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Sequential Validation of Blood-Based Protein Biomarker Candidates for Early-Stage Pancreatic Cancer

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Abstract

Background: CA19-9, which is currently in clinical use as a pancreatic ductal adenocarcinoma (PDAC) biomarker, has limited performance in detecting early-stage disease. We and others have identified protein biomarker candidates that have the potential to complement CA19-9. We have carried out sequential validations starting with 17 protein biomarker candidates to determine which markers and marker combination would improve detection of early-stage disease compared with CA19-9 alone.

Methods: Candidate biomarkers were subjected to enzyme-linked immunosorbent assay based sequential validation using independent multiple sample cohorts consisting of PDAC cases (n = 187), benign pancreatic disease (n = 93), and healthy controls (n = 169). A biomarker panel for early-stage PDAC was developed based on a logistic regression model. All statistical tests for the results presented below were one-sided.

Results: Six out of the 17 biomarker candidates and CA19-9 were validated in a sample set consisting of 75 PDAC patients, 27 healthy subjects, and 19 chronic pancreatitis patients. A second independent set of 73 early-stage PDAC patients, 60 healthy subjects, and 74 benign pancreatic disease patients (combined validation set) yielded a model that consisted of TIMP1, LRG1, and CA19-9. Additional blinded testing of the model was done using an independent set of plasma samples from 39 resectable PDAC patients and 82 matched healthy subjects (test set). The model yielded areas under the curve (AUCs) of 0.949 (95% confidence interval [CI] = 0.917 to 0.981) and 0.887 (95% CI = 0.817 to 0.957) with sensitivities of 0.849 and 0.667 at 95% specificity in discriminating early-stage PDAC vs healthy subjects in the combined validation and test sets, respectively. The performance of the biomarker panel was statistically significantly improved compared with CA19-9 alone ($P < .001$, combined validation set; $P = .008$, test set).

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Conclusion: The addition of TIMP1 and LRG1 immunoassays to CA19-9 statistically significantly improves the detection of early-stage PDAC.

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal types of cancer, with a five-year survival rate of only 8% and a mortality rate closely approaching the incidence rate (1). Although resectable PDAC is associated with better survival, only 15% to 20% of PDAC patients present with localized disease (2). Imaging modalities, notably endoscopic ultrasound and magnetic resonance cholangiopancreatography, are currently used in the workup of subjects with suspected PDAC or at high risk for the disease (3). However, known risk factors have only a modest effect on PDAC incidence (4).

At present, biomarkers have limited utility for detecting early-stage PDAC. Carbohydrate antigen 19–9 (CA19-9) has shown potential as a diagnostic biomarker for both preclinical and early-stage PDAC (5,6). However, CA19-9 performance characteristics as a PDAC biomarker are limited as its accuracy varies with disease stage (7). Moreover, CA19-9 is not detectable in 5% to 10% of patients with fucosyl transferase deficiency with inability to synthesize antigens of the Lewis blood group (8). As a result, there is substantial ongoing effort to identify additional serological biomarkers that complement CA19-9 for the detection of early-stage PDAC. Markers investigated include proteins (9–14), metabolites (15–17), autoantibodies (18,19), miRNAs (20–22), markers with aberrant glycosylation (CA19-9 and Tn antigens) (6,23,24), and exosomes (25). In view of the wide array of potential biomarkers, there is a need for systematic evaluation of candidates using CA19-9 as an anchor marker given its established performance. We aimed in this study to validate circulating proteins, for which there is supportive evidence as PDAC biomarker candidates, to determine their potential to yield a marker panel with improved performance for detection of early-stage PDAC compared with CA19-9 alone.

We previously applied in-depth quantitative proteomic technologies using mass spectrometry for the identification of potential PDAC biomarkers (26). Additional ranking of promising biomarkers from the literature yielded a total 17 candidates which were subjected to sequential validation in multiple independent sample sets.

