New MUC1 Serum Immunoassay Differentiates Pancreatic Cancer From Pancreatitis

David V. Gold, David E. Modrak, Zhiliang Ying, Thomas M. Cardillo, Robert M. Sharkey, and David M. Goldenberg

ABSTRACT

Purpose
To evaluate a new immunoassay for identification and quantitation of MUC1 in the sera of patients with pancreatic cancer or pancreatitis. The sensitivity and specificity of the assay are examined and compared to results from a CA19-9 immunoassay.

Methods
An in vitro enzyme immunoassay was established with monoclonal antibody PAM4 as the capture reagent, and a polyclonal anti-MUC1 antibody as the probe. Patient sera were obtained from healthy, adult patients with acute and chronic pancreatitis, and those with pancreatic and other forms of cancer, and were measured for PAM4-reactive MUC1.

Results
At a cutoff of 10.2 units/mL, 41 (77%) of 53 pancreatic cancer patients, none of the healthy, adult patients with acute and chronic pancreatitis, and those with pancreatic and other forms of cancer, and were measured for PAM4-reactive MUC1.

Conclusion
The high sensitivity and specificity observed suggest that the PAM4-based immunoassay of circulating MUC1 may be useful in the diagnosis of pancreatic cancer.

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INTRODUCTION

Pancreatic cancer is considered one of the most lethal forms of cancer in the United States. Although only the 10th most common form of cancer, with an estimated 32,000 new cases diagnosed in the United States annually, it is the fourth and fifth leading cause of cancer deaths in men and women, respectively. In large part, this is a result of its nonsymptomatic growth, but also the fact that the major complaints at presentation (ie, weight loss, abdominal pain, nausea, and, frequently, jaundice), are not sufficiently diagnostic, but are consistent with benign, inflammatory, and other malignant disease processes. Complicating the matter still further is the potential for pancreatic cancer to arise within a setting of chronic pancreatitis. Unless there is a great deal of suspicion on the part of the physician, a response, perhaps, to a family history of pancreatic cancer or other risk factors, the likelihood of correctly diagnosing pancreatic cancer at an early stage is low. Thus, with most pancreatic cancer presenting at late stages of disease, treatment procedures currently available are not able to achieve a cure, nor improve survival substantially, except in rare cases. Surgical resection has been the only modality that can offer a chance at cure, but because of a large tumor burden and/or anatomic considerations at presentation (eg, involvement of major blood vessels), only 10% to 25% of patients are candidates for curative resection. For those patients undergoing surgical treatment, the 5-year survival rate is still poor, averaging only approximately 10%. Frontline treatment for nonresectable disease is usually chemotherapy with the pyrimidine antimetabolite gemcitabine or radiotherapy. Although long-term survival is disappointing with either treatment,
glycosyltransferases. Differences—normal versus malignant, dependent, at least in part, on the expression and activity of specific neomarkers such as sialyl-Lea (CA19-9), sialyl-Lex, and sialyl-Tn—resulting in an aberrant oligosaccharide profile giving rise to the expression of these markers released into a body fluid. The gold standard for detection of pancreatic cancer has been the immunoassay for the CA19-9 antigen, an oligosaccharide (sialyl-Lea) present within the MUC1 mucin-type glycoprotein. Originally, elevated levels of the CA19-9 antigen were found in 70% of pancreatic cancer patients, and in none of the pancreatectomy specimens examined. In further studies, the sensitivity was reported in the range of 70% to 95%, and the specificity in the range of 72% to 90%. CA19-9 levels are known to be elevated in a number of benign and malignant conditions, so that currently, the assay is not used for initial diagnosis. However, the assay has proved to be useful for disease management, with the continued increase in CA19-9 serum levels post-therapy indicative of a poor prognosis.

