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Detection of Early-Stage Pancreatic Adenocarcinoma

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Abstract

Background—Pancreatic adenocarcinoma is an almost universally lethal disease, in large part, due to our inability to detect early-stage disease. Monoclonal antibody PAM4 is reactive with a unique biomarker expressed by greater than 85% of pancreatic adenocarcinomas. In this report, we examined the ability of a PAM4-based immunoassay to detect early-stage disease.

Methods—The PAM4-based immunoassay was used to quantitate antigen in the serum of healthy volunteers (N=19), patients with known pancreatic adenocarcinoma (N=68), and patients with a primary diagnosis of chronic pancreatitis (N=29).

Results—Sensitivity for detection of pancreatic adenocarcinoma was 82%, with a false-positive rate of 5% for healthy controls. Patients with advanced disease had significantly higher antigen levels than those with early-stage disease ($P<0.01$), with a diagnostic sensitivity of 91%, 86%, and 62% for stage 3/4 advanced disease, stage-2, and stage-1, respectively. We also evaluated chronic pancreatitis sera, finding 38% positive for antigen; however, this was discordant with immunohistochemical findings that suggest the PAM4-antigen is not produced by inflamed pancreatic tissue. Furthermore, several of the serum-positive pancreatitis patients, for whom tissue specimens were available for pathological interpretation, had evidence of neoplastic precursor lesions.

Conclusions—These results suggest the use of the PAM4-serum assay to detect early-stage pancreatic adenocarcinoma, and that positive levels of PAM4-antigen are not derived from inflamed pancreatic tissues, but rather may provide evidence of subclinical pancreatic neoplasia.

Impact—The ability to detect pancreatic adenocarcinoma at an early stage could provide for early therapeutic intervention with potentially improved patient outcomes.

Keywords

pancreatic adenocarcinoma; early detection; immunoassay; biomarker; mucin

Introduction

The number of patients who succumb to pancreatic cancer each year continues to rise, unlike other leading cancers, where surveillance and/or screening technologies have led to a decrease in cancer-related mortality rates (1). For pancreatic cancer, the overall survival rate is only

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20% after one year and less than 4% after 5 years. The two major reasons for this poor prognosis are: 1) The inability to detect the disease at an early-stage, when curative measures may have greater opportunity to provide successful outcomes, and 2) there is no effective treatment for advanced disease. In general, patients with early-stage disease have better survival rates than those with late-stage disease; those with surgically resected localized disease have a 5-year relative survival of 22% *vs* 1–2% for patients with unresectable advanced metastatic disease (2). Although early detection provides a higher probability for successful therapeutic intervention, a 22% 5-year relative survival rate translates to an unacceptably high mortality rate of 78% for localized disease (3). Thus, early detection, accurate staging, and improved therapeutic procedures are related, and each is in vital need of improvement for successful management of the patient with this disease.

Over the past several years, our group has provided immunohistochemical evidence that the PAM4 monoclonal antibody (MAb) identifies a unique biomarker expressed by more than 85% of invasive pancreatic adenocarcinomas, including early stage-1 disease and the precursor lesions, pancreatic intraepithelial neoplasia (PanIN), intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystic neoplasms (MCNs) (4,5). The specific epitope detected by MAb-PAM4 is absent from normal pancreas and, for the most part, pancreatitis and other normal and malignant tissues. Therefore, immunohistochemical detection of the epitope is likely to indicate the presence of pancreatic neoplasia. In our first report of a PAM4-based serum enzyme-immunoassay (EIA), a sensitivity of 77% for detection of advanced, late-stage pancreatic adenocarcinoma and a specificity of 95% were observed (6). We now provide evidence that the serum-based PAM4-EIA can correctly predict the presence of early-stage pancreatic adenocarcinoma.

Materials and Methods

Human Specimens

Sera (N=68) were obtained from patients with a confirmed diagnosis of pancreatic adenocarcinoma being treated at the Johns Hopkins Medical Center, Baltimore, MD, and stored frozen <5 yrs. Each of these patients underwent surgical resection of the pancreas, providing an opportunity for accurate diagnosis and staging. For stage-1 disease, no neoplastic cells were observed outside of the pancreas. However, we appreciate that patients with pancreatic adenocarcinoma are likely to have undetected micrometastatic disease at presentation, including those patients reported with stage-1 disease. For this reason, we evaluated follow-up survival data. All patients described as having stage-1 disease survived at least 1 year (time to last recorded follow-up visit), with a median survival time of 2.70 years (25^{th} percentile = 1.32 years) in comparison to the latest SEER data (2002–2006), which reports a 1.42-year median survival for patients having stage-1 disease treated by surgical resection (2). These samples were collected with approval of the Johns Hopkins Institutional Review Board. A total of 29 sera from patients with a diagnosis of chronic pancreatitis were obtained from the Johns Hopkins Medical Center and Zeptometrix Corp. (Franklin, MA). Healthy volunteers (N=19) provided blood for control specimens under a New England Institutional Review Board approved protocol at the Center for Molecular Medicine and Immunology. All specimens were de-identified, with the only clinical data provided to the investigators being the diagnosis, stage of disease, follow-up survival time, and size of the primary tumor.

