Early Detection and Biomarkers in Pancreatic Cancer

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Abstract
Major advances in cancer control will be greatly aided by early detection for diagnosing and treating cancer in its preinvasive state before metastasis. Unfortunately, for pancreatic ductal adenocarcinoma (PDAC), which is the fourth leading cause of cancer-related death in the United States, effective early detection and screening are currently not available and tumors are typically diagnosed at a late stage, frequently after metastasis. Partly because of low sensitivity/specificity, existing biomarkers such as CA19-9 are not adequate as early detection markers of pancreatic cancer. Thus, a great need exists for new biomarkers for pancreatic cancer. This article focuses on recent developments in the identification of new serum protein biomarkers that are useful in the early detection of PDAC. (JNCCN 2007;5:1034–1041)

Major advances in cancer control will be greatly aided by early detection for diagnosing and treating cancer in its preinvasive state before metastasis. Unfortunately, for pancreatic ductal adenocarcinoma (PDAC), which is the fourth leading cause of cancer-related death in the United States, effective early detection and screening are currently not available and tumors are typically diagnosed at a late stage, frequently after metastasis. PDAC is generally considered to be incurable by available treatment modalities, with a 5-year survival rate of less than 4%.

PDAC is the most lethal of all cancers by anatomic site, with 33,730 new cases and equivalent mortality levels expected in the United States in 2006.1 Existing biomarkers for this disease are inadequate.2 CA19-9 has been tested for its usefulness as an early detection marker in pancreatic cancer.2–6 However, the sensitivity and specificity of this biomarker are not high, and serum levels are significantly increased in inflammatory diseases of the pancreas and biliary tract. Therefore, CA19-9 is not useful for early diagnosis, mass screening, distinguishing between PDAC and chronic pancreatitis, or the targeting of therapeutics. Thus, a great need exists for new biomarkers for PDAC. In the absence of good biomarkers, 80% to 90% of PDAC cases are diagnosed too late in the disease process for surgical resection to be an effective option. Among the 10% to 20% of PDAC cases for which surgical resection is an option, most patients ultimately die of recurrent or metastatic disease.7,8 Resistance to chemotherapy and radiation, whether intrinsic or acquired, is a major cause of treatment failure in PDAC. Specific chemotherapeutic agents, such as gemcitabine, are able to induce significant sensitization of the cancer cells to radiation. This has made gemcitabine combined with radiation the best nonsurgical therapy for PDAC.9 However, few patients with PDAC benefit from the chemo sensitizing effects of gemcitabine,10 and no way currently exists to predict which patients will benefit from combined treatment.

Early detection of cancer has improved survival for several cancers, including breast,11 colon,12–14 prostate,15,16 and cervical cancers.17 Early detection of PDAC may be possible through both noninvasive (i.e., imaging technologies) and invasive means (analysis of pancreatic juice and patient serum profiling). Given the relative complexity and high cost-to-benefit ratio of imaging methodologies (e.g., helical computed tomography [CT], magnetic resonance imaging, positron emission tomography scan,
endoscopic ultrasound) and endoscopic retrograde cholangiopancreatography for obtaining pancreatic juice for analysis, these technologies are not likely to be adopted clinically for mass screening purposes. Therefore, much interest has been shown in developing and validating serum-based biomarkers for early detection of PDAC. This article focuses on recent developments in identifying new serum protein biomarkers with potential use in the early detection of PDAC (Table 1).

Harnessing the Humoral Immune Response to Cancer

The humoral immune response to cancer in humans has been well demonstrated through the identification of autoantibodies to several different intracellular and surface antigens in patients with various tumor types. Tumor-specific humoral immune responses directed against oncoproteins, mutated proteins such as p53, or other aberrantly expressed proteins have all been described. Although it is currently unknown whether the occurrence of these antibodies is beneficial, knowledge of potential tumor antigens that may evoke tumor-specific immune responses may be useful in early cancer diagnosis, establishing prognosis, and immunotherapy against the disease. In pancreatic cancer, autoimmunity has been shown against several cellular proteins, including MUC1, p53, Rad51, and DEAD-box protein 48. MUC1 is a transmembrane glycoprotein involved in cell–cell and cell–extracellular matrix interactions, and MUC1 autoantibodies have been observed in sera from patients with various different tumors. In pancreatic cancer, the presence of MUC1 IgG autoantibodies has been shown to be associated with a favorable prognosis. Although p53 autoantibodies have been observed in 18.2% of patients with pancreatic cancer, these autoantibodies were also found in 5.3% of patients with acute pancreatitis and 12.1% of patients with chronic pancreatitis, indicating that the humoral response to p53 was not specific to malignancy. The recombination factor Rad51 is highly expressed in pancreatic adenocarcinoma, and Rad51 autoantibodies have been observed in 7% of patients with pancreatic cancer. Autoantibodies to DEAD-box protein 48, a member of the DEA(D/H)-box RNA helicase family, were observed in serum from 63.6% of patients with

