation of *p16* may be demonstrated in PanIn 2 lesions, with subsequent loss of BRCA2, p53, and DPC4 expression identified with greater frequency in PanIn 3 specimens.<sup>4</sup> IPMN is a radiographic diagnosis characterized by mucin production, cystic dilatation of the pancreatic duct, and intraductal papillary growth; histologic findings in resected specimens range from lowgrade dysplasia to invasive malignancy. The molecular changes associated with high-grade dysplasia and invasive malignancy in IPMN are not yet well characterized; KRAS and *p53* mutations appear to occur with less frequency. Inactivating mutations in STK11/LKB1, a tumor suppressor gene mutated in Peutz-Jaeger syndrome, have also been found in a minority of malignant IPMN.<sup>5</sup> Mucinous cystic neoplasms occur predominantly in women, and are

**Table 1.** Common Genetic Alterations in Pancreatic

 Adenocarcinoma<sup>4,81</sup>

Genetic Alteration	Frequency
KRAS mutation	>95%
<i>p53</i> inactivation	50–75%
<i>p16</i> inactivation	>95%
DPC4 inactivation	55%
BRCA2 inactivation	7%
STK11/LKB1 mutation	5%

characterized by a desmoplastic ovarian-type stroma, with a similar spectrum of dysplasia to IPMN.<sup>6</sup>

#### Potential Utility of Tissue Biomarkers in PAC Screening

As the survival of patients diagnosed with invasive PACeven at early stages-remains poor, research efforts are now focused on methods of identifying premalignant pancreatic lesions in patients at high risk for PAC. A comprehensive genomic analysis of matched samples from resected and metastatic sites of PAC, which were obtained at autopsy series with computational modeling based on the rate of accumulation of passenger (non-carcinogenic) mutations, was performed. The analysis estimated the timeframe from initial carcinogenic mutation in the normal pancreatic ductal cell to patient death from metastatic disease to be 21 years.7 This finding indicates a potential lengthy window for screening and intervention to prevent the development of invasive carcinoma, but it is at odds with the clinical observation that PAC is rarely an incidental diagnosis and frequently presents at an advanced stage. Several screening programs aiming to identify premalignant pancreatic lesions in patients at risk of PAC based on family history or known genetic predisposition syndrome have published initial results. The yield of screening healthy individuals has varied considerably, mainly due to differing inclusion criteria and screening modalities.<sup>8-11</sup> Our institution recently published results of screening performed on healthy at-risk relatives of patients with PAC, as determined by having at least 1 first-degree relative (FDR) with PAC diagnosed before age 50, 2 or more relatives with PAC at any age (1 being an FDR), 3 or more second-degree relatives with PAC, or a known BRCA1/2 mutation.<sup>12</sup> Screening was performed with magnetic resonance cholangiopancreatography, followed by endoscopic ultrasound if an abnormality was identified. The diagnostic yield of screening in this broadly defined group of healthy relatives was 8.3%. Larger studies screening high-risk individuals are needed to determine the impact on PAC mortality.

#### Molecular Analysis of Pancreatic Cyst Fluid

Pancreatic cystic lesions pose a particular diagnostic challenge, as it may be difficult radiographically to differentiate between a mucinous cystic lesion with the potential for malignant transformation and a benign serous cyst. Management of pancreatic cysts, the majority of which are now incidentally discovered, is an evolving and complex field. Even with the application of the best currently available consensus guidelines that utilize clinical and radiographic features to identify cystic pancreatic lesions with high risk for malignant transformation, there is potential for overtreatment of lesions with low malignant potential and for undertreatment of lesions that may already contain high-grade dysplasia or invasive malignancy. Efforts have focused on the identification of biomarkers from cyst fluid samples, frequently readily available by endoscopic assessment, which may predict for the presence or subsequent development of malignancy. Cytologic examination of cyst fluid, although highly specific for malignancy, lacks sensitivity due to low cellular content and contamination of fluid with mucin. Measurement of cyst fluid levels of the tumor marker CEA may help to differentiate IPMN from benign pancreatic cysts; a cyst fluid CEA level of greater than 200 ng/mL has a positive predictive value of up to 85% in identifying mucinous cystic pancreatic lesions.<sup>13</sup>

