

Using Quantitative Seroproteomics to Identify Antibody Biomarkers in Pancreatic Cancer

Darshil T. Jhaveri^{1,2,3,4}, Min-Sik Kim⁵, Elizabeth D. Thompson⁶, Lanqing Huang^{2,3,4}, Rajni Sharma⁶, Alison P. Klein^{2,4}, Lei Zheng^{2,3,4}, Dung T. Le^{2,3,4}, Daniel A. Laheru^{2,3,4}, Akhilesh Pandey^{2,3,4,5,6,7}, Elizabeth M. Jaffee^{1,2,3,4}, and Robert A. Anders⁶

Abstract

Pancreatic cancer is the fourth leading cause of cancer-related deaths in the United States. Less than 6% of patients survive beyond the fifth year due to inadequate early diagnostics and ineffective treatment options. Our laboratory has developed an allogeneic, granulocyte-macrophage colony-stimulating factor (GM-CSF)-secreting pancreatic cancer vaccine (GVAX) that has been tested in phase II clinical trials. Here, we employed a serum antibodies-based SILAC immunoprecipitation (SASI) approach to identify proteins that elicit an antibody response after vaccination. The SASI approach uses immunoprecipitation with patient-derived antibodies that is coupled to quantitative stable isotope-labeled amino acids in cell culture (SILAC). Using mass spectrometric analysis, we identified more than 150 different proteins that induce an antibody response after vaccination. The regulatory subunit

12A of protein phosphatase 1 (MYPT1 or PPP1R12A), regulatory subunit 8 of the 26S proteasome (PSMC5), and the transferrin receptor (TFRC) were shown to be pancreatic cancer-associated antigens recognized by postvaccination antibodies in the sera of patients with favorable disease-free survival after GVAX therapy. We further interrogated these proteins in over 80 GVAX-treated patients' pancreases and uniformly found a significant increase in the expression of MYPT1, PSMC5, and TFRC in neoplastic compared with non-neoplastic pancreatic ductal epithelium. We show that the novel SASI approach can identify antibody targets specifically expressed in patients with improved disease-free survival after cancer vaccine therapy. These targets need further validation to be considered as possible pancreatic cancer biomarkers. *Cancer Immunol Res*; 4(3); 1–9. ©2016 AACR.

Introduction

Pancreatic ductal adenocarcinoma (PDA) is notably the most aggressive and debilitating cancer, with only 1% to 4% of patients having an overall survival of more than 5 years (1, 2). These low survival statistics are due to inadequate early diagnostics and resistance to current chemoradiation therapies (3–5). Thus, alter-

native screening and treatment approaches are urgently needed for PDA.

We developed an allogeneic, granulocyte-macrophage colony-stimulating factor (GM-CSF)-secreting pancreatic cancer vaccine (GVAX), which has completed phase II clinical trials (6). A functional genomic approach identified a pancreatic cancer antigen, mesothelin, recognized by T cells (7). We reported the induction of mesothelin-specific T-cell responses only in patients with a disease-free survival (DFS) >3 years, suggesting that the vaccine induces immunologically relevant T-cell responses (6). However, finding T-cell antigens is limited by the need for patient-specific HLA reagents (8).

To circumvent this limitation, we developed a high-throughput, HLA-independent method to uncover serum antibodies induced by GVAX therapy in patients with extended DFS. Antibodies may directly or indirectly remove malignant cells via opsonization, antigen presentation to T cells, and by initiating natural killer cells or complement-dependent cell toxicity (9). Examining antibody responses can also aid in the identification of T-cell antigens and T-cell responses that could be potentially useful as predictive markers for survival or response to therapy. For example, melanocyte differentiation antigen, RAB38/NY-MEL-1, was initially identified by studying antibody responses in melanoma patients using the serologic screening of cDNA expression library (SEREX) methodology. Recent studies have shown that spontaneous CD8⁺ T-cell responses are also directed at this antigen (10). Comparing pre- and postvaccination Western blots of PDA cell line lysates, separated by two-dimensional electrophoresis (2-DE) followed by mass spectrometry analysis,