MUC1 has received a great deal of attention as a prospective biomarker because of the complex and heterogeneous nature of the epitopes expressed within the antigen. Although the antigen is found in normal, benign, and malignant conditions of both pancreatic and nonpancreatic tissues, it is usually overexpressed in cancer. Furthermore, MUC1 synthesized by cancerous tissues usually will display an aberrant oligosaccharide profile giving rise to the expression of neomarkers such as sialyl-Lea (CA19-9), sialyl-Leb, and sialyl-Tn (TAG-72), as well as the cryptic epitopes such as Tn. In addition, because of underglycosylation, the peptide core of the mucin becomes exposed such that epitopes within the core that are not accessible within normal tissue-derived MUC1 may serve as potential biomarkers (eg, tandem repeat epitopes reactive with MAbS HMFG1 and 2, and SM3). Heterogeneity in glycosylation of the peptide core is dependent, at least in part, on the expression and activity of specific glycosyltransferases. Differences—normal versus malignant, and organ versus organ—can provide for distinct epitopes that may show higher specificity for malignant tissues and/or specific organs. Thus, there have been numerous reports concerning the development and application of anti-MUC1 antibodies for several forms of cancer. Currently, several of these are available in commercial form for use in patient management (CA15-3 [Abbott Laboratories, Abbott Park, IL] and CA27.29 [Bayer Diagnostics, Tarrytown, NY] are just two) but none have proven to be of significant diagnostic value.

We have developed and characterized the anti-MUC1 monoclonal antibody PAM4 and reported its potential utility for the detection and therapy of pancreatic cancer. PAM4 demonstrates a relatively high specificity for an MUC1 produced by pancreatic cancer, as compared with MUC1 antigens derived from other types of cancer (eg, breast, ovarian, etc). Preclinical studies of radioimaging and -therapy, as well as initial clinical imaging studies, have provided evidence as to the high specificity and sensitivity of the antibody for pancreatic cancer. In this article, we extend these studies with the development of a PAM4-based blood immunoassay for quantitation of circulating antigen, and describe its potential role for identification of patients with pancreatic cancer, especially in comparison with the CA19-9 immunoassay.
RESULTS

Development and Characterization of the PAM4-Based Immunoassay

Given the nature of MUC1, it was possible that multiple copies of the epitope identified by PAM4 are expressed on MUC1. However, initial studies with a homogeneous PAM4 capture/probe sandwich assay were not successful. The immunoassay that was finally developed employed PAM4 as the capture reagent, with an unlabeled, purified IgG derived from rabbit polyclonal antipancreatic mucin antiserum as the probe, followed by peroxidase-labeled donkey antirabbit IgG as the detection reagent. Because of the considerable microheterogeneity inherent within MUC1, we chose to report our results in arbitrary units/mL based on an initial reference standard of MUC1 purified from xenografted CaPan1 human pancreatic tumor. The lower limit of detection for the immunoassay was 1.0 unit/mL, with saturation occurring at MUC1 concentrations above 100 units/mL. A linear range was determined to be 1.5 units/mL to 25 units/mL of antigen (Fig 1). Interassay (n = 5) coefficients of variation (CV) were calculated for reference standards of 20 units/mL (CV = 8.0%) and 8 units/mL (CV = 3.8%). Mean recoveries were 17.5 ± 2.8 and 7.1 ± 1.9 for the 20 and 8 units/mL standards, respectively.

Levels of PAM4-reactive MUC1 in Patient Specimens

Sera from a total of 283 patients, including 53 with pancreatic cancer, were examined for the presence of PAM4-reactive MUC1. The frequency distribution of serum MUC1 concentrations for the varying disease groups is provided in Figure 2. The receiver operator characteristic (ROC) curve was calculated (Fig 3), and the area under the curve (AUC) determined to be 0.88 ± 0.03 (95% CI, 0.84 to 0.92) with P < .0001, a highly significant difference for discrimination of pancreatic cancer from nonpancreatic cancer specimens. At a cutoff value of 10.2 units/mL, the sensitivity and specificity were calculated to be 77% and 95%, respectively, with a positive diagnostic likelihood ratio (+DLR) of 13.7, as compared with healthy, benign, and nonpancreatic cancer groups.

The data presented in Table 1 show that the median and mean values for the pancreatic cancer group were more than 10-fold greater than for all of the other groups, even though the overwhelming ma-

Fig 1. Standard curve for PAM4-reactive MUC1. The enzyme immunoassay was linear within the range of 3.1 to 25 units/mL of MUC1. The equation of the trend line and the value for R² are provided.