Reagents

Preparation of mucin standards, the PAM4 antibody, and a polyclonal, rabbit anti-mucin antiserum, IgG fraction, were described previously (6). Human IgG (purified immunoglobulin, reagent grade) was obtained from Sigma Aldrich (St. Louis, MO). Reagent grade 1-butanol and chloroform were obtained from Eastman Chemical Co. (Kingsport, TN). Murine MA5

antibody reactive with the MUC1 protein core was obtained from Immunomedics, Inc. (Morris Plains, NJ). A non-binding isotype-matched control antibody, Ag8, was purified in our laboratory from the P3X63-Ag8 murine myeloma.

Sample Preparation

All assays were performed in a blinded fashion. To prepare the specimens for immunoassay, 300 μL of serum were placed in a 2.0 mL microcentrifuge tube and extracted with an equal volume of 1-butanol. The tubes were vortexed vigorously for 2 min at which time 300 μL of chloroform were added and the tubes again vortexed for 2 min; this latter step was included in the procedure in order to invert the aqueous and organic layers. The tubes were then centrifuged in a Sorvall MC-12V microfuge at a setting of 12,000 rpm for 5 min. The top aqueous layer was removed to a clean tube and the sample diluted 1:2 in 2.0% (w/v) casein-sodium salt (Sigma Aldrich) in 0.1 M sodium phosphate buffer, pH 7.2, containing 0.15 M sodium chloride (PBS) for immunoassay.

Enzyme immunoassay

The immunoassay was performed in a 96-well polyvinyl plate that had been coated with 100 μL of humanized-PAM4 IgG at 20 μg/mL in PBS with incubation at 4°C overnight. The wells were then blocked by addition of 200 μL of a 2.0% (w/v) solution of casein in PBS and incubated for 1.5 h at 37°C. The blocking solution was removed from the wells and the plate washed 5-times with 250 μL of PBS containing 0.1% (v/v) Tween-20. The standards, or unknown specimens, 100 μL in triplicate, were added to the appropriate wells and incubated at 37°C for 1.5 h. The plate was then washed 5-times with PBS-Tween-20 as above. The polyclonal, rabbit anti-mucin antibody, diluted to 5 μg/mL in 1.0% (w/v) casein in PBS containing 50 μg/mL non-specific, human IgG, was added to each well and incubated for 1 h at 37°C. The polyclonal antibody was then washed from the wells as above, and peroxidase-labeled donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), at a 1:2000 dilution in 1.0% (w/v) casein in PBS, also containing 50 μg/mL human IgG, was added to the wells and incubated at 37°C for 1 h. After washing the plate as above, 100 μL of a 3,3',5,5'-tetramethylbenzidine substrate solution (BioFx Laboratories, Owings Mills, MD) were added to the wells and incubated at room temperature for 30 min. The reaction was stopped by the addition of 50 μL 4.0 N sulfuric acid, and the optical density read at a wavelength of 450 nm using a SPECTRA-MAX 250 spectrophotometer (Molecular Devices (Sunnyvale, CA). Because of the considerable microheterogeneity of the PAM4-mucin, we chose to report our results in arbitrary units/mL, based on an initial reference standard purified from xenografted CaPan-1 human pancreatic tumor.

Immunohistochemistry

Paraffin-embedded specimens obtained from the Cooperative Human Tissue Network were cut to 4 micron sections on superfrost plus adhesive slides (Thermo Scientific, Waltham, MA). Tissue sections were then heated to 95°C for 20 min in a pH 9.0 Tris buffer, Target Retrieval Solution (Dako, Carpinteria, CA), allowed to cool to room temperature, and then quenched with 3% H_2O_2 for 15 min at room temperature. Primary antibodies were then used at 10 μg/mL with an ABC Vectastain kit (Vector Laboratories, Burlingame, CA) for labeling the tissues. The slides were scored independently by two pathologists using a paradigm consistent with that reported for earlier studies on biomarkers in pancreatic adenocarcinoma (5): 0-negative, <1% of the tissue was labeled; 1-a weak, focal labeling of between 1%–25% of the tissue; 2-a strong, focal labeling of between 1%–25% of the tissue; 3-a weak, diffuse labeling >25% of the tissue; 4-a strong, diffuse labeling >25% of the tissue. Only the appropriate tissue components (*e.g.*, adenocarcinoma cells, normal ducts, etc.) were considered for assessment.

Statistical Analyses

Standard curves were generated from the immunoassay data, with regression analyses performed to interpolate concentrations of the unknown samples (Prism 4.0 software, GraphPad, La Jolla, CA). Receiver operating characteristic (ROC) curves were generated by use of the Med-Calc statistical software package (version 7.5) (Med-Calc, Mariakerke Belgium). Student's *t*-test was used to compare variables in any two groups. The Cochran-Armitage test was used to detect a trend between detection rates and stage of disease.

