Laminin, gamma 2 (LAMC2): A Promising New Putative Pancreatic Cancer Biomarker Identified by Proteomic Analysis of Pancreatic Adenocarcinoma Tissues*[S]

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In pancreatic cancer, the incidence and mortality curves coincide. One major reason for this high mortality rate in pancreatic ductal adenocarcinoma (PDAC) patients is the dearth of effective diagnostic, prognostic, and disease-monitoring biomarkers. Unfortunately, existing tumor markers, as well as current imaging modalities, are not sufficiently sensitive and/or specific for early-stage diagnosis. There is, therefore, an urgent need for improved serum markers of the disease. Herein, we performed Orbitrap® mass spectrometry proteomic analysis of four PDAC tissues and their adjacent benign tissues and identified a total of 2190 nonredundant proteins. Sixteen promising candidates were selected for further scrutiny using a systematic scoring algorithm. Our preliminary serum verification of the top four candidates (DSP, LAMC2, GP73, and DSG2) in 20 patients diagnosed with pancreatic cancer and 20 with benign pancreatic cysts, showed a significant ($p < 0.05$) elevation of LAMC2 in pancreatic cancer serum. Extensive validation of LAMC2 in healthy, benign, and PDAC sera from geographically diverse cohorts ($n = 425$) (Japan, Europe, and USA) demonstrated a significant increase in levels in early-stage PDAC compared with benign diseases. The sensitivity of LAMC2 was comparable to CA19.9 in all data sets, with an AUC value greater than 0.85 in discriminating healthy patients from early-stage PDAC patients. LAMC2 exhibited diagnostic complementarity with CA19.9 by showing significant ($p < 0.001$ in two out of three cohorts) elevation in PDAC patients with clinically low CA19.9 levels. ***Molecular & Cellular Proteomics 12: 10.1074/mcp.M112.023507, 2820–2832, 2013.***

Pancreatic ductal adenocarcinoma (PDAC)[1] is one of the most devastating cancers and the fourth leading cause of cancer-related deaths in North America (1). Ninety-five percent of patients will not survive beyond five years; this high mortality rate is primarily attributed to the lack of effective diagnostic techniques and treatment regimens. The hallmark features of pancreatic cancer (PC) are late presentation and aggressive metastatic progression (2, 3). The National Cancer Institute statistics estimate that approximately $1.9 billion is being spent in the United States alone each year on PC diagnosis and treatment. PDAC is classified into resectable (~10–20%), locally advanced unresectable (~30–40%), and metastatic (~50%) (3). PDAC diagnosed at resectable stage can possibly be cured with complete surgical removal. This could improve the survival rates and considerably lower treat-

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Received August 29, 2013, and in revised form, June 12, 2013
Published, MCP Papers in Press, June 24, 2013, DOI 10.1074/mcp.M112.023507

[1] The abbreviations used are: PDAC, pancreatic ductal adenocarcinoma; PC, pancreatic cancer; RP, reverse-phase; SCX, strong cation-exchange chromatography; SEC, size-exclusion chromatography; IPA, Ingenuity Pathway Analysis; GO, gene ontology; MudPIT, multidimensional protein identification technology; FPR, false positive rate; FFPE, formalin fixation with paraffin embedding; CTC, circulating tumor cell; HPDE, human pancreatic ductal epithelial; CA19.9, carbohydrate specific antigen 19–9; FXYD, FXYD domain-containing ion transport regulator 3; SPARC, secreted protein, acidic, cysteine-rich; DSP, desmoplakin; LAMC2, laminin, gamma C2; GP73, Golgi membrane protein-1; DSG-2, *desmoglein-2;* LFQ, label free quantitation; SC, spectral counts; XIC, extracted ion current; SRM, selected reaction monitoring; PA, pathway analysis; ROC, receiver operating characteristic; AUC, area under curve; SN, sensitivity; SP, specificity; CI, confidence interval; FA, formic acid; ESI, electrospray ionization; CP, chronic pancreatitis; PD, pancreatic ductal; PanIN, pancreatic intraepithelial neoplasia.

ment costs. It is projected that 20–40% of patients with resectable PDAC survive more than five years after complete surgical removal, highlighting the importance of early-stage diagnosis. Unfortunately, carbohydrate antigen 19–9 (CA19.9), the current standard serum tumor marker for PDAC, has certain limitations as an early detection biomarker (its sensitivity for small tumors {<3 cm} is ~50% and it is significantly elevated in many benign conditions (*e.g.* biliary obstruction, hepatic cirrhosis, chronic pancreatitis)) (4, 5). In light of the scarcity of other, more reliable markers, CA19.9 is currently used in the clinic as a prognostic and surveillance marker. Undoubtedly, the need for a more reliable consistent biomarker (or biomarker panel) for early PDAC diagnosis remains unmet. In pursuit of novel PDAC biomarker candidates, we have previously delineated the proteomes of malignant pancreatic ascitic fluids, pools of pancreatic juice, and pancreatic cancer cell lines (BxPC3, CAPAN, CFPAC1, MIA-Paca2, PANC1, and SU.86.86). We identified a panel of five potential candidate biomarkers, which, in combination, slightly outperformed CA19.9 in a pilot verification study (40 individuals; 20 healthy, and 20 PDAC) (6).

From a different perspective, in the current study, we deployed a comparative quantitative tissue proteomic methodology to compare the proteome of malignant pancreatic tissues with that of their adjacent normal counterparts. A total of 2190 nonredundant proteins were identified, which were further scrutinized using a systematic scoring algorithm based on their quantified cancer-*versus*-normal ratios, on their identification in malignant pancreatic ascites fluid, on their cancer-specific nature, and on their tissue-expression profiles. Our analysis resulted in sixteen promising candidate biomarkers, which fulfilled our criteria and selected for further validation studies. In a multistep validation approach, the selected candidates were first verified in serum samples obtained from 20 patients with benign pancreatic diseases and 20 patients with pancreatic cancer, using commercially available ELISA kits. The best candidate (LAMC2) was further tested in three geographically diverse cohorts from Germany, Japan, and the US composed of 435 serum samples from healthy, benign, and early and late stage cancer patients. Our approach brought to light a previously unknown, promising PDAC candidate biomarker, LAMC2.

EXPERIMENTAL PROCEDURES

Patient Population and Clinical Specimens—All tissues were obtained with informed consent and Institutional Ethics Review Board (IERB) approval from the Department of Visceral, Thoracic and Vascular Surgery, University Hospital Carl Gustav Carus, Technical University of Dresden, Germany. For all four patients included in this study, the PDAC tumors were surgically resected from the head region of the pancreas. Their age/sex/clinical diagnosis-TNM classification was: 59/F/pT3 pN1 cM0 G2 R0; 57/F/pT3 pN1 cM0 G3 R1; 75/F/pT3 pN1 cM0 G3 R1; 56/M/pT3 pN0 cM0 G2 R0. Four benign tissues were collected adjacent to their PDAC counterparts. Tissues were fixed in Tissue-TEK® shortly after their surgical removal according to manufacturer's instructions. The fixed tissues were transported on dry ice. All samples were stored at −80 °C until further use. Tissue specimens were handled in accordance with the rules and regulations of IERB at Mount Sinai Hospital, Toronto, Canada. Details about sample populations and study groups used for serum validation studies are shown in Table I. Blood was collected in ACD (anticoagulant) vacutainer tubes and plasma samples were processed within 24 h of blood draw. Blood samples were centrifuged at room temperature for 15 min at 1000 × g to pellet the cells. After centrifugation, the plasma samples were aliquoted into 1 ml cryotubes and stored at −80 °C or liquid nitrogen until use in this study. The samples were shipped to our laboratory in Toronto, Canada, on dry ice.

Tissue Protein Extraction and Trypsin Digestion—The frozen specimens were thawed to room temperature and washed several times with phosphate buffered saline (PBS-pH 7.4) to remove the TEK material. The clean tissues were transferred to sterilized 1.5 ml Eppendorf® tubes. Tissues were pulverized using a sterile glass rod and ~ 0.5 ml of T-PER® (Thermo Scientific, Mississauga, Canada) was added (T-PER is a detergent-based tissue lysis reagent). The mixture was sonicated for about 5 min on ice using MISONIX immersion tip sonicator (Q SONICA LLC, CT, USA). Tissue protein extracts were centrifuged to remove debris and subjected to overnight dialysis (1 kDa molecular weight cutoff membrane) to remove salts and surfactants. The dialyzed tissue extracts were measured for total protein content using the BCA assay (Thermo Scientific, Mississauga, Canada). Equal amounts (~500 μg) of protein from eight samples (four PDAC and four controls) were subjected to tryptic digestion, performed as described elsewhere (6, 7).