Fig 2. Frequency distribution of PAM4-reactive MUC1 in patient sera. Cutoff value = 10.2 units/mL.

Fig 3. Receiver operator characteristics curve for the performance of the PAM4-based immunoassay. Values for the area under the curve (AUC) and 95% CIs are provided.

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antigen (as did one lymphoma and three pancreatitis patients). Specificity of the immunoassay for discrimination of pancreatic cancer and these other MUC1-producing malignancies was still quite high (90%; $P < .0001$) with a +DLR of 7.8. We also examined specimens from patients with non-Hodgkin’s lymphoma, which does not normally produce MUC1. Only one of 14 patients tested positive for MUC1; however, the antigen concentration was found to be relatively high (194 units/mL). It is important to note that specificity of the immunoassay for cancer (whether pancreatic or not) was quite high (97%), with a +DLR of 11.0.

### Comparison of PAM4 and CA19.9 Immunonassays

Of the 53 pancreatic cancer specimens, only 41 were assessable for both PAM4-reactive MUC1 and CA19.9 because of insufficient volume of certain samples (Table 3). Of these, 24 (59%) were considered positive for CA19.9 (at a cutoff of 35 units/mL). As with the PAM4 immunoassay, none of the healthy specimens were positive for CA19.9. However, of the 87 pancreatitis samples, CA19.9 was positive in 37%. ROC analyses (Fig 4) for discrimination of pancreatic cancer from pancreatitis serum specimens provided an AUC of 0.67 ± 0.05 (95% CI, 0.58 to 0.75), with a specificity of 63% and a +DLR of 1.6 for the CA19-9 test. Statistical analyses for PAM4-reactive MUC1 in this same subset of pancreatic cancer and pancreatitis sera differed little from the group analyses discussed earlier; sensitivity for this subset was slightly reduced (71%), but specificity remained high (96%), as did the +DLR (15.4). There was no correlation between PAM4 and CA19-9 values. Two of the four PAM4-positive pancreatitis specimens were also positive for CA19-9. A direct pair-wise comparison of the ROC curves resulted in a statistically significant difference ($P < .003$), with the PAM4 immunoassay demonstrating a superior sensitivity and specificity for discrimination of patients with pancreatic cancer from those with pancreatitis.

### DISCUSSION

The timely and accurate diagnosis of pancreatic cancer can be difficult, even when one considers biochemical and imaging data along with presenting signs and symptoms. Compounding this challenge is the considerable overlap of symptoms and clinical data that occurs between pancreatic cancer and focal pancreatitis, worsened by the potential for pancreatic cancer to arise in a setting of pancreatitis and/or the ability of pancreatic cancer to induce secondary inflammatory processes. The importance of distinguishing these two disease entities is highlighted by reports that 5% to 10% of pancreatic resections are eventually determined to be pancreatitis rather than pancreatic cancer.

Unfortunately, there are no procedures currently available for the accurate detection and diagnosis of pancreatic cancer. It is for this reason that the NCI’s Pancreatic Cancer Progress Review Group has drawn specific attention to the “urgent need for better screening and diagnostic techniques,” with a recommendation to “delineate and validate effective molecular biomarkers for pancreatic cancer.” The search for novel, clinically useful biomarkers has included investigations of individual antigens, including MUC1 and other MUC antigens (eg, MUC4), hormones, and enzymes, among other substances, as well as, more recently, investigations of genomic and proteomic profiling.