¹Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, Maryland. ²Department of Oncology, Johns Hopkins University School of Medicine, Baltimore, Maryland. ³The Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Baltimore, Maryland. ⁴The Skip Viragh Center for Pancreatic Cancer Research and Clinical Care and The Sol Goldman Pancreatic Cancer Center, Johns Hopkins University School of Medicine, Baltimore, Maryland. ⁵McKusick-Nathans Institute of Genetic Medicine and Department of Biological Chemistry, Johns Hopkins University School of Medicine, Baltimore, Maryland. ⁶Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, Maryland. ⁷Adrienne Helis Malvin Medical Research Foundation and Diana Helis Henry Medical Research Foundation, New Orleans, Louisiana.

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Corresponding Authors: Robert A. Anders, Sidney Kimmel Cancer Center at Johns Hopkins University School of Medicine, 1550 Orleans Street, Baltimore, MD 21231; E-mail: rander54@jhmi.edu; and Elizabeth M. Jaffee, The Sidney Kimmel Cancer Center at Johns Hopkins, 4M07 Bunting Blaustein, Cancer Research Building, 1650 Orleans Street, Baltimore, MD 21231. E-mail: ejaffee@jhmi.edu

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led to the discovery of Annexin A2 as a potential therapeutic target in PDA (11). This discovery has led to a deeper insight into PDA progression and metastasis and is rapidly being translated into Annexin A2-based mAb therapy for PDA. Thus, the serum of vaccinated patients with a favorable survival profile holds promise for identifying therapeutic targets in PDA.

The major drawbacks to current sera-based screening approaches are the inability to identify cell membrane proteins, and the low-throughput and semiquantitative readouts. We therefore developed a serum antibodies-based SILAC immunoprecipitation (SASI) approach to identify proteins that elicit an antibody response after vaccination. This method takes advantage of stable isotope labeling of amino acids (SILAC) in PDA cell culture, immunoprecipitation with patient-derived antibodies, and mass spectrometric analysis. The result is the subtraction of prevaccine sera from postvaccine sera, providing a means to specifically study only vaccine-induced antibody responses.

This approach identified regulatory subunit 12A of protein phosphatase 1 (MYPT1 or PPP1R12A), regulatory subunit 8 of the 26S proteasome (PSMC5), and the transferrin receptor (TFRC) as targets of postvaccination antibodies in the sera of patients who received GVAX and showed a favorable DFS. We further analyzed MYPT1, PSMC5, and TFRC expression in two independent sets of GVAX-treated patients' normal and malignant pancreatic tumor specimens. We found statistically significant expression of these proteins in malignant compared with normal duct epithelium. The antibody responses detected to these proteins in patients with improved DFS suggests that targeting of these proteins could have antitumor potential. Overall, our data demonstrate that this type of SASI approach can selectively identify new candidate biomarkers for screening and aid in the development of better targeted therapies.

Materials and Methods

Patients, serum, and tissue samples

Patients ($N = 60$) were enrolled in a phase II study of an allogeneic GM-CSF-secreting whole-cell pancreatic cancer vaccine in compliance with the Johns Hopkins Institutional Review Board (IRB)-approved J9988 protocol (6). Blood samples were collected prevaccination, 14 days after first vaccination, and 28 days after each subsequent vaccination. Sera were collected by centrifugation, aliquoted, and stored at -80°C . Pancreatic tumor tissue samples were collected from patients at the time of pancreaticoduodenectomy and prior to vaccination. We also obtained tissue samples from a neoadjuvant study, J0810, for validation purposes (12).

Antibody purification

Antibodies were purified from pre- and postvaccination sera using a protein G column (GE Healthcare) as per the manufacturer's protocol. Quantification of purified antibodies was done with a NanoDrop spectrophotometer (Thermo Fisher Scientific).