Results

Accuracy and precision of the immunoassay

A set of control standards with nominal concentrations of 15.60, 6.20, 2.50, and 1.00 units/mL was evaluated on several nonconsecutive days (N=7) for determination of accuracy and precision. Curve fitting for the standards generally gave resultant goodness of fit values for r^2 >0.990. Accuracy was calculated to be within 8% of the nominal value for the first three concentrations, but fell to approximately 22% for the 1.00 units/mL standard. Linear regression of nominal *vs* measured units/mL in this series of controls gave a trend-line with a slope of 0.965 and y intercept of 0.174 (r^2 = 0.999), where a slope of 1.00 with a y intercept of 0.00 would constitute 100% accuracy (Figure 1). An average absolute difference between nominal and recovered mass equal to 0.190 ± 0.173 units/mL for the two lowest concentration standards suggested a minimum absolute error of approximately 0.2 units/mL for the EIA. Values for the coefficient of variation (CV) were 6.40%, 4.85%, 12.0%, and 66.4%, respectively, for the 4 control standards. Taken together, the data suggest that the PAM4-immunoassay provides a level of accuracy and reproducibility that are within the guidelines suggested for an immunoassay measurement of an analyte; accuracy and precision were within 15% for concentrations above the cutoff value (2.40 units/mL), and within 20% at the cutoff value (7). To further test this, we examined 3 sera, two of which were from healthy controls, on 3 separate days. The two healthy controls gave average results of 0.27 ± 0.06 and 0.30 ± 0.27 units/mL, each of which was close to the minimum absolute error for the EIA with consequent high CV of 21.65% and 88.19%, respectively. The other patient serum gave an average of 19.45 ± 2.51 units/mL with a CV of 12.9%.

Quantitation of antigen in patient sera

In a prior publication, we reported that the PAM4-immunoassay had a sensitivity of 77% and a specificity of 95% for pancreatic carcinoma (6). Continuing these studies, we evaluated a new group of 24 sera from patients diagnosed with pancreatic adenocarcinoma. Only two of the sera had levels of PAM4-reactive antigen considered to be positive. Therefore, we considered and evaluated several reasons why the immunoassay had not performed as expected, including the quality of the immunoassay reagents, the possibility that the antigen was being degraded and/or removed from the serum, its presence in the form of immune complexes, or being bound by a blocking substance. To summarize our experiments, we discovered there is a substance in fresh human serum and/or specimens stored frozen for short periods of time (<5 yrs) that will bind to the PAM4-reactive epitope, thus preventing its detection within the immunoassay. Percent recovery of antigen from fresh normal human serum (N=2) spiked with PAM4-antigen at concentrations from 5–20 units/mL were on the order of 33% or less.

In a series of reports, Slomiany and co-workers described that gastric mucin had covalenty bound and/or associated lipids and fatty-acids (8–10), and that these lipids and fatty acids had specific effects upon the physicochemical properties of the mucin. Furthermore, it was of interest that fatty-acid synthetase levels and activity are significantly elevated in pancreatic adenocarcinoma, as is also the case for other forms of cancer and other pathologic conditions

(11). Speculating that the blocking substance might be lipid in nature,, we performed organic extraction of sera from the group of 24 pancreatic adenocarcinoma patients that had been stored frozen for <5 years. As was noted above, without prior extraction, only 2 of the 24 specimens (8.3%) had levels of PAM4-antigen that were considered positive, whereas after organic extraction, 22 of the 24 specimens (92%) had positive levels of the PAM4-antigen.

We were also able to re-evaluate, from our initial 2006 study (6), 10 pancreatic adenocarcinoma patient sera that had been stored frozen for >15 years to confirm the prior results. With or without extraction, all 10 specimens had levels of antigen that were considered to be positive. Regression analysis to compare paired results from extracted and non-extracted sera gave a trendline with slope of 1.10 ($r^2 = 0.94$), demonstrating that with or without extraction of these long-term frozen sera, the results were similar. It is likely that long-term storage of the specimens provided opportunity for degradation of the inhibiting substance or, at the very least, unmasking of the epitope. All further testing of sera was performed with organic extraction of specimens prior to immunoassay.

Specimens evaluated for PAM4-reactive antigen included 68 patients with confirmed pancreatic adenocarcinoma divided by stage: 21 from stage-1; 14 from stage-2; and 33 from stages-3 and -4 (advanced). In addition, 19 sera collected from healthy adult volunteers and 29 patients diagnosed with chronic pancreatitis were included as control groups. The maximum concentration shown in the dot-plot (Figure 2) is 80 units/mL, because there were insufficient volumes of sera to perform additional dilution studies. Although a cutoff value of 10.2 units/mL had been reported previously (6), because of the use of an organic extraction procedure, as well as minor, yet potentially significant differences in the current EIA protocol (reagent concentrations, inclusion of human IgG in buffers, etc.), we chose to treat the current data set independently of prior results. A positive cutoff value of 2.4 units/mL was calculated by ROC curve statistics (Figure 3) for the comparison of all pancreatic adenocarcinoma specimens *versus* healthy adults. The overall sensitivity for detection of pancreatic adenocarcinoma was 82%, with an area under the curve of 0.92 ± 0.03 (95% CI = 0.84–0.97). At this level of sensitivity, a false-positive rate of 5% was observed for the healthy control group, the single positive case having 3.65 units/mL of circulating antigen, just above the cutoff value. Unfortunately, insufficient volumes of sera prevented our performing CA19-9 immunoassays for comparison to the PAM4-immunoassay results. Furthermore, the number of CA19-9 values available from medical records were too few to be useful for statistical comparisons.