Our laboratory has generated and characterized the PAM4 anti-MUC1 monoclonal antibody and examined its potential utility for...
imaging and therapy of pancreatic cancer.\textsuperscript{30-34} Although several antibodies have been described as reactive with MUC1 derived from various types of cancer,\textsuperscript{28,42,43} PAM4 demonstrates a more restricted reactivity with pancreatic cancer, as compared with other MUC1-producing tumors. For the present study, we developed a PAM4-based immunoassay for the detection and differential diagnosis of pancreatic cancer. Overall, the immunoassay provided high sensitivity and specificity, with a value $\geq 10.2$ units/mL indicating a high likelihood of pancreatic cancer ( + DLR = 13.7), as compared with normal and benign disease groups, as well as non-pancreatic cancers. Also of significance, the immunoassay showed high specificity (97%) for identification of cancerous specimens (both pancreatic and nonpancreatic) in comparison to noncancerous specimens, with a + DLR of 11.0. This observation will certainly need to be examined in greater detail by further comparison of cancer with benign and inflammatory diseases of several tissues, in addition to the pancreatitis specimens analyzed within the present study.

Notably, the nonpancreatic cancer group found to have the greatest number of positive cases was colorectal cancer. This is not surprising, given that colorectal tumors express MUC1 and our original studies on PAM4 documented weak positive immunohistochemical staining of goblet cells within the normal GI tract, as well as positive staining of approximately 40% of colorectal tumors.\textsuperscript{29} However, identification of PAM4+ pancreatic cancer and PAM4+ colorectal cancer should not be difficult when follow-up imaging studies are performed, including the potential use of PAM4-based imaging procedures. In two separate reports,\textsuperscript{33,34} we described clinical targeting and imaging of pancreatic cancer using $^{131}$I- and $^{99m}$Tc-labeled murine PAM4. Definitive tumor targeting was observed in eight of 10 patients. Of the two patients for whom tumor imaging was not observed, one was later confirmed to have had chronic pancreatitis rather than pancreatic cancer, providing further evidence for the specificity of PAM4, whereas tumor tissue from the other patient was later found to be a poorly differentiated tumor that was nonreactive with PAM4 by immunohistology. No accumulation of radiolabeled PAM4 was observed in healthy tissues known to express PAM4-defined MUC1 (eg, the GI tract). Future research in this area includes development of PAM4-based nuclear imaging agents that we hope will prove useful for follow-up scanning of patients who have positive results by immunoassay.

Currently, the biomarker CA19-9 is considered to be the standard for monitoring patients with pancreatic cancer even though the assay is not useful for the initial diagnosis of the disease. Positive CA19-9 results have been reported for substantial numbers of patients with benign pancreatic disease, as well as malignancies of nonpancreatic origin (colorectal, gastric, ovarian, lung, and hepatobiliary, among other cancers).\textsuperscript{12-17} Although not specific for pancreatic cancer, CA19-9 is usually one of the first clinical assays performed as part of the diagnostic work-up.

As already noted, an important consideration for diagnosis of pancreatic cancer is the ability of any assay or procedure to distinguish between pancreatic cancer and pancreatitis. In this regard, the CA19-9 immunoassay fails to provide adequate diagnostic accuracy, with reports of positive results for pancreatitis in the range of 13% to greater than 31%.\textsuperscript{12-17,41,44} In our studies, a direct pair-wise comparison of results from PAM4-based and CA9-9 immunoassays demonstrated a significant and substantially superior performance of the PAM4-based immunoassay to distinguish pancreatic cancer from pancreatitis ($P < .003$). Sera from only four (5%) of 87 pancreatitis patients were positive by use of the PAM4-based immunoassay. In contrast, positive levels of CA19-9 were found in 37% of the pancreatitis specimens examined.