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Methods

Blood Samples

All human blood samples were obtained following institutional review board approval and informed consent. For initial discovery studies using in-depth quantitative mass spectrometry, a pool of plasma was constituted from six prediagnostic PDAC cases (male sex, median age = 66.5 years, range = 62–76 years) and six matched controls (male sex, median age = 67.0 years, range = 61–76 years). These samples were collected from subjects who were subsequently diagnosed with stage IA (n = 1), IB (n = 2), and IIB (n = 3) PDAC an average of 9.3 months (range = 8–12 months) after sample collection as part of the Carotene and Retinol Efficacy Trial (27) and from six controls from the same cohort who were matched for age, sex, and smoking history and who were not diagnosed with cancer over a four-year follow-up period. Candidate biomarkers were subjected to enzyme-linked immunosorbent assay–based sequential validation using independent multiple sample cohorts consisting of PDAC cases (n = 187), benign pancreatic disease cases (n = 93), and healthy controls (n = 169). Plasma samples obtained from the University of Michigan Comprehensive Cancer Center under the auspices of the Early Detection Research Network, consisting of 75 PDAC cases, 27 healthy controls, and 19 chronic pancreatitis cases, were used for initial validation and biomarker selection (triage set). An additional set (combined validation set) of plasma samples from 73 patients with early-stage PDAC, 60 healthy controls, 60 patients with chronic pancreatitis, and 14 patients with benign pancreatic cysts from Evanston Hospital (validation set 1), the University of Utah (validation set 2), and the University of Texas MD Anderson Cancer Center (validation set 3) was used for biomarker sequential validation and panel development. All chronic pancreatitis samples were collected in an elective setting in the clinic in the absence of an acute flare-up. An additional independent plasma sample set for testing the combined biomarker panel was obtained from the International Agency for Research on Cancer, consisting of 39 early-stage PDAC patients and 82 healthy controls (test set). Study flow diagram and clinical characteristics of the patients in the validation sets and test set are presented in Supplementary Figure 1, Supplementary Table 1, and Supplementary Table 2 (available online).

Mass Spectrometry Analysis of Human Plasma Samples

Quantitative mass spectrometry analysis of human plasma samples was done as previously described (26). Details on plasma sample preparation and mass spectrometry analysis are provided in the Supplementary Methods (available online).

Enzyme-Linked Immunosorbent Assay

Circulating protein concentrations were determined using the following enzyme-linked immunosorbent assay (ELISA) kits: ALCAM (R&D Systems, Minneapolis, MN), CA19-9 (Alpha Diagnostic International, San Antonio, TX), COL18A1 (R&D Systems), WFDC2 (IBL-America, Minneapolis, MN), IGFBP2 (R&D Systems), LCN2 (Bioporto, Hellerup, Denmark), LRG1 (IBL-America), LYZ (ALPCO, Salem, NH), PARK7 (R&D Systems), pro-CTSS (R&D Systems), REG3A (DYNABIO, Marseille, France), SLPI (R&D Systems), THBS1 (R&D Systems), TIMP1 (R&D Systems), TNFRSF1A (R&D Systems), total-CTSS (R&D Systems), and CHI3L1 (Quidel, San Diego, CA). The assay for NPC2 was developed in-house. For all ELISA experiments, each sample was assayed in duplicate, and the absorbance or chemiluminescence was measured with a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA). An internal control sample was run in every plate, and each value of the samples was divided by the mean value of the internal control in the same plate to correct interplate variability. Additional details on ELISA assays are provided in Supplementary Methods (available online).

Statistical Analysis

Raw assay data were $\log_2$-transformed after imputation of the lowest detected value for each assay to the values below limit of detection. A one-sided Wilcoxon rank-sum test was used to compute *P* values comparing PDAC cases with healthy controls,