| Table 1 Current Promising Biomarkers for the Detection of Pancreatic Cancer |
|-----------------|-----------------|-----------------|
| Name of the Biomarker | Technology Used for Discovery | Type | Study |
| p53 | Humoral response | Autoantibody | Raedle et al. |
| MUC1 | Humoral response | Autoantibody | Hamanaka et al. |
| Rad51 | Humoral response | Autoantibody | Maacke et al. |
| DEAD-box protein 48 | Humoral response | Autoantibody | Xia et al. |
| Calreticulin | Humoral response | Autoantibody | Hong et al. |
| Vimentin | Humoral response | Autoantibody | Hong et al. |
| Osteopontin | Serum profiling | Serum protein | Koopmann et al. |
| MIC-1 | Serum profiling | Serum protein | Koopmann et al. |
| PGK 1 | Serum profiling | Serum protein | Hwang et al. |
| CEACAM1 | Serum profiling | Serum protein | Simeone et al. |
| HIP/PAP | Pancreatic juice profiling | Pancreatic juice protein | Gronberg et al. |
| Lipocalin 2 | Pancreatic juice profiling | Pancreatic juice protein | Gronberg et al. |
| pg96 | Pancreatic juice profiling | Pancreatic juice protein | Gronberg et al. |
| PAP-2 | Pancreatic juice profiling | Pancreatic juice protein | Gronberg et al. |
| RNase 1 | N-linked glycan profiling | Serum protein | Peracaula et al. |
| Hemopexin | N-linked glycan profiling | Serum protein | Zhao et al. |
| Kininogen-1 | N-linked glycan profiling | Serum protein | Zhao et al. |
| Anti-thrombin-III | N-linked glycan profiling | Serum protein | Zhao et al. |
| Haptoglobin-related protein | N-linked glycan profiling | Serum protein | Zhao et al. |
| Plasma protease C1 inhibitor | N-linked glycan profiling | Serum protein | Zhao et al. |
pancreatic cancer, whereas these autoantibodies were only found in the serum from 1.9% of normal subjects.28

Several approaches are currently available for identifying tumor antigens. In contrast to identifying tumor antigens based on analysis of recombinant proteins (which do not contain posttranslational modifications found naturally), a proteomics-based approach to identifying tumor antigens that facilitates the identification of autoantibodies to proteins as they occurred in their natural states, in lysates prepared from tumors, and tumor cell lines may be preferable. This technology may uncover antigenicity associated with aberrant posttranslational modification of tumor cell proteins. A proteomics approach was implemented to identify PDAC tumor antigens that elicit a humoral response against proteins that are expressed in the Panc-1 pancreatic adenocarcinoma cell line. Two-dimensional polyacrylamide gel electrophoresis was used to simultaneously separate individual cellular proteins from the Panc-1 cell line. The separated proteins were transferred onto polyvinylidene fluoride membranes. Sera from patients with cancer were screened individually for antibodies that reacted against the separated proteins through Western blot analysis. Proteins specifically reacting with sera from cancer patients were identified with mass spectrometry. One study11 showed that a humoral response directed against calreticulin isoform 1 or 2, or both, occurred in 58.3% of patients with pancreatic cancer. One of 18 patients with chronic pancreatitis (5.6%) and 1 of 15 healthy controls (6.6%) showed autoantibodies to calreticulin isoform 1; none showed autoantibodies to isoform 2. None of the sera from patients with colon cancer exhibited reactivity against these proteins. Only 1 of 14 (7.1%) samples of sera from patients with lung adenocarcinoma showed autoantibodies to calreticulin isoform 1, whereas 2 of 14 (14.3%) sera samples showed autoantibodies to isoform 2. In a second study,11 the authors showed a humoral response directed against a single isoform of vimentin in 44.4% patients with pancreatic cancer. One of 18 (5.6%) patients with chronic pancreatitis and none of the noncancer controls exhibited reactivity against the antigenic vimentin isoform.