Recent elucidation of the most common acquired molecular changes associated with pancreatic carcinogenesis has led to examination of cyst fluid DNA as a potential predictive biomarker of malignancy. The PANDA (Pancreatic Cyst DNA Analysis) trial examined cyst fluid DNA from patients with indeterminate pancreatic cystic lesions enrolled in a prospective multicenter study. Although 391 patients were enrolled in the trial, only the 113 patients in this unblinded study who proceeded to surgery or subsequently developed a pancreatic malignancy were included in the final analysis.<sup>14</sup> DNA extracted from cyst fluid was quantified and examined for KRAS mutation status and loss of heterozygosity (allelic imbalance). The presence of a KRAS mutation alone was predictive of a mucinous cyst, with a sensitivity of 45% and a specificity of 96%, although it was not helpful in differentiating between benign and malignant mucinous cysts. This was consistent with the observation that KRAS mutation occurs early in the process of malignant transformation. The combination of KRAS mutation followed by allelic loss was predictive for malignancy, as was the amount of DNA present in cyst fluid and the presence of high allelic loss amplitude. A subsequent study compared the predictive ability of a commercially available molecular analysis of cyst fluid alone in determining mucinous histology or malignancy versus the clinical assessment as determined by histology of a resected specimen or from 2 of 3 concordant preoperative tests (EUS features, CEA cyst fluid analysis, and cytology).<sup>15</sup>

Concordance between the diagnoses, as determined by molecular analysis and clinical assessment, was high, lending further support to the use of *KRAS* mutation assessment, loss of heterozygosity, and yield of cyst fluid DNA for detection of malignant or premalignant pancreatic cysts. However, the additional value of molecular analysis over radiographic and endoscopic cyst assessment, in combination with fluid CEA level and cytologic assessment, remains unclear. Further prospective studies are required to validate the clinical utility of this approach before it enters routine clinical practice. As techniques for molecular analysis improve and become more widely available, we anticipate the identification of novel pancreatic cyst fluid biomarkers predictive of malignant transformation.

# Genetics and Potential Therapeutic Implications of Hereditary PAC

Although the majority of cases of PAC are sporadic, up to 10% occur in the setting of a strong family history of pancreatic malignancy or a known inherited cancer predisposition syndrome.<sup>16</sup> Several hereditary conditions have been identified as carrying a significantly increased risk of developing PAC, including germline BRCA1/2 mutations, familial adenomatous polyposis (FAP), hereditary non-polyposis colorectal cancer (HNPCC), Peutz-Jaeger syndrome, familial atypical multiple mole melanoma syndrome (FAMMM), and hereditary pancreatitis.<sup>17-23</sup> In a further subset of patients with PAC, a significant family history of PAC is seen, but no genetic abnormality can be identified; this likely represents cases associated with germline mutations of as-yet unidentified genes and/or shared environmental factors.<sup>24</sup> The study of the pathologic and clinical characteristics of hereditary forms of PAC offers the potential for the development of a molecularly targeted therapeutic approach in a genetically selected subset of patients, most notably in BRCA1/2 mutation-associated cases.

Loss of function of the BRCA1 or BRCA2 protein interferes with the ability of cells to repair doublestranded DNA breaks (DSB) by homologous recombination<sup>25</sup>; exposure of *BRCA1/2*-mutant cancer cells to DSB, such as those induced by alkylating agents or ionizing radiation, results in hypersensitivity of cells unable to repair this DNA damage. Poly (ADP-ribose) polymerase (PARP)1 and PARP2 are key components of the DNA repair mechanism for cells with single-strand breaks and nucleoside base damage.<sup>26</sup> Inhibition of PARP in BRCA1or BRCA2-deficient cells leads to transformation of background single-strand breaks into DSB, which are lethal in cells unable to repair DSBs by homologous repair.<sup>27</sup> This effect of synthetic lethality has led to the development of PARP inhibitors in *BRCA1-* or *BRCA2-*mutation–associ-