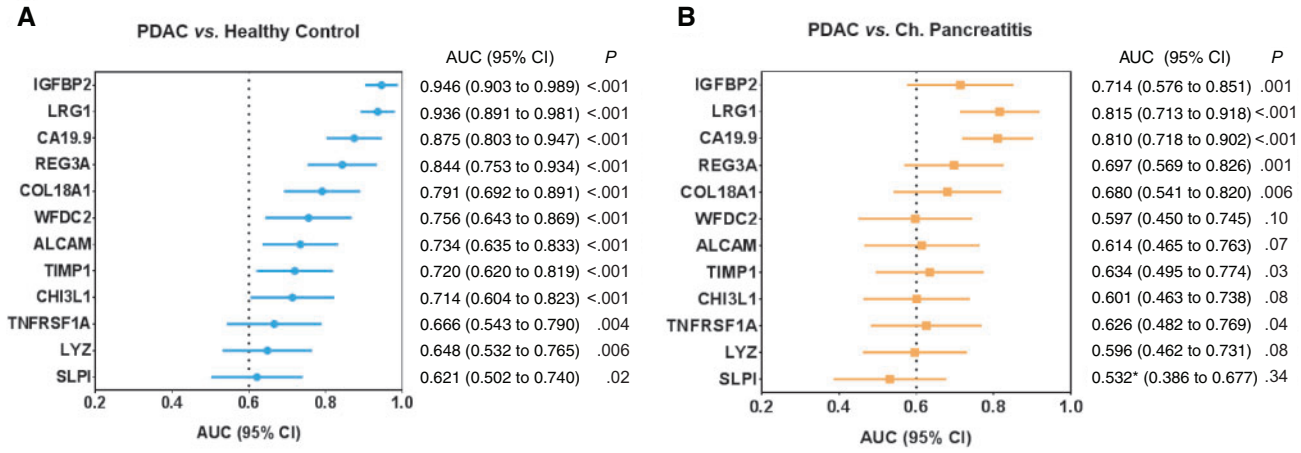

Figure 1. Figure resolution doesn't look optimal, should I send original TIFF files? Biomarker candidates with statistically significantly higher levels in pancreatic ductal adenocarcinoma (PDAC) than healthy controls in the triage set. Performance of the biomarker candidates in the comparison of **(A)** PDAC (n = 75) vs healthy controls (n = 27) and **(B)** PDAC vs chronic (ch.) pancreatitis patients (n = 19) in the triage set. **Bars** indicate area under the curve (95% confidence interval). *P* values were calculated using a one-sided Wilcoxon rank-sum test. *Indicates that the reverse ordering was used. AUC = area under the curve; CI = confidence interval.

chronic pancreatitis cases, and pancreatic cyst cases. The applied test was one-sided as it aimed to test the null hypothesis of AUC = 0.50 vs the alternative hypothesis AUC > 0.50. Receiver operating characteristic (ROC) curve analysis was performed to assess the performance of biomarkers in distinguishing PDAC cases from healthy controls, chronic pancreatitis cases, and pancreatic cyst cases. Owing to the small sample size of each set, validation sets 1, 2, and 3 were merged for model development by standardizing the data such that the mean was 0 and standard deviation was 1 for healthy controls. Because validation set 3 did not include healthy controls, the results were standardized such that the benign pancreatic cyst samples had the same mean and standard deviation as chronic pancreatitis samples. Detailed methods for the development of the biomarker panel are included in the Supplementary Methods (available online). Statistical analyses were performed using MATLAB R2014b and SAS version 9.3. A *P* value of less than .05 was considered statistically significant in all the analyses.

Results

Selection of Biomarkers for Testing in a Triage Set

Potential circulating biomarkers for sequential validation studies were selected among protein candidates who met two or more of the following criteria: 1) inclusion in the compendium of potential biomarkers of pancreatic cancer (28); 2) identified as elevated by in-depth quantitative proteomic analysis of a pool of six plasma samples from prediagnostic stage IA–IIB PDAC cases compared with six matched control samples; 3) previously found to be elevated in the plasma of PDAC tumor-bearing mice at an early stage (26). As a result, a total of 17 potential biomarkers were selected, in addition to CA19-9, for further testing. The selected markers consisted of ALCAM, CHI3L1, COL18A1, IGFBP2, LCN2, LRG1, LYZ, NPC2, PARK7, REG3A, SLPI, pro-CTSS, total-CTSS, THBS1, TIMP1, TNFRSF1A, and WFDC2 (Supplementary Table 3, available online).

We first evaluated the individual performance of the 17 potential biomarkers and CA19-9 in plasma samples from a triage set consisting of 75 PDAC cases, 27 healthy controls, and 19 chronic pancreatitis cases (triage set). The levels of 12 biomarkers were statistically significantly higher in PDAC compared with healthy controls, each with an area under the curve (AUC) greater than 0.60 and a *P* value of less than .05 (Wilcoxon rank-sum test) (Figure 1A). The levels of seven of these biomarkers (IGFBP2, LRG1, CA19-9, REG3A, COL18A1, TIMP1, and TNFRSF1A) were also statistically significantly higher in PDAC cases compared with chronic pancreatitis cases ($P < .05$, Wilcoxon rank-sum test) with greater than 0.60 of AUC (Figure 1B). We chose the seven biomarker candidates (IGFBP2, LRG1, CA19-9, REG3A, COL18A1, TIMP1, and TNFRSF1A) for further evaluation in validation sets 1, 2, and 3.