As shown in Table 1, sensitivity for detection of early, stage-1 pancreatic adenocarcinoma was relatively high, with 13 of 21 (62%) specimens above the cutoff value. As expected, this detection rate was lower than that observed for the stage-2 (86%) and advanced stage-3 and -4 (91%) patient groups. A statistically significant trend ($P < 0.01$) was noted for detection rate *vs* stage of disease. We considered that this was most likely due to tumor size or burden; the average tumor sizes for stage-1, stage-2, and stage-3/4 groups were 2.14 ± 1.02 cm^3, 3.36 ± 1.18 cm^3, and 3.45 ± 1.06 cm^3, respectively. While there was no statistically significant difference in tumor size between the stage-2 and -3/4 groups ($P > 0.41$), a statistically significant difference was observed for each of these two groups when compared to stage-1 tumor size ($P < 0.004$ or better). However, it should be noted that individual tumor size did not correlate with antigen concentration in the serum ($r^2 = 0.0065$).

Specimens reported as Stage-1 could be divided into stage-1A (N=13) and stage-1B (N=8) subgroups based on tumor size, with detection rates of 54% and 75%, respectively; however, caution is emphasized since the number of patients in each subgroup is small. The average tumor size for stage-1A was 1.41 ± 0.58 cm^3 (range: 0.4 cm^3–2.0 cm^3) and for stage-1B was 3.15 ± 0.44 cm^3 (range: 2.5 cm^3–4 cm^3); $P < 0.001$ for comparison of the two groups. While, on the whole, tumor sizes were smaller in stage-1A disease than in stage-1B, there was no

apparent statistical correlation between individual tumor size and concentration of the PAM4-antigen in the blood ($r^2 = 0.03$). Furthermore, it is important to note that of the 13 stage-1A specimens, 4 of the 7 positive cases had PAM4-antigen levels considerably higher than the cutoff value, with a range of 17.65–32.65 units/mL.

We also evaluated a set of 29 patient sera with the primary diagnosis of chronic pancreatitis. At the 2.4 units/mL cutoff established by ROC evaluation of normal and pancreatic adenocarcinoma patients, 11 pancreatitis patients (38%) were positive. ROC curve analysis of pancreatitis sera compared directly to the pancreatic adenocarcinoma specimens gave an area under the curve of 0.77 ± 0.05 (95% CI = 0.68–0.85). The median value for the pancreatitis group was 1.28 units/mL, comparable to the healthy volunteer group (1.18 units/mL), but considerably lower (3.5-fold) than the stage-1 pancreatic adenocarcinoma group (4.53 units/mL). It should be noted that our prior results for pancreatitis specimens suggested a considerably lower false-positive rate, only 5%; however, this was likely due to the pancreatitis specimens being stored frozen for less than 5 years, and should have required organic extraction to provide correct analyses.

Biopsy and/or surgical specimens were available from 14 of the chronic pancreatitis specimens, 6 of which were from patients who were considered positive for circulating PAM4 antigen. In 3 of these 6 positive cases, precursor lesions were identified within the tissue sections. The question was then considered whether the positive serum test was due to pancreatitis or the presence of neoplastic precursor lesions. We performed immunohistochemistry on an additional 30 biopsy specimens from patients diagnosed with pancreatitis. Of the 30 specimens, one frank invasive pancreatic adenocarcinoma and one large PanIN-2-3 lesion were identified (in separate specimens) by use of PAM4 staining, while surrounding acinar-ductal metaplasia (ADM) and normal tissues were negative (Figure 4). Of the remaining 28 specimens, 19 had sufficient parenchyma to be evaluated, 16 of which had evidence of ADM. PAM4 was negative in all but two of these cases, and in each of these gave only a very focal, weak labeling of ADM within the specimens (Figure 5).

Discussion

Prior studies employing both immunohistology of tissue specimens and EIA of circulating antigen have demonstrated that the PAM4-reactive epitope is a biomarker for invasive pancreatic adenocarcinoma (4–6), and is expressed at the earliest stages of pancreatic neoplasia (i.e., PanIN-1). It is not detectable within normal pancreatic tissues (ducts, acinar and islet cells), nor the majority of non-pancreatic cancers examined (breast, lung, gastric, and others). Thus, an elevation of the PAM4-epitope concentration in the serum provided a high positive likelihood ratio of 16.8 for pancreatic adenocarcinoma (6). Missing from the prior study was clinical information regarding the stage of disease. Consequently, we could not evaluate the value of the immunoassay for detection of potentially curable early disease until now, where we report that the PAM4-based EIA can detect patients having early-stage pancreatic adenocarcinoma, and can provide accurate discrimination from disease-free individuals. The assay's sensitivity for detection of early pancreatic adenocarcinoma was 62% for patients with stage-1 and 86% for patients with stage-2 disease; serum levels generally increased with advancing stage of disease. It is noteworthy that a high percentage of patients with stage-1 and -2 disease are asymptomatic, and that detection of tumor growth at these early stages can provide improved prospects for survival.