ated malignancies, with tumor responses seen in phase I and II clinical trials of BRCA-mutant breast and ovarian cancer.28,29 Demonstration of the activity of PARP inhibitors in BRCA1/2 mutation-associated breast and ovarian cancer has sparked interest in the evaluation of these agents in BRCA1/2 mutation-associated PAC. A recent series from our group,<sup>30</sup> along with several anecdotal clinical reports,<sup>31</sup> indicate increased sensitivity to platinum chemotherapy and PARP inhibitors in patients with PAC arising in a setting of known BRCA1/2 germline mutation. In collaboration with investigators and in partnership with the Cancer Therapeutic and Evaluation Program (CTEP) and the Lustgarten Foundation, we plan to conduct a randomized phase II trial evaluating the addition of PARP inhibition to platinum-based therapy in a selected genetic population of BRCA1, BRCA2, or PALB2 mutation carriers with PAC. Table 2 outlines some of the biomarkers that have been shown to be predictive of benefit and resistance to therapy in PAC.

# Molecular Markers in PAC With Potential Therapeutic Implications

#### DPC4 Gene

Germline mutations in the DPC4 gene result in juvenile polyposis syndrome, while acquired homozygous mutation or deletions are found in 53% of PAC.32 DPC4 (SMAD4) codes for a transcription factor centrally involved in intracellular mediation of response to transforming growth factor receptor B (TGF $\beta$ ) as well as bone morphogenic protein (BMP) and activin signaling.<sup>33,34</sup> TGFB plays a dual role in tumorigenesis. Although it initially functions as a tumor suppressor gene by inhibiting cell growth and differentiation, in established tumors TGF $\beta$  signaling actually promotes tumor growth, dissemination, and the epithelial to mesenchymal transition. Initial studies assessing the prognostic implications of SMAD4 loss of expression in PAC reported conflicting results. Biankin and colleagues reported loss of DPC4 in 63 of 119 (53%) PAC samples, and found no benefit to operative resection in patients whose tumors expressed DPC4/SMAD4 (P=.5).35 In contrast, a significant survival benefit to resection was seen in patients who had lost DPC4/SMAD4 expression (median survival, 14.2 months vs 3.1 months; P<.0001). Moreover, survival for patients with resected DPC4/SMAD4-negative tumors was significantly longer than survival for all other groups combined. These results are in contrast to those published by Tascilar and associates, who used immunohistochemistry (IHC) to examine the SMAD4 status of 249 resected PAC specimens. They reported significantly improved survival in patients whose tumors retained DPC4 staining com**Table 2.** Potential Biomarkers Predictive of Benefit/Resistanceto Therapy in Pancreatic Adenocarcinoma (All RequiringProspective Validation)

Molecular Marker	Therapy
BRCA1/2 mutation	DNA damaging chemotherapy, radiation, PARP inhibitors <sup>30</sup>
C-met expression	C-met inhibition <sup>51</sup>
SPARC expression (Tumor and stroma)	Nab-paclitaxel
hENT, CDK, CDA expression	Gemcitabine <sup>54,63</sup>
DPC4 retention	Chemoradiation <sup>38</sup>
KRAS wild-type status	Erlotinib <sup>40</sup>

BRCA1/2=breast cancer gene 1/2; CDA=cytidine deaminase; CDK=cyclindependent kinase; PARP=poly ADP ribose polymerase; SPARC=secreted protein rich in cystein.

pared to those that did not-a finding that persisted on multivariable analysis (median survival, 19.2 months vs 14.7 months; P=.03).<sup>36</sup> More recently, the association between pattern of disease spread and DPC4 tumor status in PAC has been examined.<sup>37</sup> A phase II trial of induction chemotherapy followed by chemoradiation in patients with localized PAC examined DPC4 expression in 29 pretreatment biopsy samples. Although 71% (10/14) of patients with loss of DPC4 expression had distant progression as site of first treatment failure, 73% (11/15) of patients with retention of DPC4 expression had predominantly local disease progression (P=.016). These findings concur with results from a rapid autopsy series, which examined DPC4 status in PAC samples taken from patients both with an extensive burden of metastatic disease and from patients with predominantly local disease at time of death. Loss of DPC4 expression was seen in 75% of metastatic cases, as compared to 22% of localized cases.38 These findings suggest that retention of DPC4 expression in PAC may predict for a local pattern of disease progression, and thus allow for selection of patients most likely to benefit from local treatment strategies such as chemoradiation. While this hypothesis awaits prospective validation in several planned trials, the use of DPC4 status in clinical decision making for patients with localized PAC cannot yet be recommended.