SASI sample preparation

Panc 10.05 cells (ATCC line CRL-2574) were developed (13) in E.M. Jaffee's laboratory and the cells were authenticated using short tandem repeat analysis in the Johns Hopkins Genetic Resource Core Facility at 6-month intervals. Panc 10.05 cells were grown in either light ($^{12}\text{C}_6\text{-Lys}$, $^{12}\text{C}_6\text{-Arg}$) or heavy ($^{13}\text{C}_6\text{-Lys}$, $^{13}\text{C}_6\text{-Arg}$) RPMI1640 media containing 10% FBS and antibiotics

in a humidified incubator at 37°C with 5% CO_2 . Stable isotope containing amino acids, $^{13}\text{C}_6\text{-arginine}$ and $^{13}\text{C}_6\text{-lysine}$, were purchased from Cambridge Isotope Laboratories. Arginine and lysine-free RPMI1640 media, FBS, and antibiotics (penicillin and streptomycin) were purchased from Invitrogen. The light and heavy cells were washed with PBS and harvested using mammalian protein extraction reagent buffer (M-PER; Thermo Fisher Scientific) in the presence of cocktail protease inhibitors (Thermo Fisher Scientific). Protein was quantified using the Lowry method.

Immunoprecipitation for mass spectrometry

Equal amounts (10 mg) of light and heavy cell lysates were incubated with purified pre- and postvaccination antibodies at 4°C overnight, respectively. On the following day, the two sets of lysate:antibody mixture were each incubated with protein G beads (Invitrogen) and washed using M-PER buffer. The immunoprecipitates were eluted by boiling in NuPAGE LDS sample buffer (Invitrogen). The light and heavy eluted lysates were mixed 1:1. The mixture was concentrated and resolved by 10% SDS-PAGE. The gel was stained using a Coomassie dye staining kit (Invitrogen) prior to in-gel tryptic digestion for preparation of liquid chromatography tandem mass spectrometry (LC/MS-MS) samples.

LC/MS-MS and data analysis

In-gel digestion and LC/MS-MS analysis were performed as described previously (14). The stained gel was excised into 18 bands and each band was destained in a 40-mmol/L ammonium bicarbonate/40% acetonitrile solution. The samples were reduced with a 5-mmol/L dithiothreitol/20% acetonitrile solution, alkylated with 10 mmol/L of iodoacetamide, and digested with trypsin. Sequencing grade-modified porcine trypsin was purchased from Promega. The peptides were extracted, desalted, dried, and reconstituted in 0.1% formic acid. The peptides were analyzed by reverse-phase LC/MS-MS. Briefly, the peptides were separated using online reverse-phase nano-high-performance liquid chromatography with a C18. Peptide samples related to patients 1 and 3 were analyzed on EASY-nLC (Thermo Scientific) coupled online to an LTQ-Orbitrap Elite mass spectrometer (Thermo Scientific), whereas peptide samples related to patient 6 were analyzed on the Eksigent Nano 2D high-performance liquid chromatography (HPLC) pumping system [Eksigent interfaced directly with an LTQ-Orbitrap XL mass spectrometer (Thermo Electron)]. Isolated proteins from each band were identified using an automated database search algorithm, MASCOT, within the Proteome Discoverer software platform (Thermo Electron) and processed in Perseus software. Our data were searched at a mass tolerance of 10 ppm for precursor peptide ions and with carbamidomethylation of cysteine as a fixed modification and oxidation of methionine as a variable modification (14). The SILAC labels (6 Da) are a variable modification to arginine and lysine. The proteolytic enzyme indicated was trypsin and we allowed up to two missed cleavage events.