The cancer patients in this study all underwent surgical resection, providing an opportunity to accurately stage each patient. However, we appreciate that many patients with pancreatic cancer are suspected of having micrometastatic disease at presentation, even if they do not have histologically-apparent regional lymph node involvement. This highlights a general

problem in the study of early detection, particularly with a low-incidence disease such as pancreatic adenocarcinoma. The accrual of specimens that are well-defined is problematic. Further complicating the issue is that many of these pancreatic cancers occur in the presence of chronic pancreatitis, cholecystitis, and neoplastic precursor lesions, amongst other conditions.

Of 29 sera with a primary diagnosis of chronic pancreatitis, 38% were identified as positive for PAM4-antigen. However, at the present time we are unsure of the biological and clinical significance of this 38% positive rate. Several of these serum-positive patients, for whom tissue specimens for pathological interpretation were available, had evidence of neoplastic precursor lesions. Furthermore, a discrepancy was observed in the comparison of tissue reactivity by immunohistology and serum levels of antigen by immunoassay. By immunohistochemistry, only 10% of the evaluable specimens showed evidence of PAM4 staining within the ADM, although this was at considerably lower intensity than observed for the overwhelming majority of pancreatic adenocarcinoma specimens (5). Therefore, the results suggest that positive levels of PAM4-antigen within the serum may not be derived from inflamed pancreatic tissues, but rather could provide evidence of subclinical pancreatic neoplasia, such as PanIN lesions, and that, at the very least, positive results provide the rationale for clinical follow-up of these patients. Since there is detectable PAM4-antigen in normal controls, it is also possible that the elevations observed in pancreatitis patients are from a source outside of the pancreas.

Also, findings from genetically-engineered animal models of pancreatic adenocarcinoma suggest that human pancreatic neoplasia may arise before the PanIN-1 lesion (12). ADM was the earliest change observed in the mutant *KRAS* targeted model described by Zhu et al. (13). On the other hand, Shi et al. reported that although *KRAS* gene mutations can occur within ADM, they occur predominantly within ADM that are associated with PanIN lesions (14). The authors suggest this may occur by retrograde extension of the PanIN to the surrounding ADM. As yet, there is no conclusive evidence that ADM progress to PanIN. The fact that PAM4 is reactive with ADM in two patients with pancreatitis is of interest. Therefore, we are currently evaluating a larger sampling of pancreatitis tissue and blood specimens derived from the same patients to identify further specimens of PAM4-positive ADM that can be evaluated for *KRAS* mutation and alterations of other oncogenes.

Although the results reported here suggest that we are able to detect early-stage pancreatic adenocarcinoma, the fundamental question remains whether the immunoassay can influence patient outcome. At the present time, screening the general population for pancreatic cancer is not considered medically or economically worthwhile, because the disease is simply too infrequent. However, there is considerable interest in screening patients predicted to have an increased risk of developing pancreatic adenocarcinoma. Several studies have demonstrated that screening individuals with strong family histories of pancreatic cancer can identify precursor neoplasms of the pancreas that are amenable to surgical resection (15–17). For example, relatives of pancreatic cancer patients have a significantly higher risk of developing pancreatic cancer than the general population (18). Patients with germline *BRCA2* mutations are also at increased risk for developing pancreatic cancer (19,20), and many of these patients do not have a family history of breast and ovarian cancer (21). A small percentage of patients with familial pancreatic cancer harbor mutations of *PALB2* (partner and localizer of BRCA2), a susceptibility gene for pancreatic cancer (22–24). Similarly, patients with long-standing chronic pancreatitis are at increased risk of developing pancreatic cancer, and the risk is very high, over 30%, among patients with early-onset (teenage) hereditary pancreatitis (25,26). A 20- to 34-fold higher risk has been observed in individuals with familial atypical multiple mole (FAMMM) syndrome (27). Also, several studies have shown a significantly increased risk of developing pancreatic cancer in diabetic individuals who meet certain criteria (28,29). Longitudinal surveillance of these patients by use of the PAM4-immunoassay may provide for

early detection of neoplasia. A second potential use of the immunoassay could be as a means to detect recurrence of disease post-therapy, and in particular, following surgical resection for those patients where the tumor is supposedly confined to the pancreas.