#### KRAS/EGFR

The modest survival benefit achieved with the addition of erlotinib (Tarceva, Genentech/OSI Pharmaceuticals) to gemcitabine, in patients with advanced PAC in the

PA.3 trial led to the approval of erlotinib for treatment of advanced PAC, but also fueled a search for potential biomarkers of response to epidermal growth factor receptor (EGFR)-targeted therapy in PAC.<sup>39</sup> Just over a quarter of patients on the original trial had tissue samples available for correlative studies, a problem frequently encountered in trials of advanced PAC. In this limited number of samples, no correlation between EGFR copy number or KRAS mutation status and response to therapy was identified, although there was a non-significant trend towards improved survival in KRAS wild-type patients who received erlotinib. A similar association between KRAS status and survival was seen in a recently published phase III trial of gemcitabine in combination with capecitabine (Xeloda, Genentech) or erlotinib in patients with advanced disease; patients were allowed to cross over at time of disease progression. Almost three quarters of the 281 patients on this trial had tissue samples available for biomarker analysis. Lack of KRAS mutation was associated with increased survival on univariate analysis, whereas EGFR status was not predictive of response or outcomes.<sup>40</sup> The development of a grade II or greater skin rash is consistently reported as predictive of a survival benefit in patients with PAC treated with erlotinib, but has not been shown to correlate with EGFR or KRAS expression of the mutation status of the tumor. Activating mutations in the tyrosine kinase domain of the EGFR receptor, which are predictive of response to anti-EGFR therapy in lung adenocarcinoma, are rarely found in PAC.<sup>41</sup> Ongoing adjuvant trials of anti-EGFR therapy in PAC, including the currently enrolling adjuvant RTOG 0848 trial, which is assessing whether the addition of erlotinib to gemcitabine and the incorporation of combined fluoropyrimidine-based radiation improve survival (NCT01013649), will provide the opportunity to correlate clinical outcomes with tissue biomarkers in resected PAC samples.<sup>42</sup>

#### Secreted Protein Rich in Cystein (SPARC)

SPARC is a protein involved in multiple key biologic processes, including cell proliferation.<sup>43</sup> Overexpression of SPARC is frequently seen in peritumoral fibroblasts of pancreatic cancer stromal tissue; this is in contrast to the adenocarcinoma cells, where expression of SPARC is commonly downregulated by promoter methylation. Increased expression of SPARC in the tumor microenvironment has been proposed as a negative predictor of survival in patients with resected PAC.<sup>44,45</sup> Nab-paclitaxel is an albumin-bound nanoparticle form of paclitaxel that is currently undergoing prospective evaluation in patients with advanced PAC. Preclinical data indicate that increased expression of SPARC in the interstitial space increases tumor uptake of nab-paclitaxel; this

is explained by the high affinity with which albumin binds to the SPARC protein.46,47 A phase I/II trial of gemcitabine in combination with nab-paclitaxel showed that expression of SPARC by pancreatic stromal cells and PAC cells was predictive of response to the drug combination.<sup>48</sup> The encouraging overall survival of 12.2 months and response rate of 44% observed in patients with advanced PAC treated with combination therapy (at the maximum tolerated dose of nab-paclitaxel) in this trial has prompted phase III investigations. Recruitment is currently ongoing for a randomized phase III trial that is comparing the combination of nab-paclitaxel and gemcitabine to single-agent gemcitabine in patients with advanced disease. This trial will also prospectively examine SPARC expression as a predictive marker of clinical benefit to therapy with nab-paclitaxel.49