Strong Cation Exchange Chromatography—Tryptic-peptides were acidified in buffer A (0.26 M FA in 5% acetonitrile) and loaded onto a PolySULFOETHYL aspartamide strong cation exchange (SCX) column (4.6 mm x 50 mm, 200A and 5 μm) (The Nest Group, Inc., MA, USA) and fractionation was performed using an Agilent 1100 HPLC system. A 60 min linear gradient method was operated with buffer A→B (B: 0.26 M formic acid (FA) in 5% acetonitrile and 1 M ammonium formate) at a flow rate of 250 μl/min. Fractions were collected in 250 μl aliquots,, which were later pooled into 12 fractions. To evaluate column performance, a peptide cation exchange standard mixture was applied before and after the chromatographic analysis.

LC-MS/MS Analysis on LTQ-Orbitrap®—The peptides from SCX fractions were desalted using the Omix C18 tips (Varian Inc., Palo Alto, CA). Samples were diluted with Buffer A (0.1% FA in water) and injected onto a C_{18} trap column (150 μm; packed in-house) using the EASY-nLC system (Proxeon Biosystems, Odense, Denmark). Peptides were eluted from the trap column with an increasing concentration of Buffer B (0.1% FA in acetonitrile) onto a resolving 5 cm long PicoTip Emitter (75 μm inner diameter, 8 μm tip, New Objective) packed in-house with 3 μm Pursuit C_{18} (Varian Inc.). Peptides were resolved using gradient reverse phase (RP) liquid chromatography at a flow rate of 400 nL/min for 90 min. The chromatography system was connected online to an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, San Jose, CA) via a nano-ESI source (Proxeon). The capillary temperature was 160 °C and spray voltage was 2 kV. The mass spectra were acquired in data-dependent mode. The first scan event was a full MS scan from 450–1450 m/z and the next six scans were MS/MS scans on the six most intense parent ions observed in the first scan. These scan events were alternated between each other for 90 min MS acquisition time. Collision dissociation energy for MS/MS was set at 35%. Dynamic exclusion, monoisotopic precursor selection and charge state screening were enabled.

Database Searching and Bioinformatics—The MS spectra were searched against the nonredundant IPI human database (version 3.71 containing 86745 forward and 86745 reverse protein sequences) using two search engines, separately: Mascot, version 2.1.03 (Matrix Science) and the Global Proteome Machine manager, version 2006.06.01 (GPM X! Tandem; Beavis Informatics Ltd., Canada). The

TABLE I

Demographics and clinical characteristics and LAMC2 and CA19.9 levels according to the cohorts. PDAC, pancreatic ductal adenocarcinoma; CP, Chronic pancreatitis; PD, pancreatic ductal, S.E., standard error, x; data not available

Variable	US cohort				German cohort				Japanese cohort		
	Control	Benign	PDAC-stage IIB	PDAC-stage IV	Control	Benign	PDAC-operable	PDAC-inoperable	Control	Benign	PDAC
Number of subjects	20	15	25	25	50	50	50	50	50	50	50
Age (years Mean ± S.E.)	59.8 ± 4.39	55.6 ± 4.74	64.4 ± 1.93	62.1 ± 1.92	58.3 ± 2.4	49.8 ± 1.4	67.3 ± 1.1	63.7 ± 1.4	64.1 ± 1.1	60 ± 1.2	63.4 ± 1.2
Sex (M/F)											
Male	5	8	18	15	31	31	30	31	26	x	x
Female	15	7	7	10	19	19	20	19	24	x	x
Race											
White	20	14	24	23	x	x	x	x	x	x	x
Others	x	1	1	2	x	x	x	x	x	x	x
Risk factors											
Smokers	7	11	18	10	x	x	x	x	x	x	x
Diabetes	4	7	8	4							
Chronic pancreatitis	x	14	x	x	x	50	x	x	x	7	x
Pancreatic cyst	x	1	x	x	x	x	x	x	x	35	x
PD dilation	x	x	x	x	x	x	x	x	x	8	x
Serological concentrations											
LAMC2-ng/ml (Mean ± S.E.)	130.6 ± 25.4	244.7 ± 63.2	489.6 ± 69.7	510 ± 62	179.2 ± 32.7	261.2 ± 36.5	487.7 ± 51.3	394.1 ± 45.6	87 ± 6.9	140 ± 20	382.2 ± 44
CA19.9-U/ml (Mean ± S.E.)	12.8 ± 2.8	61.1 ± 15.7	804.15 ± 415.3	4167.9 ± 879.5	7.6 ± 0.9	27.3 ± 12.7	332.3 ± 100	872.3 ± 290.5	21.6 ± 4.6	101.2 ± 56.5	1699.7 ± 437.9

following parameters were used: (1) enzyme: trypsin; (2) one missed cleavage allowed; (3) fixed modification: carbamidomethylation of cysteines; (4) variable modifications: oxidation of methionines; (5) MS^1 tolerance, 7 ppm; and (6) MS^2 tolerance, 0.4 Da. The resulting Mascot DAT and X! Tandem XML files were merged using Scaffold® (version 2.06, Proteome Software Inc., Portland, Oregon) with MudPIT (multidimensional protein identification technology) option checked. Scaffold data was filtered using the X! Tandem Log E (min 3.0) and Mascot ion-score filters [ion score 15, 30(+2) and 40 (+3)] to obtain a protein false-positive rate (FPR) of ≤ 1%. FPR = 2 × (number of proteins identified by searching the reverse sequences)/(the total number of identified proteins). Scaffold® protXML reports were exported and uploaded into Protein Center (Proxeon Biosystems, Odense, Denmark) to create Venn diagrams.

Label-free Quantitation—Thermo RAW files from Orbitrap MS analysis (SCX fractions from eight tissue samples; four PDAC and four adjacent benign) were uploaded into MaxQuant v. 1.1.1.25 (www.maxquant.org) and searched with Andromeda (built into MaxQuant) against the nonredundant IPI.Human v.3.71 database. Data was initially searched against a "human first search" database with a parent tolerance of 20 ppm and a fragment tolerance of 0.5 Da in order to calculate and adjust the correct parent tolerance to 5 ppm for the search against the IPI Human v. 3.71 fasta file. During the search, the IPI v.3.71 Human fasta database was randomized and FPR was set to 1% at the peptide and protein levels. Data was analyzed with "Label-free quantification" checked and the "Match between runs" interval set to 2 min. Label free quantitation (LFQ) intensity values corresponding to each protein from four benign and four PDAC samples were averaged and used to calculate PDAC/Benign fold change ratios.

Prioritization of Biomarker Candidates for Verification—Proteins identified exclusively in tumor tissue samples were uploaded to protein center v. 3.5.2. (Proxeon Bioinformatics, Odense, Denmark) to retrieve GO annotations. The protein center annotations were verified with GeneCards website (http://www.genecards.org/) to pinpoint the cellular localization. Proteins that were found to be extracellular, membrane bound, or secreted were prioritized using the scoring system outlined below. First, the proteins were scored based on the number of tissues they were identified in. Proteins identified in all four pancreatic cancer tissues received two points and those identified in two or three tissues received one point. Next, proteins were scored based on their average LFQ intensity (sum of LFQ intensities ÷ number of tissues identified in). Proteins with an average LFQ intensity in the top 10% were given two points, whereas those within the top 50% received one point. Next, we scored proteins based on differences in mRNA overexpression in pancreatic cancer *versus* normal pancreatic tissues (using a pancreatic cancer microarray database for mRNA expression compiled in our lab (Dimitromanolakis *et al.* unpublished data); a fold change greater than five was assigned two points, whereas a fold change greater than two was given one point. Finally, proteins identified in the proteomic analysis of ascites from pancreatic cancer patients were given an additional point. The maximum score a candidate biomarker could obtain is seven points. All of the proteins with a final score greater than three were searched using PubMed and excluded if they had been previously studied as pancreatic cancer serum biomarkers.

ELISA—Enzyme-linked immunosorbent assays for LAMC2, DSG2, DSP, and GP73 were purchased from USCN Life Sciences and used with serum samples, according to the manufacturer's protocols. The ELECSYS CA19.9 immunoassay from Roche Diagnostics was used to measure serum CA19.9 levels. All validation assays were performed in a randomized fashion; cancer, healthy, and benign serum samples were randomly loaded on ELISA plates to reduce bias, and analyzed in duplicates. Within-run and day-to-day precision of all

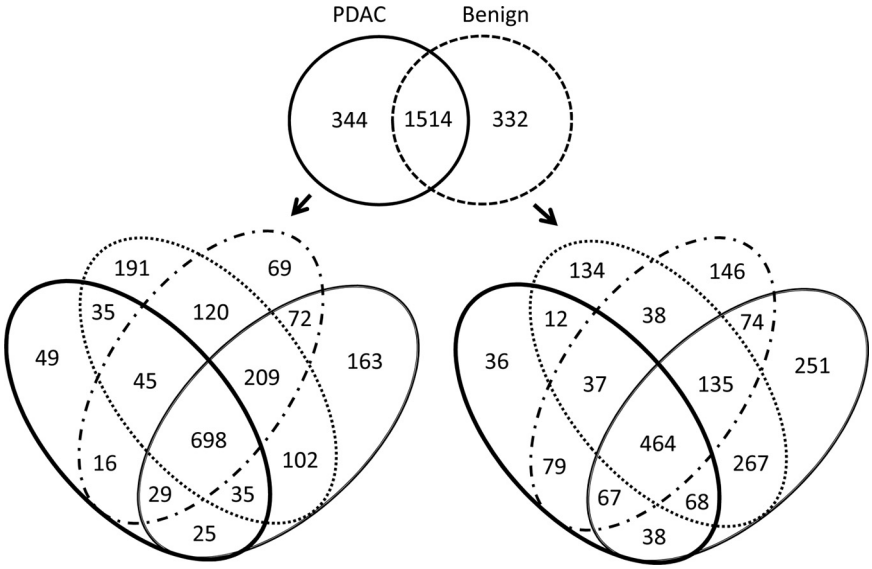

FIG. 1. **Total nonredundant proteins identified.** Venn diagram showing the distribution of the identified 2,190 tissue proteins between and within the four PDAC and four benign samples. PDAC, pancreatic ductal adenocarcinoma. Numbers indicate the number of proteins.