\begin{table}[h]
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\begin{tabular}{lccc}
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 & Cutoff Value & AUC* & +DLR \\
\hline
PAM4 & 10.2 & 0.85 $\pm$ 0.04 & 71 & 96 & 15.4 \\
CA19.9 & 35.0 & 0.67 $\pm$ 0.05 & 59 & 63 & 1.6 \\
\hline
\end{tabular}
\caption{Comparison of PAM4 and CA19.9 Immunoassays for Differential Diagnosis of Pancreatic Cancer}
\footnotesize{NOTE. Of the 53 pancreatic cancer specimens shown in Tables 2 and 3, 41 were assessable for both PAM4 and CA19-9 antigens. All of the pancreatitis specimens (87) were examined by both immunoassays. Abbreviations: AUC, area under the curve; +DLR, positive diagnostic likelihood ratio. *$P$ for comparison of AUC for PAM4 and CA19-9 immunoassays is $< .003$.}
\end{table}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{Comparison of PAM4 and CA19.9 receiver operator characteristics curves for discrimination of pancreatic cancer and pancreatitis. Values for the area under the curve (AUC) are provided. A statistically significant difference ($P < .003$) was calculated for the comparison of AUCs.}
\end{figure}
To date, no single biomarker has been proven to have sufficient diagnostic accuracy so as to provide a stand-alone means for early detection and diagnosis of cancer. However, it is unlikely that a diagnosis and follow-up therapeutic plan would be made on the basis of a single test result. Thus, biomarkers can prove to be of significant clinical value by providing evidence to suspect cancer or, when combined with other clinical data, to aid in differential diagnosis. This has been the case for the most widely used tumor markers, CEA, PSA, and of course CA19-9, for pancreatic cancer. None is specific for its intended malignancy; however, each has found its use as part of a paradigm for diagnosis and management of cancer.

The question, then, is whether a PAM4-based immunoassay can provide added clinical value as compared with the assay currently used for CA19-9. Our results suggest this will be the case. However, we are aware that sensitivity and specificity are dependent on the range of pathologic conditions and numbers of specimens chosen for the study. It will be necessary to corroborate these initial results with larger numbers of specimens of both early and advanced disease, as well as a number of benign diseases and cancer types that are known to be either MUC1 or non-MUC1 producers. Furthermore, it will be important to obtain clinical data to determine the potential role of this immunoassay for early detection (small tumors) and, thus, its potential use in screening of patients at risk for development of pancreatic cancer and for disease management. If confirmed, the PAM4-based immunoassay could provide a cost-effective means for noninvasive detection of pancreatic cancer, and importantly, a means for discrimination of malignant and inflammatory disease processes within the pancreas.

**REFERENCES**

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Authors’ Disclosures of Potential Conflicts of Interest

Although all authors completed the disclosure declaration, the following authors or their immediate family members indicated a financial interest. No conflict exists for drugs or devices used in a study if they are not being evaluated as part of the investigation. For a detailed description of the disclosure categories, or for more information about ASCO’s conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

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GLOSSARY

Epitope: Region within an antigen that has the potential to give rise to an antibody response. With respect to protein antigens, epitopes may be defined on the basis of primary, secondary, or tertiary structure of the molecule and, consequently, may be exposed or hidden within the molecule.

Glycosyltransferase: Class of enzymes that transfer sugar molecules (eg, glucose, galactose) to amino acids in proteins, several different lipid molecules, and carbohydrates, with specificity of the enzyme residing in the sugar molecule transferred, molecule glycosylated, and coenzymes that act as carriers of the sugar molecule.

MUC1: A transmembrane-anchored mucin-type glycoprotein, MUC1 is present on the surface of normal epithelial cells of organs such as the breast, ovary, pancreas, and colon. It is also a tumor marker because it is overexpressed in similar malignant tissues. Structurally, MUC1 has an extracellular carbohydrate-rich domain, a transmembrane and a cytoplasmic domain. Human MUC1, a membrane-bound glycoprotein, is a major component of the ductal cell surface of normal glandular cells probably involved in intercell adhesion. MUC1 is overexpressed and aberrantly glycosylated in carcinoma cells, promoting tumor metastases and inducing immune suppression.

Positive diagnostic likelihood ratio (+DLR): Diagnostic likelihood ratios use the sensitivity and specificity of the test used in disease diagnosis to provide a direct estimate of how a test result will change the odds of having a disease. The +DLR indicates the odds of having the disease when the test is positive and is mathematically defined as the following: +DLR = test sensitivity / (1 - test specificity)

ROC (receiver operating characteristic) curves: ROC curves plot the true positive rate (sensitivity) against the false-positive rate (1-specificity) for different cut-off levels of a test. The area under the curve is a measure of the accuracy of the test. An area of 1.0 represents a perfect test (all true positives), whereas an area of 0.5 represents a worthless test.

Xenograft: Host graft from a species that is not related to the recipient.