Validation of Biomarker Candidates in Three Independent Sets of Early-Stage PDAC Plasma Samples and Development of a Biomarker Panel

We subjected seven biomarker candidates to validation using three independent plasma sample sets—validation set 1 consisted of stage IB–IIB PDAC cases (n = 10), healthy controls (n = 10), and chronic pancreatitis cases (n = 10); validation set 2 consisted of early-stage (IA–IIA) PDAC cases (n = 42), healthy controls (n = 50), and chronic pancreatitis cases (n = 50); and validation set 3 consisted of resectable PDAC cases (n = 21) and benign pancreatic cyst cases (n = 14) (Supplementary Table 1, available online). AUC values for all seven biomarkers selected in the triage set indicate that their plasma levels were consistently elevated in PDAC patients compared with matched controls in validation sets 1, 2, and 3 (Supplementary Table 4, available online). The AUCs for these seven markers, except for IGFBP2 in the comparison of PDAC vs chronic pancreatitis cases in validation set 2, were greater than 0.60 in discriminating PDAC cases from healthy controls as well as chronic pancreatitis cases in both validation set 1 and set 2. In addition, four biomarkers (CA19-9, TIMP1, LRG1, and IGFBP2) also yielded AUCs greater than 0.60 in plasma samples from PDAC cases compared with benign pancreatic cyst cases in validation set 3 (Supplementary Table 4, available online).

To develop a biomarker panel for early-stage PDAC, we standardized and combined the results of validation sets 1, 2, and 3. In the combined validation set, the levels of all seven biomarkers were statistically significantly higher (AUC > 0.60,

Table 1. Performance of biomarkers in combined validation set

	Marker	*P*	AUC (95% CI)	Sensitivity at 95% specificity	Specificity at 95% sensitivity
Pancreatic cancer vs healthy control	CA19-9	<.001	0.882 (0.809 to 0.956)	0.726	0.228
	TIMP1	<.001	0.880 (0.805 to 0.956)	0.411	0.500
	LRG1	<.001	0.847 (0.768 to 0.926)	0.425	0.250
	REG3A	<.001	0.819 (0.735 to 0.903)	0.452	0.094
	IGFBP2	<.001	0.800 (0.715 to 0.885)	0.425	0.333
	COL18A1	<.001	0.749 (0.660 to 0.837)	0.329	0.233
	TNFRSF1A	<.001	0.692 (0.597 to 0.788)	0.206	0.150
Pancreatic cancer vs ch. pancreatitis	CA19-9	<.001	0.819 (0.743 to 0.895)	0.288	0.243
	TIMP1	<.001	0.732 (0.644 to 0.821)	0.219	0.333
	LRG1	<.001	0.682 (0.592 to 0.771)	0.110	0.117
	REG3A	<.001	0.656 (0.563 to 0.749)	0.219	0.094
	IGFBP2	.005	0.624 (0.529 to 0.719)	0.274	0.167
	COL18A1	.005	0.628 (0.531 to 0.725)	0.082	0.133
	TNFRSF1A	.002	0.643 (0.548 to 0.738)	0.096	0.100
Pancreatic cancer vs benign pancreatic disease*	CA19-9	<.001	0.831 (0.754 to 0.907)	0.288	0.259
	TIMP1	<.001	0.742 (0.657 to 0.828)	0.206	0.324
	LRG1	<.001	0.679 (0.580 to 0.772)	0.110	0.135
	REG3A	<.001	0.651 (0.560 to 0.743)	0.192	0.090
	IGFBP2	.002	0.632 (0.542 to 0.722)	0.219	0.189
	COL18A1	.004	0.627 (0.534 to 0.719)	0.082	0.149
	TNFRSF1A	.001	0.643 (0.551 to 0.736)	0.082	0.122

*Benign pancreatic disease (chronic pancreatitis cases and benign pancreatic cyst cases). AUC = area under the curve; CI = confidence interval; ch. = chronic.

Table 2. Performance of biomarker panel in the combined validation set

Model	AUC (95% CI)	Sensitivity at 95% specificity	Sensitivity at 99% specificity	Specificity at 95% sensitivity	Specificity at 99% sensitivity	CV-AUC (SD)	*P* (vs CA19-9)	
							Bootstrap	Likelihood ratio test
Pancreatic cancer vs healthy control								
TIMP1+LRG1+CA19-9 (linear)	0.949 (0.917 to 0.981)	0.849	0.658	0.633	0.367	0.936 (0.030)	.003	<.001
TIMP1+LRG1+CA19-9 ("OR" rule)	0.955 (0.890 to 1)	0.849	0.575	0.667	0.389	0.968 (0.022)	<.001	<.001
Pancreatic cancer vs benign pancreatic disease*								
TIMP1+LRG1+CA19-9 (linear)	0.846 (0.781 to 0.911)	0.356	0.110	0.351	0.108	0.830 (0.049)	.18	.02
TIMP1+LRG1+CA19-9 ("OR" rule)	0.890 (0.802 to 0.978)	0.452	0.123	0.541	0.282	0.887 (0.041)	<.001	<.001

*Benign pancreatic disease (chronic pancreatitis cases and benign pancreatic cyst cases). AUC = area under the curve; CI = confidence interval; CV-AUC = cross-validation related average AUC.