ELISAs was <10% within the measurement range. The precision at the limit of quantitation was around 20%.

Statistical Analysis—All scatter plots were generated by Graph Pad Prism 4 Software. Comparisons between biomarker levels across different groups were performed using Mann Whitney-Wilcoxon test. Comparisons between means were performed by a *t* test or ANOVA test where appropriate. The discriminative ability of biomarkers was assessed by building receiver operating characteristic curves (ROC) for individual markers and combined predictors. The diagnostic value of the markers was evaluated based on area under the curve (AUC) calculations and evaluation of sensitivity at predetermined specificity thresholds of 80 and 90%. Confidence intervals (95%) for areas under the curve and *p* values for comparison between two correlated ROC curves were performed using the method described by DeLong (8). Multiparametric models for combinations of markers were constructed by fitting a logistic regression model on the marker concentrations. The estimated coefficients of the model were used to construct a combined score for each observation which was then used for the evaluation of the multiparametric model. All hypothesis testing was two-tailed, and *p* values of less than 0.05 were considered to indicate statistical significance. Statistical analysis was performed in the R environment (version 2.15.2) available from http://www.R-project.org. ROC curve construction and performance evaluation was performed using the pROC package (9).

Size-exclusion Chromatography—A 5 μl aliquot of a PDAC serum sample was injected onto a size exclusion chromatography (SEC) column (TSK GEL G3000 SW; 5 μm, 60 cm $\times$ 7.8 mm; Tosoh Bioscience LLC, Montgomeryville, PA) connected to an Agilent 1100 series HPLC system (Santa Clara, CA) equipped with a diode array detector. The chromatography was performed at a flow rate of 0.5 ml/min for 65 min and the mobile phase used was 0.1 M NaH_2PO_4/0.1 M Na_2HPO_4, 150 mM NaCl, pH 6.8. Size exclusion standards were chromatographed before and after the serum analysis to establish the elution zones corresponding to their molecular weights. One min fractions were collected from retention time 15 min to 65 min. The elution profile of LAMC2 in the fractions was determined using ELISA.

RESULTS

Liquid Chromatography-Tandem MS (LC-MS/MS) Proteomic Analysis—Our in-depth MudPIT-LC-MS/MS proteomic analysis coupled with MASCOT, X! Tandem, and Andromeda (MaxQuant) search engines identified 2190 nonredundant proteins from four benign and four PDAC tissues. To the best of our knowledge, this represents one of the largest proteomic datasets from PDAC tissue specimens. Among these 2190 proteins, 344 were exclusively detected in PDAC, 332 were unique to benign tissues, and 859 proteins were detected with two or more identified peptides (Fig. 1). To reduce the number of false positives in proteins with one-peptide hit, the false discovery rate was adjusted to <1%. We compared the 2190 identified proteins with our pancreatic ascites proteomic database (7) containing 818 proteins; 303 proteins were also found in PDAC and 321 were also found in benign tissues. Among the 1858 PDAC proteins (Fig. 1), 1340 were identified in at least one of the six pancreatic cancer cell lines, and 976 in the HPDE near normal cell line analyzed previously in our laboratory (6). We also compared our pancreatic tissue proteome with the HIP-2 online healthy plasma protein database; 1068 PDAC proteins were identified in the serum along with 1100 proteins from benign tissues. The comparative PDAC proteomic data with the seven pancreatic cell lines, ascitic fluid, and 150 of the most abundant plasma proteins is presented as supplemental Table S1. Label free quantitation (LFQ) was performed using MaxQuant software to ascertain proteins that are differentially expressed in PDAC tissue samples based on XIC. The normalized XIC intensities are represented in terms of LFQ intensities, which were averaged (four benign and four PDAC) to calculate the fold change (ratio). Only 738 proteins, which were identified in all eight tissues, were considered for quantitative analysis. A *t* test was performed to assess the statistical significance and only those proteins (120 proteins) with $p \leq 0.05$ were considered in the bioinformatic filtering process (supplemental Table S2).

GO Analysis—Proteins of extracellular, secretory, and membranous origins possess the highest possibility of entering the circulation, and therefore, are ideal candidates for

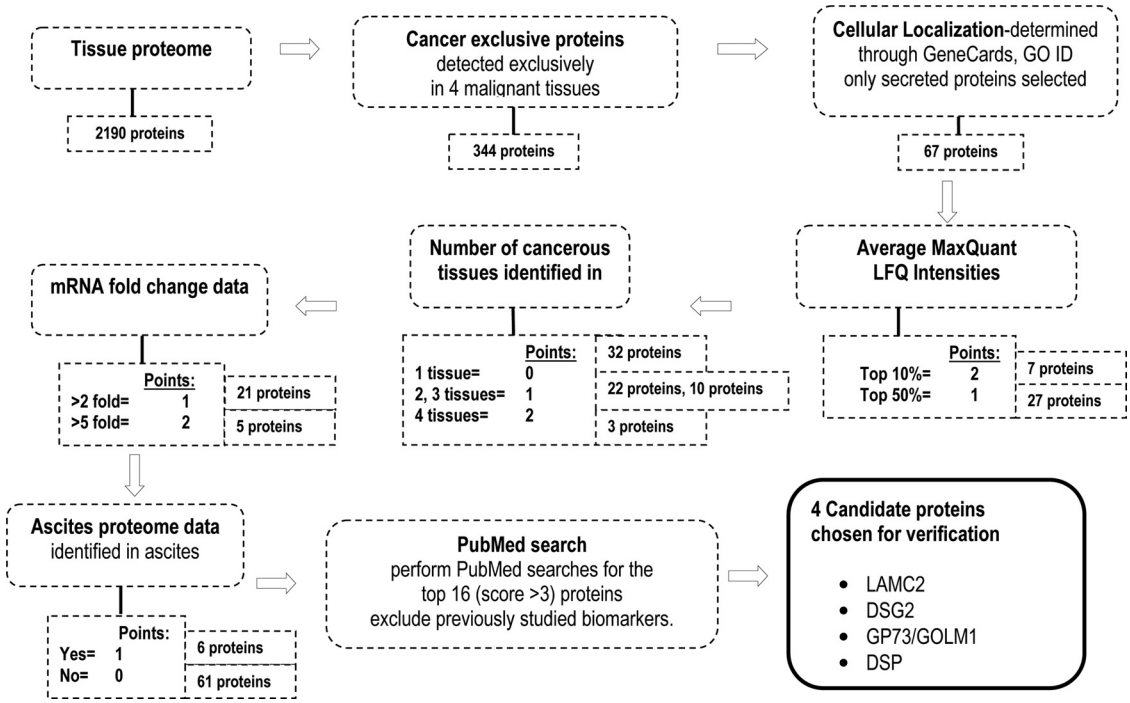

FIG. 2. **Scoring system for candidate biomarker selection.** Flow diagram showing the filtering algorithm used to identify the 16 prospective candidate biomarkers from a pool of 2190 proteins. From 344 PDAC-specific proteins, 67 were identified as membranous and extracellular by Gene Ontology (GO) and GeneCards analysis. The 67 proteins were scored based on Average LFQ (label free quantification) values, number of tissues identified in, pancreatic cancer specific mRNA fold change (increase), and identification in pancreatic malignant ascitic fluid. Only proteins with a score ≥3 (out of maximum score of 7; see text) were considered and four were verified in serum samples by ELISA.

biomarker verification studies. Thus, we relied on GO ontology analysis to classify proteins based on their cellular localization and molecular functionalities. Of the 2190 identified proteins, 74% were of cytoplasmic origin, 46% were membranous, 36% were nuclear, and 23% were extracellular proteins (some proteins were classified in more than one compartment). One caveat of tissue proteomic studies is that the cell lysis process releases the intracellular proteins, which may mask the detection of low abundance secretory proteins. However, circulating tumor cells (CTC) that are detached from primary tumors might discharge key intracellular proteins into serum (10). As expected, the majority of proteins identified were intracellular, and only a small proportion (23%) of the identified proteins was of extracellular and secretory origin. The top three molecular functions of identified proteins were protein binding and catalytic and metal ion binding (Ingenuity pathway analysis; data not shown). A high proportion of tissue proteins, as expected, were involved in metabolic processes (65%), cell organization and biogenesis (50%), and response to stimuli (42%).