The relatively high specificity of the PAM4 antibody provides a means to target both imaging and therapeutic agents with high tumor uptake and high tumor/nontumor ratios. In several preclinical reports, we have demonstrated PAM4's potential as both a directly-radiolabeled (30,31) or bispecific, pretargeting reagent (32,33) for nuclear imaging and radioimmunotherapy of pancreatic cancer. Also, initial results of a clinical phase 1b trial to evaluate a fractionated dosing of ^{90}Y-PAM4 whole IgG (clivatuzumab tetraxetan), in combination with a radiosensitizing regimen of gemcitabine, were reported recently (34). Of 22 patients with stage-3/4 disease (mostly stage-4), 68% showed evidence of disease control, with 23% of patients having partial responses based on RECIST criteria. Thus, positive results by the PAM4-based immunoassay can provide the rationale to pursue PAM4-targeted imaging and therapy, thus perhaps providing a personalized therapy.

The PAM4-based immunoassay can identify the majority of pancreatic adenocarcinoma patients of all stages. Although a direct comparison with CA19.9 was not possible in the current study, a prior comparison of the two biomarkers in a limited set of pancreatic adenocarcinoma sera (N=41) demonstrated a statistically significant difference ($P<0.01$) with PAM4-antigen levels positive in 71% of patient specimens and CA19.9-antigen levels positive in 59% of specimens (6). In general, it is thought that CA19.9 lacks the sensitivity and specificity to provide for early detection and/or diagnosis of pancreatic adenocarcinoma. However, the assay does have its use for management with continued elevation in CA19.9 serum levels post treatment indicative of a poor prognosis. Similarly, we recently reported in abstract form (35), the use of circulating PAM4-antigen levels for prediction of anti-tumor response. Further detailed studies in this regard will be presented in a separate publication.

It is noteworthy that the conditions under which specimens are stored (*e.g.*,, the length of time they are kept frozen) can have significant effects upon accessibility of the epitope under study. For the PAM4-based immunoassay, we have speculated that a fatty acid or lipid substance was able to bind the specific epitope; however, it is also possible this material was a low-molecular weight peptide or other substance soluble in organic solvents. The ability to remove this substance by organic extraction of the serum makes the PAM4-immunoassay reproducible. Our hypothesis had been based upon the studies of Slomiany et al., who reported that fatty acids are both non-covalently associated and/or covalently linked to gastric mucins (8.9). We are currently investigating the structure of the PAM4-epitope, as well as the mucin-species within which the epitope exists. In addition, the question is raised as to the biological significance of the circulating fatty-acid:PAM4-mucin interaction, if any. However, one fortuitous effect is that when using the PAM4 antibody as an *in vivo* targeting agent (*e.g.*, radioimmunotherapy), the presence of circulating PAM4-antigen is not a factor, since targeting of radiolabeled-PAM4 to sites of tumor growth has been observed in the majority of patients evaluated to date. Thus, it appears that the PAM4-antigen within tumor is free of the blocking substance.

In summary, the results suggest continued study of the PAM4-immunoassay to include evaluation of serum specimens from patients with known diagnoses of neoplastic precursor lesions (PanIN, MCN, IPMN) and benign cystic disease, amongst other pancreatic and non-pancreatic disorders.

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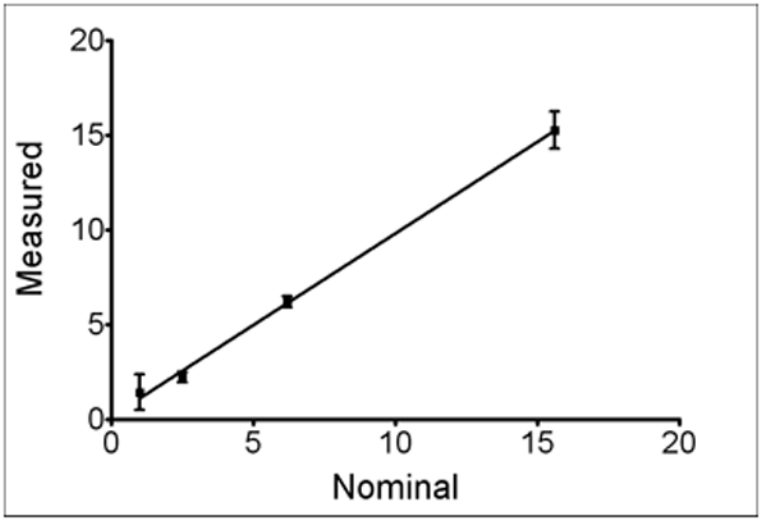

Figure 1.
Accuracy of the PAM4-immunoassay was determined to be within 10% of the nominal concentrations examined at or above the cutoff value of 2.40 units/mL. A linear trend was calculated with an equation of $y = 0.965x + 0.174$, and goodness of fit $r^2 = 0.999$.