$P < .05$, Wilcoxon rank-sum test) in PDAC cases than in healthy controls or benign pancreatic disease cases (chronic pancreatitis and benign pancreatic cyst cases combined) (Table 1). Next, we sought to develop a biomarker panel for early-stage PDAC based on a logistic regression model. The leave-m-out cross-validation technique was applied to validate the resulting logistic regression model. In the comparison of PDAC cases with healthy controls, the resulting panel consisted of TIMP1, LRG1, and CA19-9, yielding an AUC of 0.949 (95% confidence interval [CI] = 0.917 to 0.981) and a cross-validation-related average AUC of 0.936, which was statistically significantly greater than the AUC of CA19-9 alone (AUC = 0.882, 95% CI = 0.809 to 0.956; $P = .003$, bootstrap; $P < .001$, likelihood ratio test) (Table 2 and Figure 2A). The panel yielded sensitivities of 0.849 and 0.658 at 95% and 99% specificities, respectively, whereas sensitivities at 95% and 99% specificities for CA19-9 alone were 0.726 and 0.411, respectively. A statistically significant improvement over CA19-9 alone in the comparison of PDAC cases with healthy controls was also observed when a model based on the same biomarker combination (TIMP1, LRG1, and CA19-9) was trained in validation set 2 and tested with fixed coefficients in validation set 1 ($P = .04$, bootstrap in training set; $P = .02$, bootstrap in test set) (Supplementary Table 5, available online). In validation set 2, for which tumor size was available, we observed that the panel-based biomarker score was not statistically significantly correlated with tumor size, suggesting the ability of the biomarker

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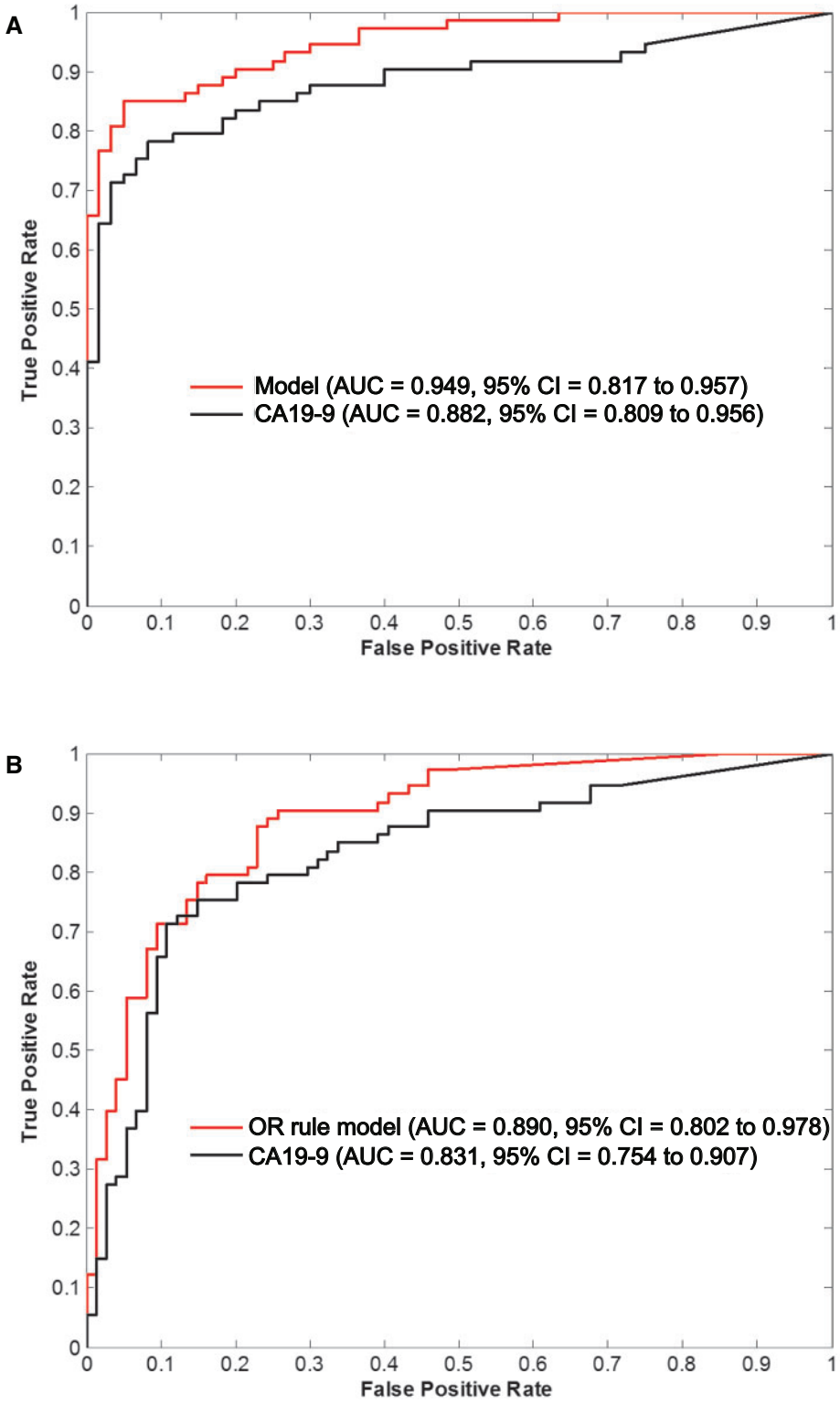