Biomarker Candidate Selection—We employed a systematic scoring system (Fig. 2) to segregate 16 candidates from ~2000 proteins based on their (1) cellular origin (extracellular/membranous); (2) mRNA expression levels by microarray in pancreatic cancer *versus* normal tissues, derived from our in-house developed database; (3) average LFQ values in PDAC tissues (ratio of cancer/normal) obtained from MaxQuant label free quantification; and (4) identification in pancreatic malignant ascitic fluids. Among the identified 2190 proteins, 344 proteins were identified exclusively in PDAC tissues. The failure to detect these proteins in each one of the four benign tissues does not endorse their absence; however, it does imply that these proteins could be present at significantly lower amounts than in their adjacent malignant tissues. Membranous and secretory proteins shed from extracellular receptors are preferred to intracellular proteins as serological biomarker candidates; therefore, we prioritized only 67 proteins from the 344, based on their cellular localization. These 67 proteins were scored according to the degree of pancreatic cancer specific mRNA overexpression. A recent study concluded that ~40% of the variation in protein level is directly correlated to mRNA expression (11). Therefore, it is reasonable to speculate that an increase in mRNA expression could lead to overexpression of the protein, and our scoring of candidates based on their cancer-specific mRNA expression is justified. Further, we presumed that if a protein (from the 67 extracellular and membranous groups) is identified in two or more PDAC tissues, its expression should be more frequent, and therefore, it will be more likely to enter the peripheral circulation. We also credited proteins that were identified in the malignant ascites proteome. Among the 67 extracellular proteins, 42 proteins were found in two or more malignant

TABLE II
List of sixteen candidate biomarkers with score ≥ 3[a]

Protein Names	Gene Name	MW kDa	PDAC Tissues[b]	mRNA fold ↑	Ascites[c]	LFQ score[d]	Tissue score	mRNA score	Ascites score	Total score
Desmoplakin	DSP	332	3	0.94	13	2	1	0	1	4
Laminin subunit gamma-2	LAMC2	131	2	8.36		1	1	2	0	4
Golgi membrane protein 1	GP73	46.3	2	2.61	4	1	1	1	1	4
Desmoglein-2	DSG2	122	2	2.32	10	1	1	1	1	4
Tetraspanin-1	TSPAN1	26.3	2	4.85	1	1	1	1	1	4
Mesothelin	MSLN	71.5	2	6.12		1	1	2	0	4
Alkaline phosphatase, placental-like	ALPPL	57.4	2	0.77		2	1	0	0	3
Cadherin-17	CDH17	92.2	2	1.94		2	1	0	0	3
Mucin-13	MUC13	54.6	1	2.45		2	0	1	0	3
S100 calcium-binding protein A14	S100A14	11.7	3	2.46		1	1	1	0	3
FXYD ion transport regulator 3	FXYD3	15.2	3	4.67		1	1	1	0	3
Afadin	AF6	208	4	1.30		1	2	0	0	3
C-type lectin domain family 13 member B	CLEC13B	216	3	4.00		1	1	1	0	3
Tetraspanin-8	TSPAN8	26	2	3.32		1	1	1	0	3
Trophoblast glycoprotein	TPBG	46	4	3.16		0	2	1	0	3
Mucin-4	MUC4	542	2	5.35		0	1	2	0	3

[a] For scoring systems see text and Fig. 2.
[b] Identified in PDAC tissues out of 4 tested.
[c] Number of unique peptides identified in ascites by proteomic analysis (see Ref. 7).
[d] LFQ, label-free quantitation; the definition of LFQ and calculation of ratio of cancer/normal is described in the text.

tissues, six were identified in ascitic fluid, and 26 proteins had >twofold mRNA overexpression in cancer *versus* normal tissues. Finally, the candidates were ranked according to their average LFQ values. This strategy identified 16 promising biomarker candidates (Table II) and the top four were verified in the current study (Fig. 3).

Verification of ELISA Performance Using SEC-HPLC—Before the application of commercial ELISA assays to the analysis of valuable serum samples, the ELISA reagents were verified by a SEC-ELISA approach. In this method, the elution of LAMC2 from an SEC column (from a pancreatic cancer serum sample) was monitored using the ELISA. Under the same chromatographic conditions, the retention time of LAMC2 (25 min) was compared with the elution profiles of molecular weight standards to calculate the molecular mass of LAMC2. The SEC-calculated molecular mass was close to the theoretical monomeric mass of LAMC2 (130 kDa) and the single peak verifies the ELISA specificity (supplemental Fig. S1).

Candidate Verification in PC Patients—The preliminary verification was performed on 20 benign (chronic pancreatitis and pancreatic cysts) and 20 PDAC (unknown stage) serum samples. The concentration medians for DSP and GP73 cancer samples were not significantly ($p > 0.05$) elevated from those of benign cysts (Fig. 3). The concentration medians for LAMC2, DSG2, and CA19.9 in PDAC samples were 1.8, 1.4, and 62- fold higher than benign sera, respectively, with p values <0.05. Of the four candidates, only the performance of LAMC2 was promising in comparison to CA19.9 in terms of sensitivity (SN) and specificity (SP). At cutoff values of 37 U/ml for CA19.9 (6) and 150 ng/ml for LAMC2, the sensitivity/specificity (SN/SP; %) for CA19–9 and LAMC2 were 80/70 and 66/90, respectively.

LAMC2 Elevation in PDAC Sera From a Japanese Cohort—To further evaluate the potential of LAMC2 as a serum PDAC biomarker, its serum concentration was determined in 50 healthy subjects, 50 with benign pancreatic conditions, and 50 with PDAC (unknown stage). These samples were obtained from Hamamatsu University School of Medicine Handa-yama, Hamamatsu, Japan. Mean age was higher ($p = 0.047$) in the PDAC group {(means ± S.E. (S.E.) 63.4 ± 1.2 years)} *versus* the benign group (60 ± 1.2 years), whereas gender information was not available. Among benign patients, 35 patients had pancreatic cyst, eight had PD dilation, and seven had chronic pancreatitis. LAMC2 and CA19.9 levels were found to be significantly elevated ($p < 0.0001$) in PDAC sera compared with both healthy and benign sera. The mean concentrations were 382.2 ± 44 ng/ml (PDAC), 140 ± 20 ng/ml (benign), and 87 ± 6.9 ng/ml (healthy/control) (Fig. 4*A*). The mean ratio of the concentrations between healthy/control, benign, and PDAC was 1:1.5:4.5. The CA19.9 mean ratio of the concentrations between healthy/control, benign, and PDAC sera were 1:5:80. Discriminative ability of CA19.9 (ROC curve AUC = 0.84) surpassed LAMC2 (AUC = 0.78) when comparing benign and PDAC patients (Fig. 4*C*); whereas LAMC2 (AUC = 0.87) outperformed CA19.9 (AUC = 0.82) in distinguishing healthy from cancer patients (data not shown). None of these differences were statistically significant. LAMC2 was found to be significantly elevated among patients with clinically low CA19.9 levels (<37 IU), in both the healthy *versus* PDAC ($p = 4.379e-04$, AUC 0.81) and the benign *versus* PDAC comparison ($p = 8.367e-03$, AUC = 0.78). We