## C-MET

PAC is an inherently chemoresistant and radiationresistant disease that has a high propensity for metastatic spread and early relapse following surgical resection. One explanation proposed for these characteristics is the presence of a population of cancer stem cells, which are characterized by resistance to therapy and a capacity for auto renewal. These cells are hypothesized to be responsible for cancer recurrence and metastatic spread.<sup>50</sup> In vitro models of PAC have shown enrichment of a stem-cell population following treatment with gemcitabine, suggesting that these cells are selected for survival during conventional systemic therapy.<sup>50</sup> C-MET has recently been identified as a stem-cell marker in PAC, in which c-MET overexpression is common.<sup>51,52</sup> In xenograft models of PAC, inhibition of c-MET was shown to slow tumor growth and to prevent the development of metastases following cardiac injection of cancer cells. Several other cancer stem-cell markers have been identified, including CD44, CD24, and ESA. The development of therapeutic strategies specifically targeting cancer stem cells is a promising strategy to prevent the development of metastases and to overcome chemoresistance in PAC.

## CD40

The CD40 receptor is a member of the tumor necrosis factor (TNF) receptor super family involved in the regulation of T-cell dependent antitumor immunity. Stimulation of the CD40 receptor with CP-870, 893, a fully humanized monoclonal agonist antibody, is therefore anticipated to enhance tumor-specific T-cell priming and activation by antigen-presenting cells (APCs).<sup>53</sup> This strategy was evaluated in 21 patients with chemotherapy-naïve, inoperable PAC who were treated with gemcitabine in combination with CP-870, 893. Median overall survival was 7.4 months; tumor regression was seen in 4 patients

(19%). Of note, several patients had a dramatic response to therapy, with complete remission in a patient with a 7.6-cm liver metastasis, and complete resolution of all hepatic metastases in a patient who ultimately underwent surgical resection of the primary tumor. Biopsies obtained from responding lesions demonstrated the surprising absence of infiltrating lymphocytes, but identified tumor-infiltrating macrophages. Further studies in a transgenic mouse model of PAC demonstrated tumor regression induced by treatment with a CD40 agonist even in mice deficient in CD4-positive and/or CD8-positive T cells. Examination of treated tumors demonstrated involution of the tumor stroma and infiltration of tumor tissue by CD40-activated macrophages. However, when systemic macrophages were depleted by treatment with clodronate-encapsulated liposomes, no tumor regression in response to CD40 activation was seen. Targeting the CD40 pathway therefore offers the potential for harnessing of inflammatory cells to achieve stromal depletion and tumor regression in a subset of patients with PAC. Ongoing studies will focus on identifying molecular markers associated with the significant responses to therapy observed in a subset of patients in this trial. Moreover, this highlights the value of accurate mouse models of PAC to guide the development of novel therapeutic strategies and identify mechanisms of response and resistance.

# Molecular Markers Predictive of Clinical Benefit to Gemcitabine Therapy in PAC

The pyrimidine analog gemcitabine remains a backbone of systemic therapy for all stages of PAC. It requires transportation across the cell membrane by a nucleoside transporter, followed by intracellular phosphorylation to form the active metabolite 2',2'-difluorodeoxycytidine-5'-triphosphate (dFdCTP). The inactive metabolite 2'2'-difluorodeoxyuridine is renally excreted. This process involves the nucleoside transporter hENT1, the phosphorylating enzyme deocoxycitidine kinase (DCK), and the metabolizing enzyme cytidine deaminase (CDA), all of which have been examined as potential predictive biomarkers of response to gemcitabine.