Why only a subset of patients with a particular tumor type develop a humoral response to a particular antigen is unclear. Immunogenicity may depend on the level of expression, posttranslational modification, or other types of protein processing, the extent of which may be variable among tumors of a similar histologic type. Other factors that may influence the immune response include variability among tumors and individuals regarding major histocompatibility complex molecules and antigen presentation. Although several autoantibodies have been identified in PDAC, in most cases they occur in less than 50% of patient's sera. Therefore, they are not effective individually for early detection of PDAC, but may show efficacy if used as a panel of biomarkers.11

Detection of Altered Plasma Protein Expression for Identification of PDAC-Specific Biomarkers

Great interest has been shown in the hypothesis that tumor-specific proteins may be found in patients' circulation and may be useful in the early detection of cancer. For example, proteins such as CA125 in ovarian cancer and prostate-specific antigen (PSA) in prostate cancer have been used clinically as diagnostic markers of cancer. CA125 is a mucin commonly used as a diagnostic marker for epithelial ovarian cancer. PSA is secreted primarily by prostate epithelial cells into the seminal plasma and is one of the best-characterized examples of a secreted glycoprotein used in cancer diagnostics. Several recent reports have described aberrantly expressed proteins in the serum of patients with pancreatic cancer. In one report, Koopmann et al.29 evaluated osteopontin as a serum biomarker of pancreatic adenocarcinoma. Although osteopontin expression was not observed in pancreatic cancer cells, normal pancreata, or macrophages distant from the infiltrating cancer, a strong osteopontin mRNA signal was observed in tumor-infiltrating macrophages in 8 of 14 pancreatic adenocarcinomas. Elevated levels of serum osteopontin were observed in the sera of 50 patients with resectable pancreatic adenocarcinoma (482 ± 170 ng/mL) compared with sera from 22 healthy subjects (204 ± 65 ng/mL). However, the investigators did not evaluate serum osteopontin levels in patients with inflammatory diseases of the pancreas (i.e., chronic pancreatitis). Thus, whether the increased serum osteopontin levels are cancer-specific or caused by the associated extensive desmoplastic response seen in pancreatic adenocarcinoma is unknown.

Serum macrophage inhibitory cytokine 1 (MIC-1) has been shown to have potential usefulness
as a serum biomarker of pancreatic cancer. The enzyme-linked immunosorbent assay (ELISA) has shown serum MIC-1 levels to be significantly higher in patients with PDAC and those with ampullary and cholangiocellular carcinomas than in those with benign pancreatic neoplasms or chronic pancreatitis, or healthy controls. Serum MIC-1 has been shown to be useful within a panel of protein biomarkers.

Serum levels of phosphoglycerate kinase 1 (PGK1) also have been shown to have potential usefulness as a serum biomarker of pancreatic cancer. Hwang et al. identified PGK1 as being overexpressed in PDAC compared with adjacent nontumor pancreata. They subsequently found that serum levels of PGK1 were significantly elevated in patients with PDAC compared with normal controls and those with other cancer types.

In a recent study, Simeone et al. observed PDAC-specific overexpression of CEACAM1, a member of the human carcinoembryonic antigen family. They subsequently measured CEACAM1 serum levels in patients with PDAC and chronic pancreatitis and in normal subjects using a double determinant ELISA. CEACAM1 was found to be expressed in the sera of 91% (74/81) of patients with PDAC, 24% (15/61) of normal subjects, and 66% (35/53) of patients with chronic pancreatitis, with a sensitivity and specificity superior to CA19-9. Unfortunately, however, none of the above protein biomarkers (osteopontin, MIC1, PGK1, and CEACAM1) have the requisite sensitivity/specificity to be useful individually as a biomarker for the early detection of pancreatic cancer, but may be useful within a panel of protein biomarkers.

Use of Mass Spectrometric Methodologies for Identification of PDAC-Specific Biomarkers
Methodologies have been developed to directly analyze the proteins contained within complex protein mixtures, such as that found within human biofluids (e.g., plasma, serum, saliva, urine). Among these technologies, some, such as surface-enhanced laser desorption/ionization (SELDI), are mass spectrometry–based. In one study, serum samples from patients with and without PDAC were analyzed using SELDI protein chip mass spectrometry. Using a case-control study design, serum samples from 60 patients with small resectable PDAC (early lesions) were compared with samples from 60 age- and sex-matched patients with nonmalignant pancreatic diseases and 60 age- and sex-matched healthy controls. To increase the number of identifiable proteins, patients’ serum was fractionated using anion exchange chromatography and profiled on 2 ProteinChip surfaces (metal affinity capture and weak cation exchange). The authors determined the minimum set of protein peaks able to discriminate between patient groups and used the unified maximum separability algorithm to compare the performance of the individual marker panels alone or in conjunction with CA19-9.