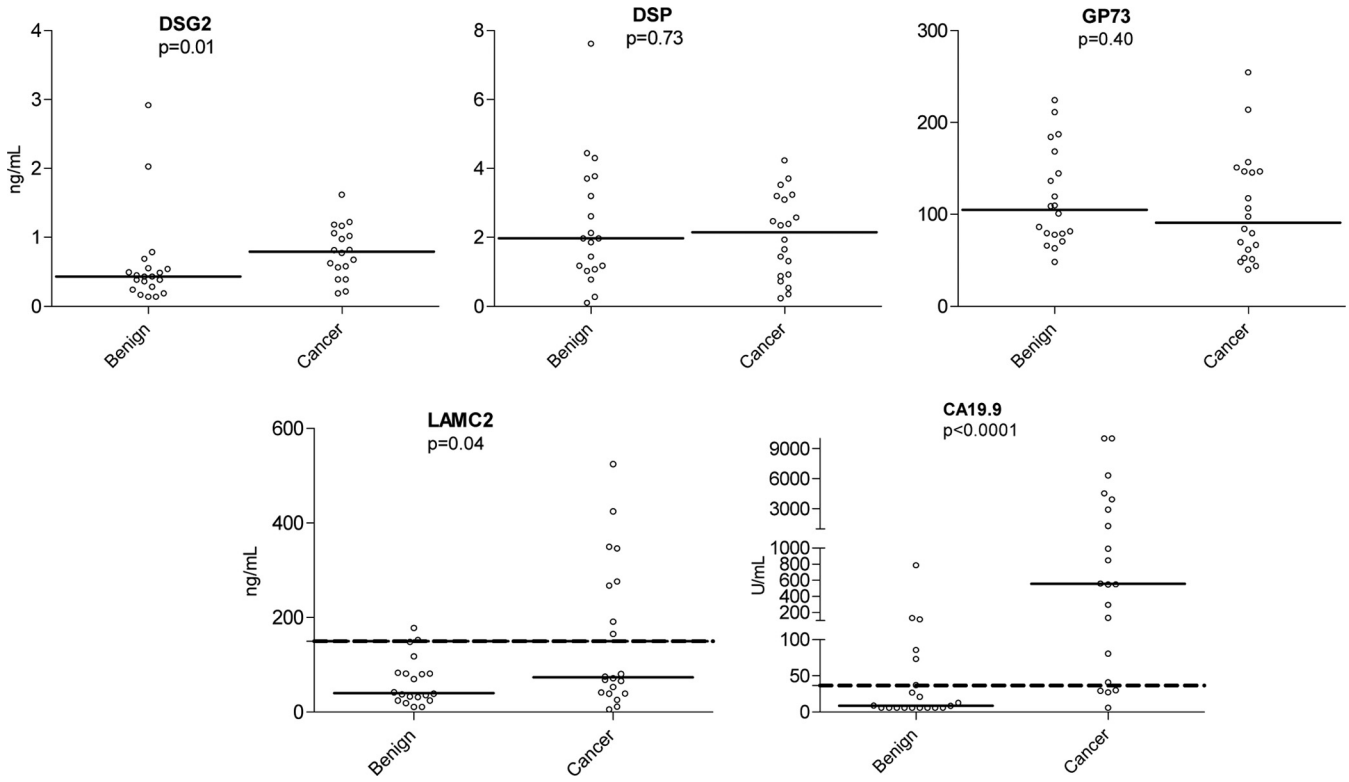

FIG. 3. **Preliminary verification of DSG2, LAMC2, GP73, and DSP.** Serum concentrations of the four candidates and CA19.9 in 20 patients diagnosed with PDAC and 20 with benign pancreatic cysts were measured using ELISA. The corresponding concentration medians are represented by a horizontal line. The dotted line denotes the cut-off values for LAMC2 (150 ng/ml) and CA19.9 (37 U/ml). *p* values were calculated by Mann-Whitney U-tests. The most promising candidate, LAMC2, was verified with additional samples (see Figs. 4 - 6).

built a predictor combining CA19.9 and LAMC2 information by using a logistic regression model. The reduced model combines the two markers in a single predictor by using the formula: 0.176*LAMC2 + CA19.9. The combined predictor showed marginal improvement of CA19.9 in AUC ($p = 0.04$) in this cohort (0.91 *versus* 0.84).

Diagnostic Performance of LAMC2 in Patients with Operable and Inoperable Tumors (German Cohort Study)—In a second validation study, we examined LAMC2 performance in stage-classified PDAC patients, consisting of sera from 200 individuals (50 normal/control, 50 chronic pancreatitis (CP), 50 operable (early-stage) PDAC, and 50 inoperable (late-stage) PDAC). These samples were collected at the University Hospital Carl Gustav Carus, Technical University of Dresden, Germany. Mean concentrations of LAMC2 were 179.2 ± 32.7 ng/ml for normal/control, 261.2 ± 36.5 ng/ml for CP, 487.7 ± 51.3 ng/ml for operable-stage, and 394.1 ± 45.6 ng/ml for inoperable-stage patient sera (Fig. 5*A*). Age and gender ratio was not significantly different between normal and control, benign and PDAC samples ($p > 0.1$, ANOVA test). ROC curve analysis showed significantly lower AUCs for LAMC2 in discriminating CP (AUC = 0.65, $p = 0.001$) and normal patients (AUC = 0.74, $p = 0.001$) from PDAC, in comparison with CA19.9 (AUCs of 0.82 and 0.88) (Fig. 5*C*). The combined predictor using information from both CA19.9 and LAMC2 constructed previously showed AUC of 0.88 (normal *versus* PDAC) and 0.81 (benign *versus* PDAC). No significant improvement in AUC ($p > 0.05$) compared with CA19.9 was found in this sample series. LAMC2 (cutoff = 150 ng/ml) showed higher sensitivity (76%) than CA19.9 (62%) in diagnosing PDAC in operable-stage patients. Among patients with clinically low CA19.9 levels (<37 IU), LAMC2 was elevated in the normal *versus* PDAC group ($p = 0.003$, median ratio 3.4) but not in the comparison between benign and PDAC ($p = 0.06$, median ratio 2.0). Among 19 patients who were negative for CA19.9, 11 (58%) were positive for LAMC2 (Fig. 5*B*). Overall, among the 100 patients with operable- and inoperable-stage PDAC, LAMC2 showed slightly better diagnostic sensitivity (63 were positive for CA19.9 and 71 for LAMC2). The majority of this enhancement in sensitivity comes from the fact that 23 of 37 (62%) of CA19.9 negative patients were positive for LAMC2. These data suggests that LAMC2 may have a role in the detection of early-stage PDAC and in some situations, such as in patients with Lewis antigen nonsecretor status; its measurement may be clinically useful. (Data shown in left panel of Fig. 5*B*).

Blinded Validation Study in Stage-II and Stage IV PDAC Sera (US Cohort Study)—In a third validation study, 85 serum samples (25 stage IIB, 25 stage IV, 20 normal and control, and 15 benign) were screened in a blinded fashion (sample iden-