## bENT1

Retrospective studies have reported a correlation between tumor hENT1 protein expression and survival in patients with PAC treated with gemcitabine in the adjuvant and metastatic settings. Examination of hENT1 IHC staining of 198 resected PAC samples from patients randomized post-pancreatic resection to either gemcitabine or 5-fluorouracil (5FU) on the phase III RTOG 9704 trial found that hENT1 protein expression correlated with survival in patients treated with gemcitabine but not in patients who were treated with 5FU.54 Assessment of hENT1 tumor expression in a retrospectively identified cohort of 83 patients treated with gemcitabine in either the metastatic or adjuvant setting reported significantly longer survival in patients with high hENT1 expression compared to those with low expression. hENT1 expression retained prognostic significance in a multivariable analysis (median survival, 12.4 months vs 22.3 months; P<.01).55 Although this evidence supports the hypothesis that patients who lack hENT1 expression do not benefit from gemcitabine, prospective validation is needed. Gemcitabine-5'-elaidate, a fatty-acid derivative of gemcitabine, is metabolized to gemcitabine and elaidic acid intracellularly by esterase in blood and tissue, and does not require a transmembrane transport by a nucleoside transporter. This novel compound is under prospective evaluation in a randomized phase II trial comparing it to first-line gemcitabine in patients with advanced PAC (NCT01124786),<sup>56</sup> and as second-line therapy in a single-arm, phase II trial limited to patients with advanced PAC who have progression on gemcitabine as best response to therapy and whose tumors lack hENT1 expression (NCT01233375).57

## DCK

Reduced activity of DCK leading to decreased phosphorylation of gemcitabine to its active metabolite has been suggested as a potential mechanism of resistance to gemcitabine therapy. In a small retrospective study, reduced expression of DCK was shown to correlate with reduced survival in patients with advanced PAC treated with gemcitabine. While mutations of the DCK gene are infrequent, epigenetic downregulation of DCK activity by promoter hypermethylation has been identified in other malignancies.58,59 Decreased CDA activity has been associated with increased grade 3 and 4 gemcitabine-related toxicity, which is due to reduced conversion of active dFdCTP to the inactive metabolite 2'2'-difluorodeoxyuridine.<sup>60</sup> In the Japanese population, a germline polymorphism in the CDA gene (CDA 208G>A) has been shown to result in reduced CDA activity and severe life-threatening toxicity following gemcitabine administration.<sup>61,62</sup> A subsequent study of a European population did not identify this polymorphism, although a recent genotypic assessment of patients treated on the RTOG 9704 trial with gemcitabine reported association of the CDA polymorphism CDA Lys27Gln with increased risk of severe hematologic toxicity.<sup>63</sup> Although several in vitro and in vivo studies have looked for an association between CDA activity and gemcitabine sensitivity, results have been conflicting to date.64,65

# Future Directions in PAC Biomarker Development

#### **MicroRNA**

While much progress has been made in determining the complex genetic alterations involved in pancreatic tumorigenesis, it is increasingly evident that not only genetic events but also epigenetic events play a key role in pancreatic carcinogenesis. Most recently, microRNAs have been identified as potential biomarkers and therapeutic targets in PAC. MicroRNAs are 22 nucleotide non-protein coding RNA molecules transcribed from the genome by RNA polymerase II.66 They inhibit mRNA expression of proteins by either degradation or inhibition of translation of specific mRNA, thereby exerting an effect on multiple cellular processes by reduced expression of proteins coded for by the target mRNA. In a variety of human cancers, microRNAs have been shown to be either reduced or overexpressed, suggesting a potentially significant role for these small molecules in the initiation and maintenance of malignancy.<sup>67</sup> In cancer tissues, overexpression of microRNA leads to reduced expression of a target tumor suppressor protein, whereas downregulation results in uncontrolled expression of oncogenes. Several investigators have successfully used comprehensive microRNA expression profiling to differentiate between benign pancreatic tissue, chronic pancreatitis, and PAC.<sup>68,69</sup> In particular, MiR-216 and MiR-217 have been shown to be specific for pancreatic tissue and absent in PAC cell lines and tissue; let-7 MiRNA is downregulated in PAC. The observation that in let-7 MiRNA-deficient cell lines, proliferation is reduced by transfection with let-7 MiRNA suggests a potential therapeutic relevance to these findings. Research efforts have also focused on the development of microRNA biomarkers of malignancy in pancreatic cyst fluid and pancreatic tissue obtained from endoscopic ultrasound-guided fine needle aspirate, along with PanIn, IPMN, and plasma samples.<sup>68-72</sup> A key area of interest will be the potential prognostic and predictive utility of microRNA expression in resected PAC, along with the development of antisense oligonucleotide therapies directed against key oncogenic microRNAs.