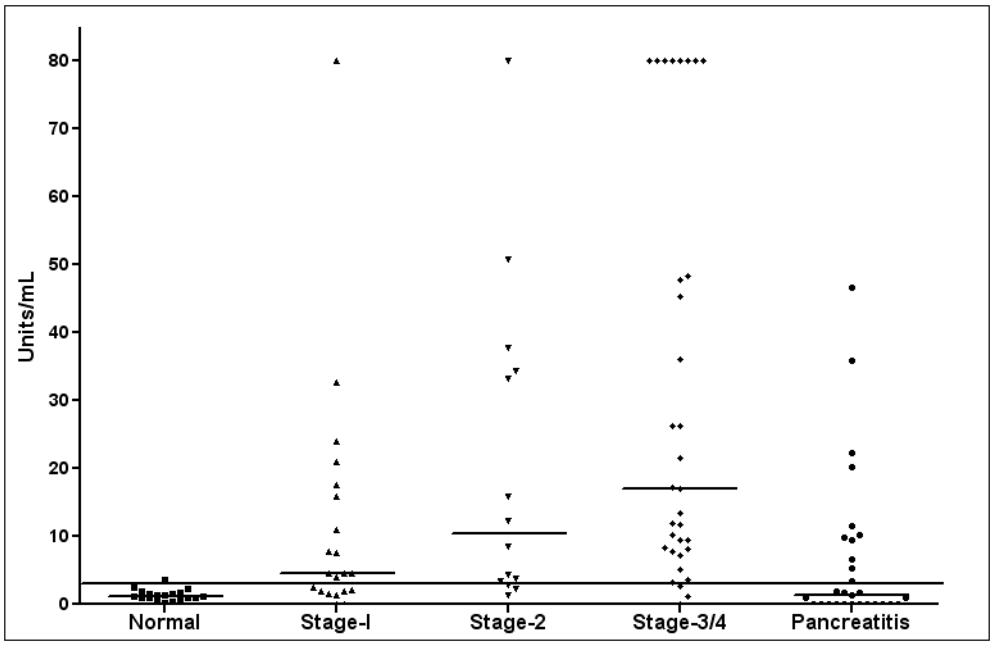

Figure 2.
Frequency distribution of PAM4-reactive antigen in patient sera by stage of disease. Cutoff value = 2.4 units/mL (red line). The median values (units/mL) are shown for each study group.

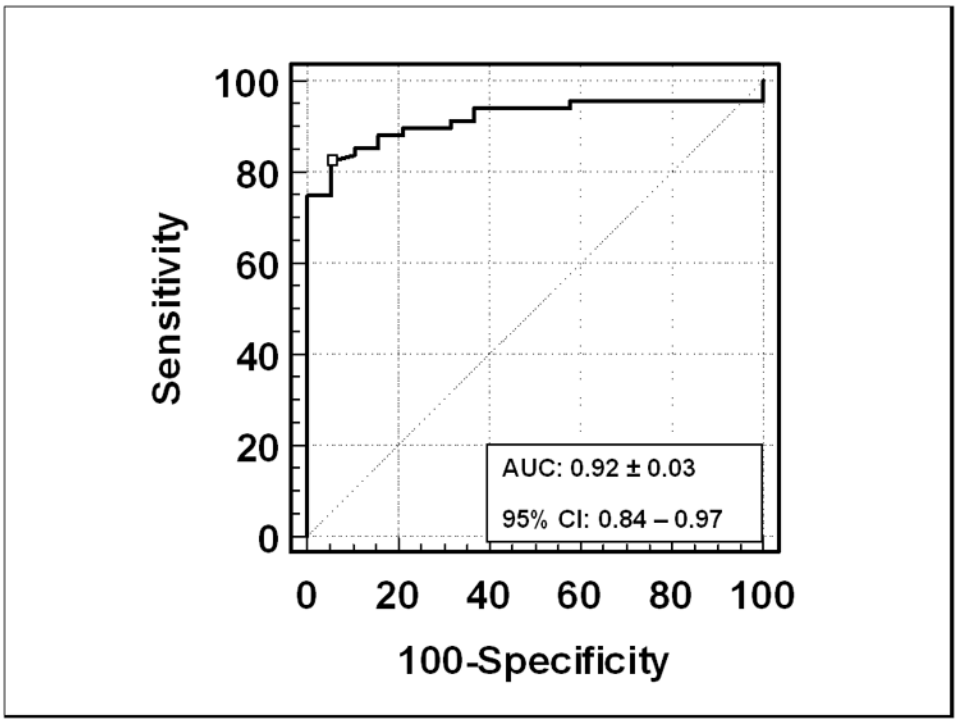

Figure 3.
Receiver Operator Characteristics (ROC) curve for the performance of the PAM4-based immunoassay; pancreatic adenocarcinoma *vs* healthy adults. Values for the area under the curves (AUC) and 95% confidence limits are provided.