Figure 2. Performance of the biomarker panel based on TIMP1+LRG1+CA19-9 in the combined validation set. Receiver operating characteristic analysis of the biomarker panel developed for **(A)** pancreatic ductal adenocarcinoma (PDAC) vs healthy control and **(B)** PDAC vs benign pancreatic disease ("OR" rule combination). AUC = area under the curve; CI = confidence interval.

Table 3. Performance of biomarker model in the test set

Marker	AUC (95% CI)	P	Sensitivity at 95% specificity	Sensitivity at 99% specificity	Specificity at 95% sensitivity	Specificity at 99% sensitivity	P (vs CA19-9)*
CA19-9	0.821 (0.736 to 0.906)	<.001	0.538	0.462	0.286	0.067	–
TIMP1	0.730 (0.626 to 0.834)	<.001	0.359	0.333	0.085	0.000	–
LRG1	0.832 (0.755 to 0.909)	<.001	0.462	0.179	0.366	0.220	–
Model (fixed coefficients): TIMP1+LRG1+CA19-9	0.887 (0.817 to 0.957)	<.001	0.667	0.410	0.220	0.207	.008

*Likelihood ratio test. AUC = area under the curve; CI = confidence interval.

combination to detect tumors of small dimension (Supplementary Figure 2, available online).

A logistic regression model based on the same biomarker combination (TIMP1, LRG1, and CA19-9) was developed to discriminate PDAC from benign pancreatic disease cases (AUC = 0.846, 95% CI = 0.781 to 0.911, and cross-validation-related average AUC = 0.830) (Table 2). We further explored whether an "OR" rule (29)–based linear regression model, whereby either CA19.9 alone or the combination of all three markers, would enable better discrimination between PDAC and benign pancreatic disease cases. The "OR" rule combination of TIMP1, LRG1, and CA19-9 yielded an AUC of 0.890 (95% CI = 0.802 to 0.978), which was statistically significantly greater than that of CA19-9 alone (AUC = 0.831, 95% CI = 0.754 to 0.907; $P < .001$, bootstrap; $P < .001$, likelihood ratio test) (Table 2 and Figure 2B). The panel yielded a sensitivity of 0.452 at 95% specificity, which represents an improvement over a sensitivity of 0.288 at 95% specificity for CA19-9 alone. The "OR" rule combination of TIMP1, LRG1, and CA19-9 resulted in high diagnostic accuracy when applied to the comparison of PDAC patients vs healthy controls yielding an AUC of 0.955 (95% CI = 0.890 to 1; P vs CA19-9: $P < .001$, bootstrap; $P < .001$, likelihood ratio test) (Table 2).

We estimated odds ratios at the Youden index-based optimal cutoff points. For the model for early-stage PDAC cases vs healthy controls, log (odds ratio) was 4.67 (95% CI = 3.29 to 6.05) at the cutoff point, with a sensitivity of 0.849 and a specificity of 0.950. For the model for early-stage PDAC cases vs benign pancreatic disease cases, log (odds ratio) was 2.98 (95% CI = 2.04 to 3.91) at the cutoff point, with a sensitivity of 0.863 and a specificity of 0.757.