## **Circulating Tumor Cells**

A consistent problem encountered in conducting translational research in PAC has been the difficulty in obtaining adequate tissue from the primary tumor for biomarker analysis; collection of viable tumor cells from the peripheral circulation offers an alternative, minimally invasive method of obtaining tumor tissue for molecular analysis. Several methods for the detection and collection of circulating tumor cells (CTCs) from the peripheral blood of patients with PAC have been described<sup>73-77</sup>; however, the overall yield of CTCs collected from an individual patient remains low, thus limiting the ability to perform detailed molecular analysis on tumor cells. With ongoing refinement of current techniques, however, collection of CTCs in PAC has potential for not only advancing our understanding of the molecular biology of PAC, but may also have a wide range of clinical applications, including screening, diagnosis, and identification of predictive and prognostic biomarkers. A prospective trial to assess the feasibility and yield of CTC collection from the serum of patients with advanced PAC, using a platform involving cell adhesion matrices, has recently opened at our institution. An exploratory objective of this study is to perform molecular profiling of CTCs and to determine if it correlates with response to therapy. The ultimate goal is to develop a noninvasive method to predict in vivo drug sensitivity.

#### Serum Proteomic Biomarkers

Significant progress in the field of proteomic biomarker discovery has been made over the last 10 years, the development of liquid chromatography-tandem mass spectrometry being one of the most important technologies to emerge.<sup>78</sup> This allows the removal of abundant serum proteins, facilitating the detection of cancer biomarker proteins present in a much lower concentration. In addition, stable isotope labeling of amino acids in cell culture (SILAC) using amino acids containing heavy isotopes may be used for metabolic labeling of peptides.<sup>79</sup> This technique may be used to generate a disease-specific, metabolically labeled proteome standard for use in quantification and analysis of disease-specific protein biomarkers in serum. While research efforts in this area have focused on the detection of protein biomarkers of early disease as a screening tool, other potential clinical applications include identification of predictive and prognostic biomarkers in patients with advanced disease.

#### Conclusion

As the search for robust, clinically relevant biomarkers in PAC continues, caution must be exercised in the interpretation of data obtained from retrospective studies and early phase clinical trials. This is increasingly important in the era of identification and integration of molecular markers and active therapies in other historically refractory solid tumor malignancies (eg, melanoma and non-small cell lung cancer), and the temptation is to also want to do so for PAC. It is clear, however, that we are entering a new era of cancer therapy, in which molecular profiling of tumor specimens is likely to become routinely performed, as the cost of genetic testing continues to fall and technology becomes more widely available. The incorporation of well-designed correlative studies into the design of therapeutic trials in pancreatic cancer therefore remains crucial to the advancement of this field.

Several potential predictive biomarkers for systemic therapy in advanced disease include tumor and stromal SPARC expression, KRAS mutation status, hENT-1, DCA, and cyclin-dependent kinase (CDK) activity. The use of DPC4 status as a predictive biomarker of localized disease may allow better selection of patients for locally directed therapies. MicroRNA and cancer stem cells have been identified as potential therapeutic targets and predictive biomarkers of clinical outcome. The ability to perform CTC collection and proteomic biomarker profiling from serum samples may overcome the traditional barriers to performing correlative studies in trials of advanced PAC. As the majority of patients still present with advanced PAC, and survival outcomes for patients even with locally resectable disease remain poor, the characterization of molecular markers to identify premalignant pancreatic lesions in high-risk patients remains a crucial goal. The improvement in survival of patients with metastatic PAC seen with the use of the 5FU, leucovorin, irinotecan, and oxaliplatin combination regimen compared to gemcitabine alone in the PRODIGE 4/ACCORD 11 (Partenarait de Recherche en Oncologie Digestive/Action Clinique Coordonnées en Cancérologie Digestive) trial offers hope for an ongoing incremental improvement in patient outcomes over the coming decade.<sup>80</sup> Although there is currently a lack of prospectively validated biomarkers for clinical application in PAC, we anticipate that this will soon evolve. The concept of personalized medicine is certainly attractive to patients and physicians alike; the challenge over the coming decade will be to integrate clinical patient information with results of molecular profiling in order to maximize the potential benefit from a given therapeutic approach.

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