Mass spectrometry data validation

Panc 10.05 cells grown in light RPMI1640 media were lysed in M-PER buffer supplemented with protease inhibitor cocktail. The lysate was immunoprecipitated with either the pre- or postvaccination purified antibodies using protein G beads. The immunoprecipitates were eluted by boiling in NUPAGE LDS sample

buffer and resolved on a NuPAGE 4%–12% Bis-Tris gel (Invitrogen). Proteins in the gel were transferred onto nitrocellulose membrane using a semi-dry apparatus (Invitrogen). The membrane was blocked in 5% bovine serum albumin (BSA, Invitrogen) in 0.1% Tween 20-PBS (PBS-T) buffer for 1 hour at room temperature and probed with the relevant primary antibody overnight at 4°C. Antibodies against galectin-3 (sc-19283), E3 ubiquitin protein ligase (sc-9561), mesencephalic astrocyte-derived neurotrophic factor (sc-34560), EGFR kinase substrate 8-like protein 2 (sc-100722), Calpain-1 (sc-81171) were purchased from Santa Cruz Biotechnology. The membrane was incubated with the corresponding peroxidase-conjugated secondary antibodies (Sigma) and then ECL Western Blotting Detection Reagents (GE Healthcare) were used for developing.

Western blot analysis for detecting antibody responses in patients

Purified recombinant proteins, PSMC5 (TP301251), MYPT1 (TP323540), and TFRC (TP300980) expressed in human HEK293 cells were purchased from Origene. One microgram of purified protein was denatured by boiling in SDS-PAGE sample buffer and resolved on a NuPAGE 4%–12% Bis-Tris gel (Invitrogen). Proteins in the gel were transferred onto nitrocellulose membrane using a semi-dry apparatus (Invitrogen). The membrane was cut into individual lanes and was blocked in 5% BSA (Invitrogen) in 0.1% PBS-T buffer for 1 hour at room temperature. After blocking, each individual lane was probed with either prevaccination or postvaccination serum at 1:1,000 dilution. A lane was used as a control and probed with mouse anti-FLAG antibody overnight at 4°C. The membrane was incubated with the peroxidase-conjugated secondary antibodies, goat anti-human IgG antibody (Sigma, A8419) for patient serum lanes, or rabbit anti-mouse IgG (Sigma, A9044) for control lane. ECL Western Blotting Detection Reagents (GE Healthcare) were used for 1 minute at room temperature for developing. χ^2 analysis was used to test for statistical significance.

IHC

Staining protocols were optimized using the pancreatic cancer cell line Panc 10.05 as a positive control and pancreatic tissue as negative control. IHC was performed on formalin-fixed paraffin-embedded 5- μ m thick sections of the available pancreatic tumor tissue samples and the tissue microarrays. The tissue samples of the patients enrolled in the study were obtained from the Department of Pathology at Johns Hopkins Medical Institutions tissue archive. The tissue microarrays (TMA) were constructed from different types of malignant tumors and their companion normal tissues (15). The diagnoses were verified by evaluation of the histopathologic and immunohistochemical stains by two reference pathologists (E.D. Thompson and R.A. Anders).

A standard IHC protocol was applied using Bond-Leica autostainer (Leica Microsystems). Briefly, tissue sections were baked for 20 minutes at 65°C followed by deparaffinization and primary antibody incubation at optimal conditions. A bond polymer detection system was applied to develop the reaction, and 3',3'-diaminobenzidine (DAB) chromogen substrate was used for the visualization of reaction as per the manufacturer's instructions (Leica Microsystems). All sections were then counterstained with hematoxylin, dehydrated, and cover slipped. Antibody details are as follows: anti-PSMC5 from Rabbit 1:150 (Sigma),

anti-PPP1R12A Rabbit (Sigma), anti-TFRC mouse clone H68.4 1:2,000 (Invitrogen).