Blinded Testing of the Biomarker Model in an Independent Set of Early-Stage PDAC Plasma Samples

Further blinded validation of the panel of three biomarkers TIMP1, LRG1, and CA19-9 was performed in an independent set of plasma samples consisting of 39 resectable PDAC cases and 82 matched healthy controls (test set) (Supplementary Table 2, available online). The levels of all three biomarkers were statistically significantly higher in PDAC cases than in healthy controls, with AUCs of 0.821 (95% CI = 0.736 to 0.906) for CA19-9, 0.730 (95% CI = 0.626 to 0.834) for TIMP1, and 0.832 (95% CI = 0.755 to 0.909) for LRG1 (Table 3). A linear combination of the three markers yielded an AUC of 0.903 (95% CI = 0.838 to 0.967), which was statistically significantly greater than the AUC of CA19-9 alone ($P = .001$, bootstrap; $P < .001$, likelihood ratio test) (Supplementary Table 6, available online). Moreover, the linear combination of TIMP1, LRG1, CA19-9 and covariates (represented by recruiting center, gender, age, smoking status, and alcohol consumption) yielded an AUC of 0.929 (95% CI = 0.878 to 0.980), which represents a statistically significant improvement over CA19-9 and covariates combination alone (AUC = 0.848, 95% CI = 0.778 to 0.920; $P = .01$, bootstrap; $P < .001$, likelihood ratio test) (Supplementary Table 6, available online). The inclusion of covariates resulted in statistically significantly improved performance compared with the three biomarker panel alone ($P = .03$, bootstrap; $P = .004$, likelihood ratio test) (Supplementary Table 6, available online).

Of note, the logistic regression model of CA19-9, TIMP1, and LRG1 with fixed coefficients, which was developed in the combined validation sets for PDAC vs healthy controls, yielded an AUC of 0.887 (95% CI = 0.817 to 0.957) ok to add, also with statistically significantly improved performance compared with CA19-9 alone ($P = .008$, likelihood ratio test) (Table 3 and Figure 3). The model yielded sensitivities of 0.667 and 0.410 at 95% and 99% specificities, respectively, whereas sensitivities at 95% and 99% specificities for CA19-9 alone were 0.538 and 0.462, respectively. The log-transformed odds ratio at the Youden index–based optimal cutoff point was 3.19 (95% CI = 2.11 to 4.26) at the cutoff point, with a sensitivity of 0.872 and a specificity of 0.780.

Discussion

The goal of our study was to determine, given a large number of promising circulating protein biomarker candidates for PDAC detection, whether a marker combination can be developed with improved performance compared with CA19-9 for detecting early-stage PDAC. Moreover, a validated protein biomarker panel may serve as an anchor marker panel to systematically determine the utility of additional marker types to yield further improvements in performance. Other marker types that may be critically tested for their comparative performance include nucleic acids (eg, mutant DNA, methylated DNA, and noncoding RNAs), metabolites, autoantibodies to tumor proteins, aberrantly glycosylated antigens, tumor-derived microparticles, and circulating tumor cells. An initial set of 17 plasma biomarker candidates selected after in-depth proteomic analysis of prediagnostic early-stage PDAC sera combined with a review of the literature was tested using ELISA to assess their performance in distinguishing PDAC cases from healthy controls and from cases of benign pancreatic disease. Seven biomarkers—IGFBP2, LRG1, CA19-9, REG3A, COL18A1, TIMP1, and TNFRSF1A—well supported by other studies (Supplementary Table 3, available online), were selected based on their performance in the triage set and subjected to validation in three independent cohorts of early-stage PDAC. A prior study tested a set of 67 biomarkers including CA19-9, TIMP1, and TNFRSF1A in prediagnostic sera from PDAC cases from the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial (30). The level of only CA19-9 was found to be statistically significantly higher in cases than in