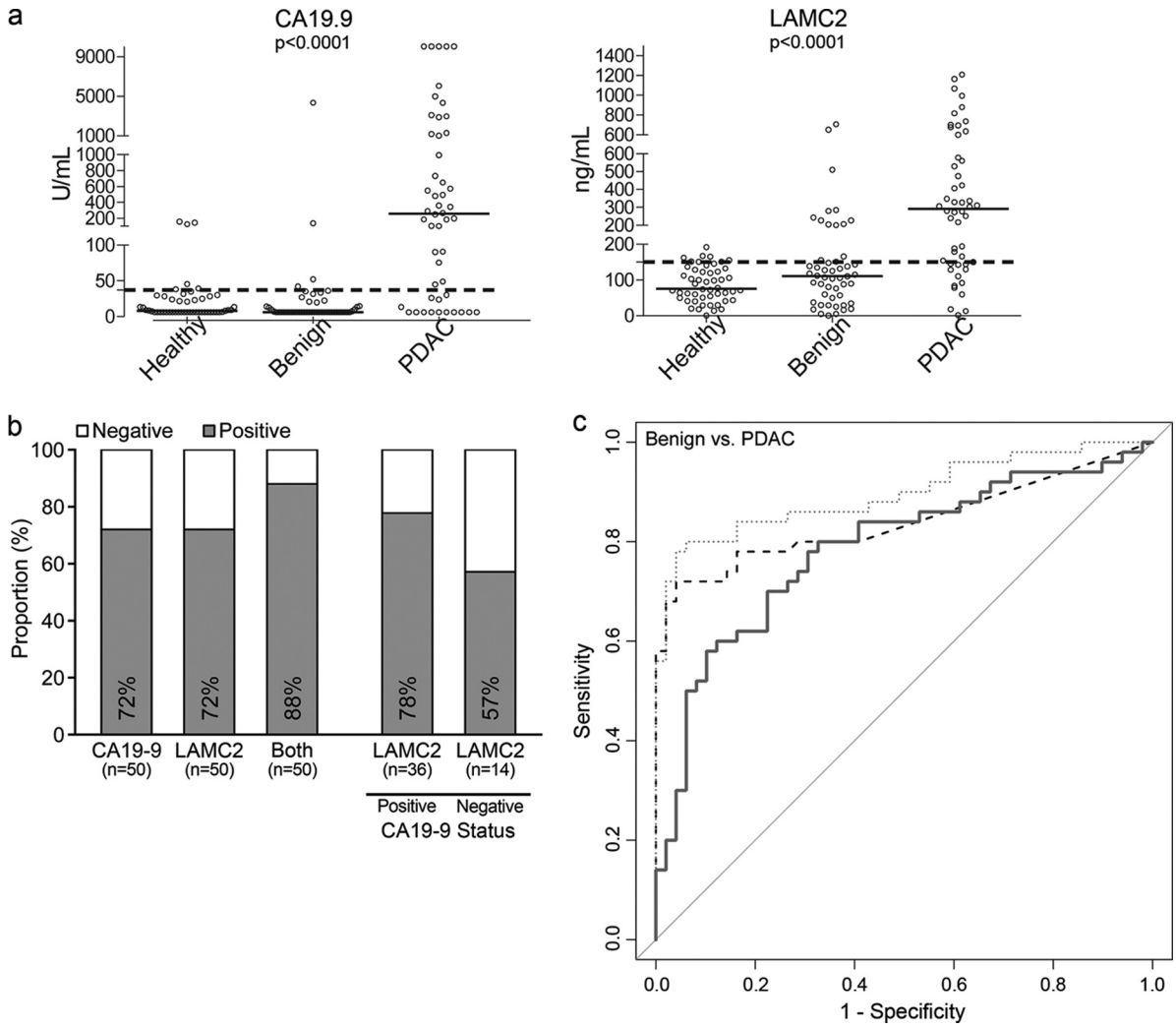

FIG. 4. **Serum LAMC2 levels in pancreatic adenocarcinoma (PDAC) and other samples from Japan.** *A*, Scatter plot of serum LAMC2 and CA19.9 concentrations determined in normal (healthy) ($n = 50$), benign disease ($n = 50$), and PDAC ($n = 50$) serum samples obtained from Hamamatsu University School of Medicine, Japan (for more details on the samples see Table I). The PDAC samples were not stage-classified. The horizontal lines represent median concentrations and the dotted lines are cut-off values for LAMC2 (150 ng/ml) and CA19.9 (37 U/ml). *p* Values were calculated by Mann-Whitney U tests. *B*, The sensitivity of CA19.9, LAMC2, and both in the diagnosis of PDAC and the proportion of patients tested positive for LAMC2 and their corresponding CA19.9 status (positive and negative). *C*, Receiver operating characteristic (ROC) curves for CA19.9, LAMC2, and both for patients with PDAC *versus* benign samples. AUC, area under the curve. The combined assessment of CA19.9 and LAMC2 surpassed the individual diagnostic ability of either of the two markers ($p = 0.035$).

tities were disclosed to our laboratory only after transmitting the ELISA data). This cohort was obtained from the University of Pittsburgh Medical Center; Pittsburgh, PA, USA. Among the 20 control sera were nine choledocholithiasis samples. Age and gender ratio was not significantly different between control, benign, and PDAC samples ($p > 0.1$, ANOVA test). Among benign patients 14 had CP and one had pancreatic cyst (please see supplemental Table S3 for more specific diagnostic information). The mean concentrations were 130.6 ± 25.4 ng/ml (normal), 244.7 ± 63.2 (benign), 489.6 ± 69.7 (stage IIB), and 510 ± 62 (stage IV) (in Fig. 6*A* medians are also presented). Both LAMC2 and CA19.9 showed significant elevation among PDAC patients (stages IIB and IV) compared with both control and benign groups ($p < 0.001$ in all cases). Both markers also showed significant elevation in early stage PDAC *versus* both the benign and normal groups ($p < 0.02$ in all cases). Discriminative ability of LAMC2 was lower than CA19.9 in the control *versus* PDAC comparison (AUC of 0.85 *versus* 0.90), whereas it was higher than CA19.9 in the benign *versus* PDAC comparison (AUC of 0.83 compared with 0.81 for CA19.9). In the benign *versus* stage IIB comparison, LAMC2 (AUC 0.840, CI: 0.699–0.981) outperformed CA19.9 (AUC 0.725, CI: 0.566–0.885), whereas in the control *versus* early stage comparison both LAMC2 (AUC 0.848, CI: 0.738–0.958) and CA19.9 (AUC 0.854, CI: 0.747–0.961) performed similarly. LAMC2 showed significant eleva-

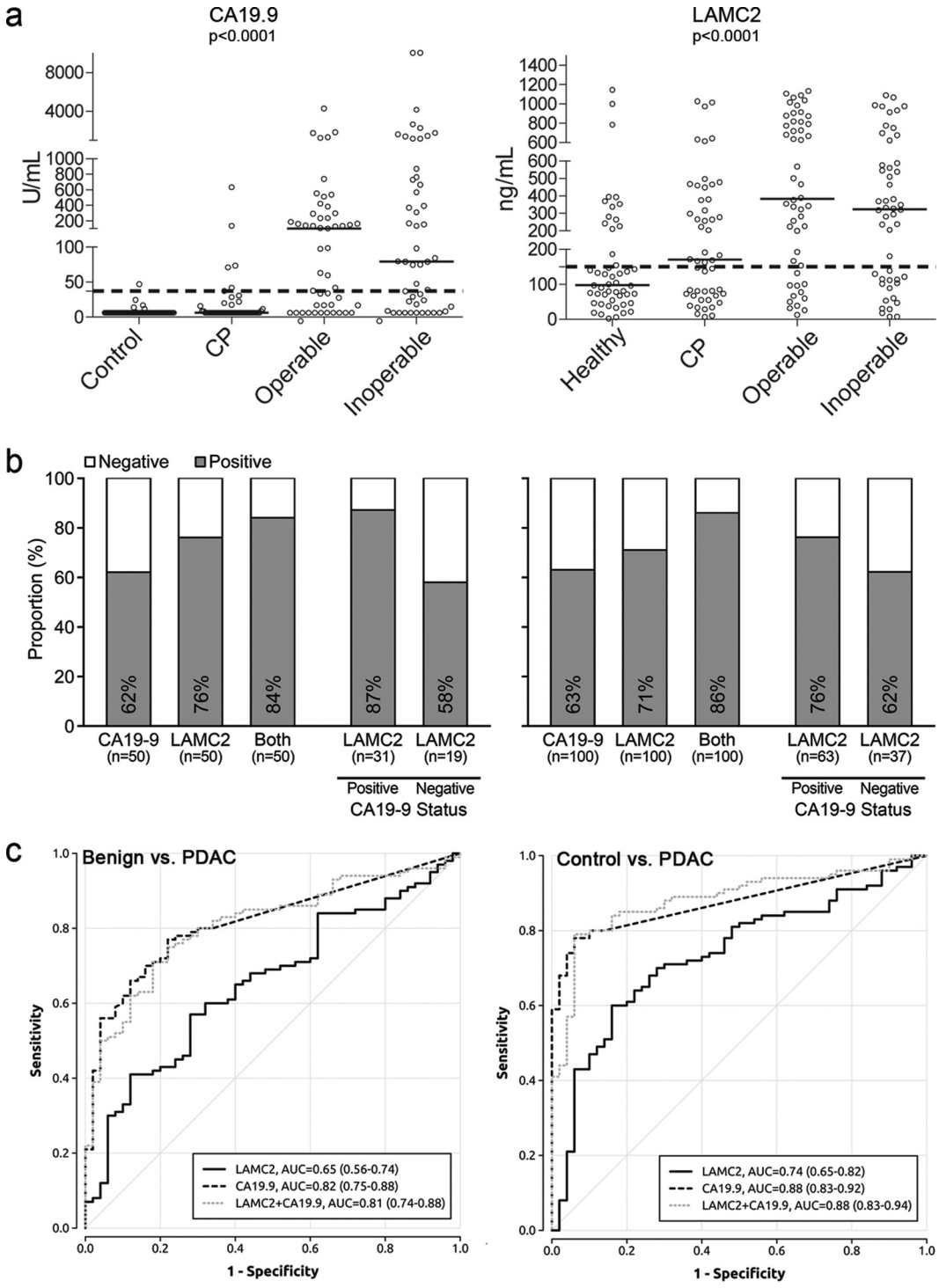

FIG. 5. **Diagnostic performance of LAMC2 in patients with operable and inoperable tumors.** *A*, Scatter plot of serum LAMC2 and CA19.9 concentrations in normal (control) ($n = 50$), benign (CP, chronic pancreatitis) ($n = 50$), and PDAC patients with operable tumors ($n = 50$) and inoperable tumors ($n = 50$). The majority of the benign samples were from chronic pancreatitis patients (CP). This validation cohort was from University of Dresden, Germany. Horizontal lines are medians and the dotted lines denote the cut-off values for LAMC2 (150 ng/ml) and CA19.9 (37 U/ml). *p* Values were calculated by Mann-Whitney U-tests (*B*) The sensitivity of CA19.9, LAMC2, and both in the diagnosis of early-stage PDAC (*left*) and early- and late-stage PDAC combined (*right*). The proportion of patients tested positive for LAMC2 and their corresponding CA19.9 status (positive and negative) is shown. The sensitivity of LAMC2 (76%) was higher than CA19.9 (62%) and the addition of LAMC2 increased the sensitivity of CA19.9 from 62% to 84% in early-stage (operable) patients. Similar data are seen for all patients. *C*, ROC curves for CA19.9, LAMC2, and both for all PDAC patients *versus* healthy and benign controls.