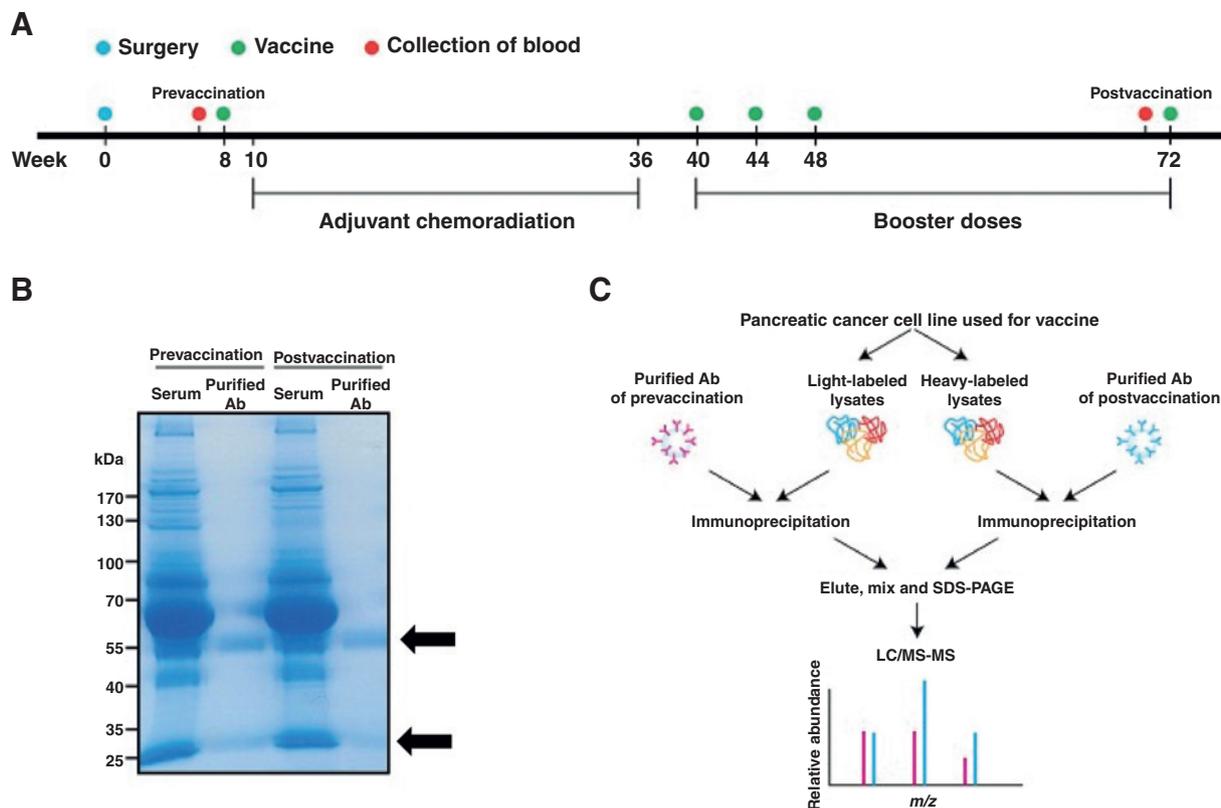
Immunohistochemically stained pancreatic malignant and companion normal tissue were independently scored by two pathologists (E.D. Thompson and R.A. Anders), who were blinded to study outcomes. Stained TMA slides were scored after conversion to digital images in a single-file pyramidal tiled TIFF format using Aperio (Leica Biosystems) software and an acquisition magnification of $\times 20$. For large tissue pancreatic cancer tissue sections, five to ten representative $\times 20$ magnification fields were scored with a compound light microscope. Scoring consisted of the percentage of staining (distribution) in each cellular compartment (membrane, cytoplasm, and nucleus) of malignant and normal cells. The staining intensity was also graded as none (0), weak (1), or strong (2). In preparation for the statistical analysis, the staining intensity and distribution data for each tissue and TMA core studied were placed into two categories (negative and positive). Natural divisions in the staining data defined "negative" (<25% distribution with intensity of 0 or 1) and "positive" (>25% with intensity 1 or 2). All *P* values were calculated using a JavaScript calculator with exact McNemar (PDA samples) or corrected χ^2 or Fisher exact test (TMA).