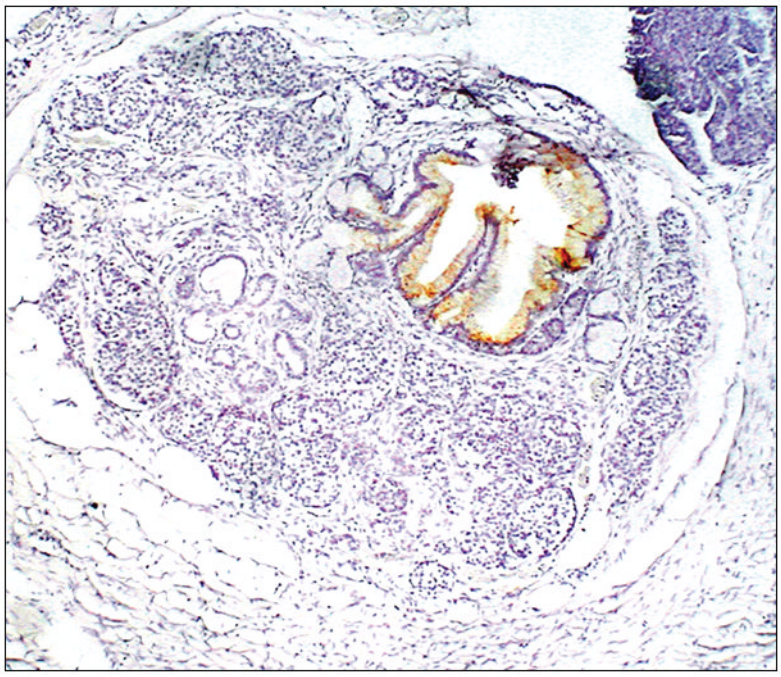

Figure 4.
Immunohistology of a PAM4-reactive PanIN-2-3 lesion identified within a biopsy section from a patient with primary diagnosis of chronic pancreatitis. (100x)

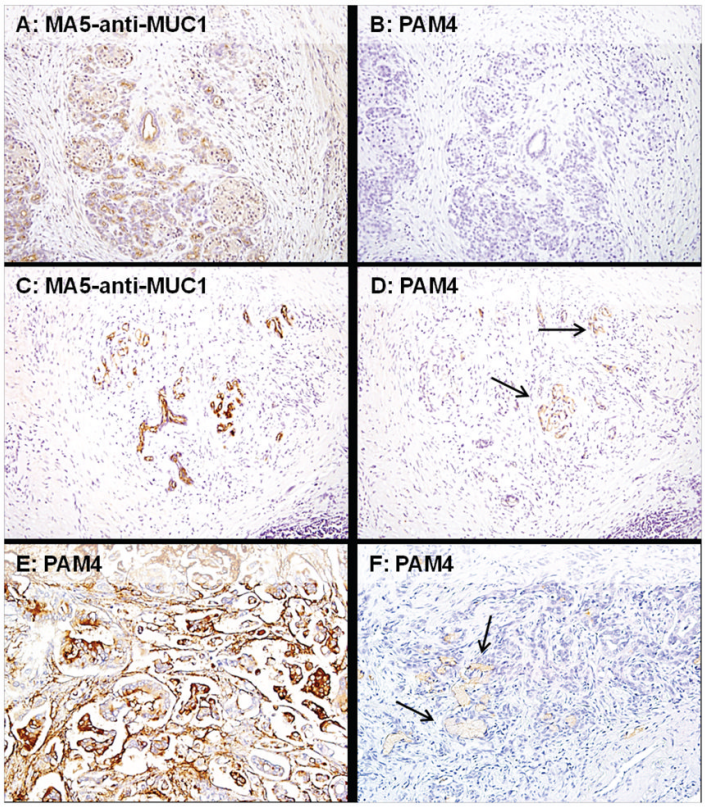

Figure 5.

Immunohistology of tissue specimens derived from patients with primary diagnoses of chronic pancreatitis and pancreatic adenocarcinoma. The upper panels (**A & B**) are from a single patient specimen and are representative of 90% of the pancreatitis specimens (18 of 20 evaluable). The MA5 anti-MUC1 (peptide core) antibody, employed as a positive control, was reactive with acinar, ductal and ADM cells (**A**), whereas the PAM4 antibody was negative for all cell types (B). The middle panels (**C & D**) are from a single patient specimen where the MA5 antibody gave an intense and diffuse labeling of the acinar, ductal and ADM cells (**C**), whereas the PAM4 antibody gave only a focal, weak reactivity with ADM (**D**). The bottom panels (**E & F)** are two individual specimens of pancreatic adenocarcinoma, each labeled with PAM4 antibody. Panel **E**, representative of the majority of pancreatic adenocarcinomas, shows an intense, diffuse labeling of adenocarcinoma cells and secreted mucin, whereas panel **F** shows a weak, focal labeling of the adenocarcinoma cells. The arrows in panels **D** and **F** point to tissues that are weakly labeled with PAM4. (200x for all magnifications)

Table 1

PAM4-reactive mucin in the sera of patients

	N	Median (units/mL)	True-Positive	T-test (P value)[a]
Total PC	68	9.85	81%	<0.001
Stage-1	21	4.53	62%	<0.002
---Stage-1A	13	3.96	54%	<0.02
---Stage-1B	8	6.05	75%	<0.02
Stage-2	14	10.39	86%	<0.005
Stage-3/4	33	13.37	91%	<0.001
Chronic Pancreatitis	29	1.28	(38% FP)	
Healthy Volunteers	19	1.18	(5% FP)	

[a] All comparisons are to healthy volunteers