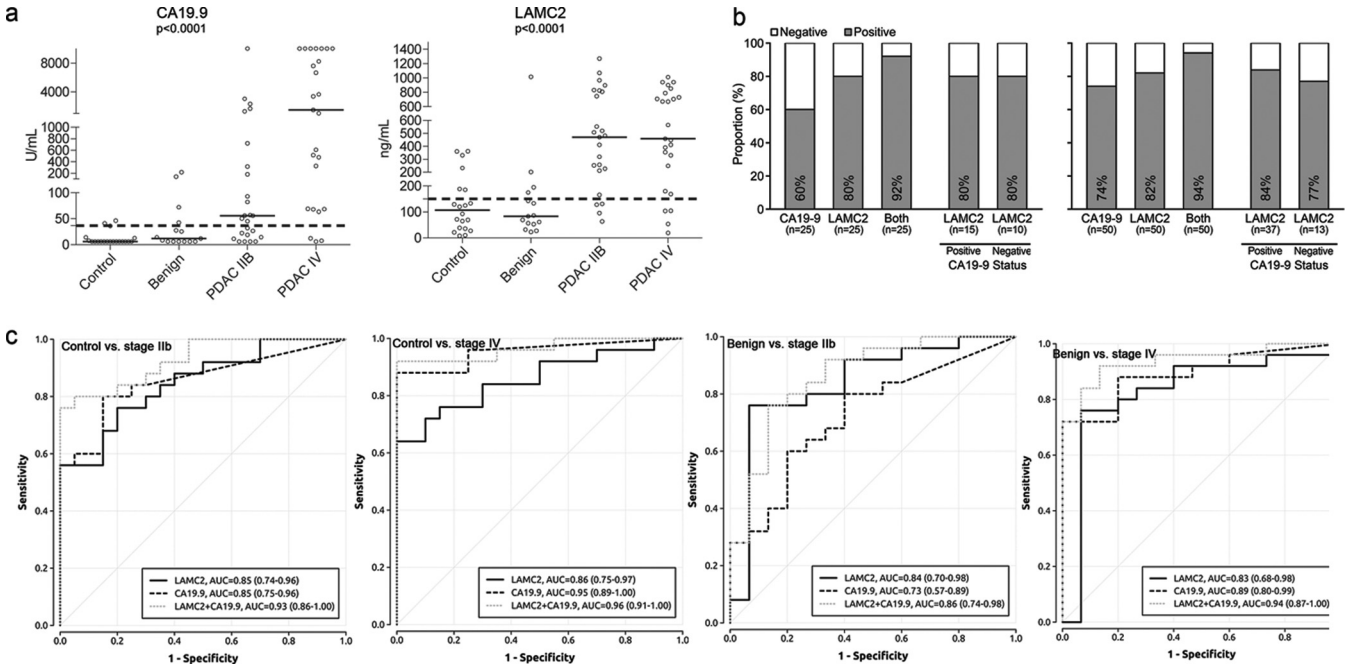

FIG. 6. **Blinded validation study in stage IIB and stage IV PDAC sera.** *A*, Scatter plot of serum LAMC2 and CA19.9 concentrations in healthy (control) ($n = 15$), benign ($n = 15$) controls, and in patients diagnosed with stage IIB ($n = 25$) and stage IV PDAC ($n = 25$). The majority of the benign samples were from chronic pancreatitis patients (Table I). The validation cohort was from the University of Pittsburgh Medical Center, USA. The study was conducted in a blinded fashion, where the sample identities were disclosed only after the sample analysis. Horizontal lines are medians and the dotted lines denote the cut-off values for LAMC2 (150 ng/ml) and CA19.9 (37 U/ml). *p* Values were calculated by Mann-Whitney U-tests (*B*). The sensitivities of CA19.9, LAMC2, and both in the diagnosis of stage IIB PDAC (*left*) and all PDAC patients (*right*). The proportion of patients tested positive for LAMC2 and their corresponding CA19.9 status (positive and negative) is shown. The sensitivity of LAMC2 (80%) was higher than CA19.9 (60%), and the addition of LAMC2 increased the sensitivity of CA19.9 from 60% to 92% in stage IIB patients. For all patients, the sensitivity of LAMC2 (82%) was better than CA19.9 (74%) and when combined, sensitivity is 94% (c) ROC curves for CA19.9, LAMC2, and both for patients with stage IIB and stage IV PDAC *versus* healthy and benign controls.

tion in PDAC samples among patients with clinically low CA19.9 levels when compared with the normal group (median ratio 4.2, $p < 0.001$) and the benign group (median ratio 4.9, $p < 0.0005$). LAMC2 also showed significant discriminative ability in stage IIB patients that were not positive for CA19.9, reaching an AUC of 0.927 (CI: 0.815–1.00). The predictor combining information from both markers as constructed previously showed improved ($p = 0.005$) ability in discriminating benign *versus* PDAC patients compared with CA19.9, reaching an AUC of 0.900 (CI: 0.816–0.984). It also showed an improved AUC of 0.946 (CI: 0.899–0.993) in the normal *versus* PDAC comparison, which was not significantly different than that of CA19.9 ($p = 0.16$). Twenty of 25 stage IIB patients were positive for LAMC2 (cutoff value 150 ng/ml), whereas, only 15 were positive for CA19.9 (Fig. 6*B*). The sensitivity of LAMC2 (80%) for stage IIB PDAC was higher than CA19.9 (60%). It is worth noting that among 10 stage IIB patients who were negative for CA19.9, eight were positive for LAMC2. Overall, for both stage IIB and stage IV PDAC, 74% of patients were positive for CA19.9, 82% for LAMC2, and 94% for either LAMC2 or CA19.9 (Fig. 6*B* right panel). The AUC from ROC analysis showed similar diagnostic performance of LAMC2 and CA19.9 in discriminating stage-IIB (0.85 and 0.85) and stage-IV (0.86 and 0.95) from healthy patients (differences were not statistically significant). Sensitivities of LAMC2, CA19.9 and LAMC2+CA19.9 at two fixed specificities (80 and 90%) are shown in Table III. The study population also included smokers and diabetes patients. Six of 18 smokers from control and benign patients were positive for LAMC2 whereas, three were positive for CA19.9. Among 11 control and benign diabetic patients, three and two patients were positive for LAMC2 and CA19.9, respectively.

DISCUSSION

The American Society of Clinical Oncology (ASCO) discourages the use of CA19.9 for pancreatic cancer diagnosis owing to: (1) false negative results in 5–10% of patients who are carriers of Lewis-negative genotype and develop tumors that do not secrete CA19.9; (2) CA19.9 overexpression is associated with nonmalignant conditions such as chronic hepatitis C infection, alcoholic liver disease, chronic hepatitis B infection, primary sclerosing cholangitis, and primary biliary cirrhosis (5); and (3) in addition to PC, increased serum CA19.9 levels were reported in colorectal, esophageal, hepatocellular, biliary, and nongastrointestinal cancers (12–13).

TABLE III
Diagnostic sensitivities of LAMC2, CA19.9, and LAMC2+CA19.9 at targeted specificities

Cohort	Comparison group	Sensitivity at 80% specificity			Sensitivity at 90% specificity		
		LAMC2	CA19.9	LAMC2+CA19.9	LAMC2	CA19.9	LAMC2+CA19.9
Japan	Benign (n = 49) vs. PDAC (n = 50)	0.62	0.78	0.84	0.54	0.72	0.80
Germany	Control (n = 50) vs. PDAC (n = 100)	0.61	0.81	0.86	0.45	0.79	0.80
	Benign (n = 50) vs. PDAC (n = 100)	0.43	0.72	0.72	0.32	0.61	0.58
US	Control (n = 20) vs. PDAC-stageIIB (n = 25)	0.72	0.80	0.80	0.56	0.60	0.68
	Control (n = 20) vs. PDAC-stageIV (n = 25)	0.76	0.88	0.90	0.68	0.88	0.88
	Benign (n = 15) vs. PDAC-stageIIB (n = 25)	0.76	0.50	0.70	0.59	0.33	0.46
	Benign (n = 15) vs. PDAC-stageIV (n = 25)	0.78	0.80	0.88	0.57	0.72	0.77

The accelerated growth of proteome wide analysis of various cancer bio-fluids and tissues using mass spectrometry has propelled investigators to propose several alternative serological biomarker candidates to overcome the limitations of CA19.9. Many of these biomarkers, including MMP-2, haptoglobin, HE4, CAM1, TIMP1, IL-8, OPN, and SAA, were shown to be promising, but failed to surpass the diagnostic performance of CA19.9 in large cohort validation studies. The dismal diagnostic performance of most of the previously proposed biomarkers requires a revisit of the PDAC biomarker discovery strategies. To this end, we performed proteomic analysis of PDAC tissues and their adjacent benign tissues using offline MudPIT (SCX and RP-LC) Orbitrap® LC-MS/MS. Our analysis identified 2190 nonredundant proteins from eight tissue samples, one of the largest PDAC tissue proteome datasets published to date (14–16). Prioritizing potential biomarker candidates from a pool of thousands of proteins and subjecting them to a serum verification phase is an ideal end point to the discovery phase.