Among the peaks identified through SELDI profiling that had the ability to distinguish between patient groups, the 2 most discriminating protein peaks could differentiate patients with PDAC from healthy controls with a sensitivity of 78% and specificity of 97%. These 2 markers performed significantly better than the current standard serum marker, CA19-9. The diagnostic accuracy of the 2 markers was improved by using them in combination with CA19-9. Similarly, a combination of 3 SELDI markers and CA19-9 was superior to CA19-9 alone in distinguishing individuals with PDAC from the combined pancreatic disease controls and healthy subject groups. SELDI markers also were better than CA19-9 in distinguishing patients with PDAC from those with chronic pancreatitis. One drawback of the SELDI technology, however, is that the direct profiling of complex protein mixtures has difficulties identifying the distinctive proteins. Furthermore, given the limited dynamic range of SELDI, distinctive features observed in serum with this approach probably represent relatively abundant proteins, not necessarily specific to PDAC.

Mass Spectrometric Profiling of Pancreatic Juice
Other mass spectrometric profiling methods have been used to profile proteins found in pancreatic juice to identify PDAC-specific biomarkers. Gronborg et al. analyzed pancreatic juice from 3 patients with PDAC using 1-dimensional gel electrophoresis, with subsequent analysis using liquid chromatography tandem mass spectrometry (MS/MS). They identified 170 unique proteins, including known pancreatic cancer tumor markers (e.g., carcinoembryonic antigen, MUC1) and proteins overexpressed in pancreatic
cancers (e.g., hepatocarcinoma-intestine-pancreas/pancreatitis–associated protein [HIP/PAP], lipocalin 2). In addition, they identified several proteins that have not been previously described in pancreatic juice (e.g., tumor rejection antigen [pg96]). Interestingly, a novel protein 85% identical to HIP/PAP was identified and designated as PAP-2. These authors, however, did not analyze pancreatic juice from normal individuals or from patients with chronic pancreatitis. Thus, conclusions cannot be drawn about the PDAC-specificity of protein expression. Chen et al. used isotope-code affinity tag technology and MS/MS to perform quantitative protein profiling of pancreatic juice from patients with pancreatic cancer and normal controls. A total of 105 proteins were identified and quantified in the pancreatic juice from a patient with pancreatic cancer, of which 30 proteins showed abundant changes of at least twofold in pancreatic cancer juice compared with normal controls.

In a subsequent study, Chen et al. used a similar approach to identify and quantify proteins from pancreatic juice isolated from patients with chronic pancreatitis. In total, 72 proteins were identified and quantified in the comparison of pancreatic juice from patients with chronic pancreatitis versus pooled normal control juice. Nineteen of the juice proteins were overexpressed and 8 were underexpressed in chronic pancreatitis juice by at least twofold compared with normal pancreatic juice. Of the 27 differentially expressed proteins in chronic pancreatitis, 9 proteins were also differentially expressed in pancreatic juice from patients with PDAC. Some of the proteins identified in these studies may be useful as PDAC-specific biomarkers. Zhou et al. collected pancreatic juice samples from patients with pancreatic cancer, benign pancreatic diseases, or cholecystitis during endoscopic retrograde cholangiopancreatography. Pancreatic juice proteins were resolved using 2-dimensional gel electrophoresis and visualized proteins were identified using matrix-assisted laser desorption ionization time-of-flight mass spectrometry. They found and identified 7 protein spots whose expression was altered in pancreatic juice from patients with pancreatic cancer. Although many of the above-identified proteins may serve as candidate biomarkers of PDAC, given the invasive nature of endoscopic retrograde cholangiopancreatography and the potential for associated morbidity, biomarkers uncovered during the profiling of pancreatic juice will probably not have much use for population-based screening for pancreatic cancer.