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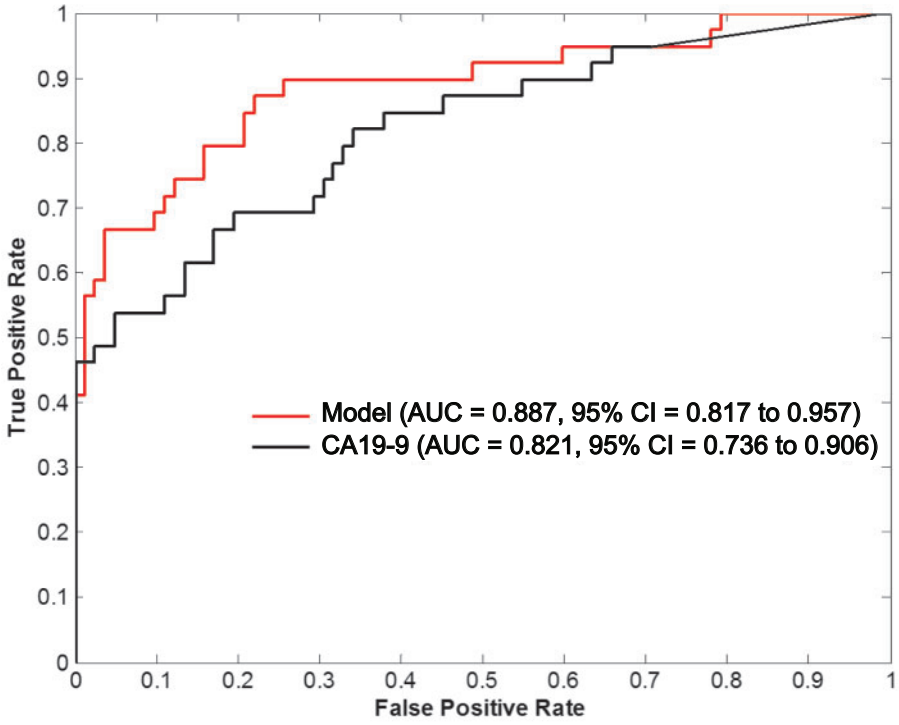

Figure 3. Performance of the biomarker model based on TIMP1+LRG1+CA19-9 in the test set. Receiver operating characteristic analysis of the combination model with fixed coefficients, which was developed in combined validation sets, for pancreatic ductal adenocarcinoma vs healthy control. AUC = area under the curve; CI = confidence interval.

controls, yielding an AUC of approximately 0.65, which is substantially reduced compared with the performance of CA19-9 in this study. The discrepancies may reflect differences in case characteristics, sample material (plasma vs serum), storage and assay conditions (choice of reagents and ELISA vs multiplexed bead-based assay). In the current study, CA19-9 performed well in distinguishing early-stage PDAC from healthy controls and from cases of benign pancreatic disease. Concordant results for CA19-9 were recently published suggesting potential utility of CA19-9 as part of a panel for preclinical PDAC (5).

TIMP1 and LRG1 best complemented CA19-9 performance in our validation studies. Increased gene expression, and/or secretion of TIMP1, has been previously observed in PDAC and found to induce tumor cell proliferation (26,31,32). Although elevated circulating TIMP1 levels have been mostly associated with PDAC (13,14,33–36), increased levels have also been found in other epithelial tumor types (37–39). A role for LRG1 has been suggested in promoting angiogenesis through activation of the TGF-β pathway (40). Apart from PDAC, increased LRG1 plasma levels have also been found in other cancer types (41–45).

The performance of the three-marker panel was statistically significantly better than CA19-9 alone in distinguishing early-stage PDAC from matched healthy subject or benign pancreatic disease controls, indicating potential relevance for the detection of early-stage PDAC. Given that any one of the three markers may be elevated in other cancer types, the marker panel is best suited for assessment of PDAC among subjects at increased risk, namely those with family history, cystic lesions, chronic pancreatitis, or subjects who present with adult-onset type II diabetes, as opposed to screening of asymptomatic subjects of average risk.

Although promising PDAC plasma protein biomarkers have been previously reported (11,12,14), this is the first study where a proteomics-based discovery, performed using both human prediagnostic and mouse early-stage PDAC plasma samples, was followed by sequential validation of the identified biomarker candidates in multiple independent sets of samples from resectable PDAC patients and matched controls. By applying a rigorous statistical method, we were able to build a model with statistically significantly improved performance than CA19-9 alone, which was blindly tested in an independent sample set.

A challenge for studies aimed at discovery and/or validation of pancreatic cancer early detection markers is the availability of a sufficient number of patients with early-stage disease, that is stage IA, or with premalignant lesions. Further validation of the three-marker panel in independent prediagnostic cohorts will be required to demonstrate utility for pancreatic cancer screening, leading to the development of an US Food and Drug Administration–approved test for targeted screening populations. Moreover, further improvement in marker performance may result from inclusion in the panel of additional marker types other than circulating proteins (15–22,46) through critical testing of marker combinations.

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