Standardized criteria for selection of candidate biomarkers do not exist. We therefore developed our own criteria, which can be considered arbitrary. The integration of pancreatic specific proteomic data and bioinformatic data mining is a reasonable way to derive highly promising biomarker candidates from thousands of proteins. In our previous publications, we used similar filtering criteria and successfully verified/validated some candidates (17, 18). The serological validation of all 16 short-listed candidates (Table II) depends on ELISA assay availability; we have verified our top four candidates: (1) desmoplakin (DSP), (2) laminin, gamma C2 (LAMC2), (3) Golgi membrane protein-1 (GP73), and (4) *desmoglein-2* (DSG2). The mRNA overexpression in PC was 8.3-fold for LAMC2 and 2.6-, 2.3-, and 1.93-fold for GP73, DSG2, and DSP, respectively. All four candidates were detected in at least two of the four PDAC tissues and in none of the four benign tissues. GP73, DSG2, and DSP were identified in our malignant pancreatic ascitic fluid proteomic analysis with 4, 10, and 13 unique peptide hits, respectively. Furthermore, GP73, DSG2, and DSP were detected after binding to a mixed lectin column consisting of concanavalin A and wheat germ agglutinin (data not shown). This confirms their N-glycosylation status, and therefore, the probability that these glycoproteins entering the peripheral blood circulation is high (7).

Hypothetically, biomarkers that do not participate in aberrant pathways will likely fail to serve as surrogate endpoints; to this end, we reviewed the literature to determine the pathobiological association of our top ranked candidates to PDAC progression. To the best of our knowledge, none of the four candidates were previously studied for pancreatic cancer diagnosis. LAMC2 was found overexpressed in PDAC tumor epithelia and inversely correlated with patient survival (19). Nerve invasion distance is an important prognostic factor in pancreatic cancer. It was shown that the overexpression of LAMC2 is associated with larger nerve invasion distance in six cancer cell lines, mouse models, and 75 patients with advanced stage pancreatic cancer (20). DSG2 is a calcium-binding glycoprotein and a key component of desmosomes. Desmoglein and desmoplakins are essential proteins in desmosomes. Desmosomes facilitate adhesion between epithelial cells (21). It was reported that up-regulation of kallikrein-7, a serine protease, in the BxPC-3 pancreatic cancer cell line is responsible for degradation of DSG2, a mechanism that reduces cell adhesion and promotes cell invasion (22). GP73 was not studied in PC; however, it is highly expressed in malignant tissues and associated with angiogenesis and tumor growth (23).

Initial serological verification of our four candidate biomarkers was performed in 20 PDAC and 20 benign serum samples. At the verification stage, LAMC2 outperformed DSG2, DSP, and GP73, and exhibited comparable SN and SP to CA19.9. The mean concentrations of LAMC2 were ~ 3.5-fold higher in cancer sera than benign sera. This is considerably higher than the cancer and benign mean ratios for previously validated candidates, such as MMP-2 (1.2), ICAM1 (1.88), TIMP1 (1.79), IL-8 (2.31), OPN (2.47), and SAA (2.6) (24–31). To further evaluate the diagnostic value of LAMC2, we analyzed serum samples from 435 patients from three geographically different cohorts (Japan, Germany, and US). To date, serological elevation of LAMC2 levels in any cancer-type has not been reported and its use for diagnostic purposes has not been explored.

In all three validation cohorts, LAMC2 was significantly ($p < 0.05$) elevated in early- and late-stage PDAC compared with both normal and benign controls. Age and gender ratio was not significantly different from each group with in cohorts ($p > 0.1$). The sensitivity of LAMC2 was comparable to CA19.9 in both early- and late-stage PDAC (Figs. 4–6). We also showed

that the combination of LAMC2 and CA19.9 could discriminate age-matched normal and benign patients from PDAC patients better than CA19.9 alone. The elevation of LAMC2 in some of the benign patients could be attributed to the fact that the majority of our benign samples were collected from chronic pancreatitis patients, who carry a 10–20% risk of developing or having early pancreatic cancer (32). Additionally, LAMC2 identified more than 50% of patients with PDAC who were CA19.9 negative. Overall, among 200 PDAC patients (from the three cohorts) 64 were CA19.9 negative; of these, 65% were LAMC2-positive. In summary, from these validation data, we foresee three potential clinical uses for LAMC2; firstly, the elevation of LAMC2 in early stage patients in the German and USA cohorts indicates that LAMC2 may be used as an aid for early-stage diagnosis; secondly, because the majority of the PDAC patients with CA19.9 negative or Lewis antigen nonsecretor status were tested positive for LAMC2, it may be used to monitor therapeutic response in these patients but currently, we do not have supportive experimental data. Third, the combination of CA19.9 and LAMC2 provides improved sensitivity for the diagnosis of early-stage PDAC , therefore, we can reasonably hypothesize that LAMC2 might prove to be an important complementary biomarker for CA19–9. Furthermore, it is worthwhile to include LAMC2 in future multipanel biomarker validation studies.

Despite promising validation data, there are still many important unanswered questions on the utility of LAMC2 as a clinically-useful pancreatic cancer biomarker. It has been recently postulated that pancreatic cancer takes a long time to develop and produce clinical symptoms; up to 20 years from initiation to death (33). Also, a premalignant lesion of pancreatic cancer has been recognized, pancreatic intraepithelial neoplasia (PanIN), as well as *in situ* pancreatic cancer. Small lesions, even with tumor sizes up to 1 cm, have a 28% chance of presenting clinically with metastasis (34). Although the long asymptomatic period offers opportunities for early detection and more effective therapeutic interventions, the highly aggressive nature of this disease requires that serum biomarkers detect such small cancers as early as possible. Absence of very early-stage or *in situ* cancer (*e.g.* T1N0M0; stage 1a) cohorts is a major limitation of this investigation. Although these samples are difficult to find, it will be important to study such patients in the future, in hopes that LAMC2, alone, or in combination with CA19–9, and other markers, can identify such early-stage and localized cancers in high-risk patients. In the US and German cohorts, most of the benign patients had chronic pancreatitis and majority of the Japanese cohort had pancreatic cysts; both of these conditions possess an uncertain risk of developing pancreatic cancer. In many instances, the serological elevations of candidate biomarkers (*e.g.* LAMC2) may not directly correlate with cancer progression but might be because of the consequences of symptoms and risk factors. Lack of precise clinical information in regards to the patient's history of chronic pancreatitis, pancreatic cysts and other potential risk factors such as diabetes, obesity, cigarette smoking, and other occupational exposures among PDAC patients may introduce some bias in this investigation. Therefore, the data presented here should be interpreted with caution. We also recognize that in forthcoming validations, we will need to include a wider variety of benign conditions in sufficiently large numbers to accurately determine the specificity of this new biomarker in comparison to CA19–9. Such comorbid patient groups may include heavy smokers, patients with diabetes, benign obstructive jaundice and other malignancies. However, because CA19–9 is currently used mainly for monitoring therapy response of pancreatic cancer patients, and, as mentioned earlier, many of those patients are negative for CA19–9, another application would be to examine the usefulness of LAMC2 for monitoring these CA19–9 negative patients and if it predicts successful or failed therapeutic responses earlier than imaging and clinical symptoms. Such studies are currently under way.

In conclusion, our comprehensive PDAC tissue proteomic study, coupled with hypothesis-driven bioinformatic analysis and a three-cohort serological validation, revealed ~2000 proteins and a highly promising biomarker, LAMC2. Our validation data warrant further investigation into the diagnostic and prognostic potential of LAMC2, and its performance in prospective biomarker panels for enhanced diagnosis of pancreatic cancer.

Proteomic Data Submission—Our PDAC/benign tissue proteomic raw data (Xcalibur raw files and Mascot DAT files) were submitted to Peptide Atlas database. Additionally, the proteomic dataset was also submitted a Scaffold® (sfd) file, which can be viewed using Scaffold® viewer that can be downloaded from http://www.proteomesoftware.com. The data file can be downloaded from the hash code: http://www.peptideatlas.org/PASS/PASS00254. Protein accession numbers, number of peptides identified per protein and sequence coverage (%) information are provided as supplementary information 1. In addition, we also provide the annotated MS spectra for all the peptides as Scaffold® (sfd) file (supplementary information 2). This file was generated using "save MCP required spectra" option with in the Scaffold 4.0 software.

Study Approval—Procedures were approved by Institutional Ethics Review Boards (IERB) from Mount Sinai Hospital, Toronto, Canada, University Hospital Carl Gustav Carus, Technical University of Dresden, Germany, Hamamatsu University School of Medicine Handa-yama, Hamamatsu, Japan and University of Pittsburgh and University of Pittsburgh Medical Center, Pittsburgh, PA, USA.

Acknowledgments—We thank Chris Smith, Ihor Batruch, and Antoninus Soosaipillai for technical assistance with mass spectrometry and ELISA assays.

* This work is supported by a grant to EPD from the Ontario Institute for Cancer Research. Dr. E.P. Diamandis is a funded Investigator of

the Early Detection Research Network (EDRN) of the National Cancer Institute (USA) (Grant # SU01CA152755).

S This article contains supplemental Data.

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Conflict of interest: The authors have declared that no conflict of interest exists.

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