N-linked Glycan Profiling for Biomarker Identification in Cancer Serum

Glycoproteins are the most heterogeneous group of posttranslational modifications known in proteins. Glycans show a high structural diversity reflecting inherent functional diversity. N- and O-oligosaccharide variants on glycoproteins (glycoforms) can lead to alterations in protein activity or function that may manifest itself as overt disease. Many clinical biomarkers and therapeutic targets in cancer are glycoproteins, such as CA125 in ovarian cancer, human epidermal growth factor receptor 2 (Her2/neu) in breast cancer, and PSA in prostate cancer. Her2/neu is a transmembrane glycoprotein, with the presence of Her2 overexpression apparently a key factor in malignant transformation and predictive of a poor prognosis in breast cancer. CA125 is a mucin commonly used as a diagnostic marker for epithelial ovarian cancer. Although CA125 has long been used as an ovarian cancer marker, many of its O- and N-glycan structures have only recently been characterized. PSA is secreted primarily by prostate epithelial cells into the seminal plasma. It is one of the best-characterized examples of a secreted glycoprotein used in cancer diagnostics, and its glycoforms have been described. The alteration in protein glycosylation that occurs through varying the heterogeneity of glycosylation sites or changing glycan structure of proteins on the cell surface and in body fluids has been shown to correlate with the development or progression of cancer and other disease states. Reports have shown that the glycosylation of PSA secreted by the tumor prostate cell line LNCaP differs significantly from that of PSA from seminal plasma (normal control). These carbohydrate differences allow a distinction to be made between PSA from normal and tumor origins and provide a valuable biochemical tool for diagnosing prostate cancer. Characterization of the N-glycans from human pancreatic ribonuclease (RNase 1) isolated from healthy pancreas and pancreatic adenocarcinoma tumor cells (Capan-1 and MDAPanc-3) showed completely different glycosylation patterns. These glycosylation changes in a tumor-secreted protein reflect fundamental changes in the enzymes involved in the glycosylation pathway. Thus, a high-throughput
Biomarkers in Pancreatic Cancer

These microarrays were used to discern differences in the glycosylation structural patterns of serum glycoproteins specific for pancreatic cancer and chronic pancreatitis. N-linked glycoproteins were enriched in immunodepleted serum samples using a general multilectin column. These enriched glycoproteins were then separated using nonporous silica reverse-phase high-performance liquid chromatography. The resolved glycoproteins were then arrayed on nitrocellulose slides and probed with various lectins to screen the glycosylation structure of the serum glycoproteins. The glycoprotein–lectin interaction was assessed using a biotin–streptavidin system that had low femtomole limits of detection. The individual glycoproteins with altered glycosylations were then identified and characterized. These glycan structural alterations may be useful for the early detection of pancreatic cancer and the differential diagnosis of pancreatic cancer and chronic pancreatitis.

One study investigated 10 normal sera, 8 chronic pancreatitis sera, and 6 pancreatic cancer sera. Data from the glycoprotein microarrays were analyzed using bioinformatics approaches, including principal component analysis and hierarchical clustering. Both normal and chronic pancreatitis sera were found to cluster close together, although in 2 distinct groups, whereas pancreatic cancer sera were significantly different from the other 2 groups. Both sialylation and fucosylation increased as a function of cancer on several proteins, including hemopexin, kininogen-1, antithrombin-III, and haptoglobin-related protein, whereas decreased sialylation was detected on plasma protease C1 inhibitor. Target alterations on glycosylations were verified through lectin blotting experiments and peptide mapping experiments using µLC-ESI-TOF. These altered glycan structures may be useful for the differential diagnosis of pancreatic cancer and chronic pancreatitis and in identifying critical differences between biologic samples from patients with different clinical conditions.

Summary

Early detection of pancreatic adenocarcinoma, so that cancer can be diagnosed and treated in its preinvasive state before metastasis, may greatly impact the treatment and prognosis of patients with this deadly malignancy. Unfortunately, suitable biomarkers have not been identified for the early detection of pancreatic cancer. Biomarker discovery for this disease is still very much in its discovery phase. Multiple approaches have been developed that are promising for the identification of serum biomarkers. However, the protein biomarkers that have been identified do not possess the requisite sensitivity/specificity to be useful individually as a biomarker for the early detection of pancreatic cancer, but ultimately may have use within a panel of protein biomarkers. Additionally, other emerging technologies, such as genetically engineered mouse models of pancreatic cancer, may be useful in identifying panels of serum biomarkers that can be further explored in human sera. To determine the usefulness of any promising protein biomarkers, the candidates will need to be tested and validated through multiple independent studies using an adequately sized test and training set of sera samples from very early-stage pancreatic cancer, a resource that does not currently exist. Development of such resources, including serum from patients with nonmalignant pancreatic lesions and prospective serum collection from individuals at high risk for being diagnosed with pancreatic cancer, and serum from patients with other malignancies, is critically needed for identifying biomarkers that are useful for the early detection of pancreatic cancer. To present, serum/plasma collection has been primarily performed in individual laboratories, using heterogeneous sample collection methods. Recently, the Human Proteome Organization conducted a study to assess efficacious serum collection methods. These
findings have led to efforts presently being made by the National Cancer Institute, through the Early Detection Research Network, to develop suitable serum resources for both the discovery phase and the subsequent validation phase of biomarkers for the early detection of cancer. With the ultimate development of these standardized resources, suitable biomarkers are expected to be validated and useful for the early clinical detection of pancreatic cancer within the next 5 to 7 years.

References
Biomarkers in Pancreatic Cancer