Results

Design and validation of quantitative proteomic approach

Serum samples used in this study were derived from a phase II, single institution study of 60 pancreatic cancer patients who underwent pancreaticoduodenectomy followed by adjuvant GVAX vaccinations integrated with 5-fluorouracil-based chemoradiation (ref. 6; Fig. 1A). The patients were divided into 3 groups based on DFS and the number of GVAX treatments. Group A had DFS > 3 years and 5 GVAX treatments ($n = 12$); group B had DFS < 3 years and had 3–5 GVAX treatments ($n = 21$); and group C had disease relapse before the second GVAX ($n = 27$). Total serum antibodies from 3 group A patients (patients 1, 3, and 6) were purified from a protein G affinity column (Fig. 1B) and used to identify the targets of vaccine-induced antibodies.

The SASI screen was designed (Fig. 1C) to detect differentially produced antibodies that were present in patient serum before and after GVAX therapy. The proteome, from the Panc 10.05 cell line used in the GVAX vaccine, was differentially labeled using the SILAC technique with "light" (^{12}C) and "heavy" (^{13}C) lysines and arginines (16). Immunoprecipitation of the isotope-labeled proteins with purified patient antibodies, coupled with high resolution and high accuracy mass spectrometry analysis, identified antibody targets (proteins) with fold changes in post- versus prevaccination patient serum (Fig. 2A–C). We then validated the SILAC data with Western blot analysis. Three proteins, galectin-3, E3 ubiquitin-protein ligase UBR5, and mesencephalic astrocyte-derived neurotrophic factor had an increased antibody response postvaccination by 15.3-, 4.0-, and 3.9-fold, respectively, whereas two proteins showed a decreased antibody response, calpain-1 (0.4-fold) and EGFR kinase substrate 8-like protein 2 (0.1-fold). By Western blot analysis (Fig. 2D), galectin-3 protein increased more than 15-fold in the postvaccination blot, whereas E3 ubiquitin-protein ligase UBR5 and mesencephalic astrocyte-derived neurotrophic factor increased by 4-fold. Conversely, calpain-1 expression decreased to 0.4-fold of prevaccination amounts after vaccination and EGFR kinase substrate 8 (EPS8)-like protein 2 decreased to 0.1-fold. These qualitative Western blot analysis

**Figure 1.**

Overview of the study design and SASI screen. A, vaccination scheme based on that of Lutz and colleagues (6). The date of surgery was set as a start date for vaccination schedule (i.e., week 0). First vaccine was administered 8 weeks prior to the adjuvant chemoradiation period (weeks 10–36). Three consecutive booster injections of the vaccine were carried out at 40, 44, and 48 weeks, and the last vaccine was administered at 72 weeks. Blood samples collected before the first injection and before the last injection were used for the SASI approach. B, purification of serum antibodies. Serum antibodies from pre- and post-GVAX of patients with favorable DFS > 3 years were isolated with a protein G affinity column. Coomassie blue staining of the SDS-PAGE gel shows high purity of antibody fragments (black arrows) from serum samples and no significant difference was observed in the antibody amounts between pre- and postvaccination serum samples. C, experimental scheme. One of the GVAX pancreatic cancer cell lines was labeled by the SILAC method to produce light-labeled cell lysates and heavy-labeled cell lysates, each of which was then incubated with purified pre- and postvaccination antibodies, respectively, for separate immunoprecipitations. Immunoprecipitated light- and heavy-labeled proteins were combined and separated on an SDS-PAGE gel. Peptides extracted from the gel were analyzed by LC/MS-MS. Ab, antibody.

results mirrored the trends we observed from the quantitative mass spectrometry–derived SILAC ratios.

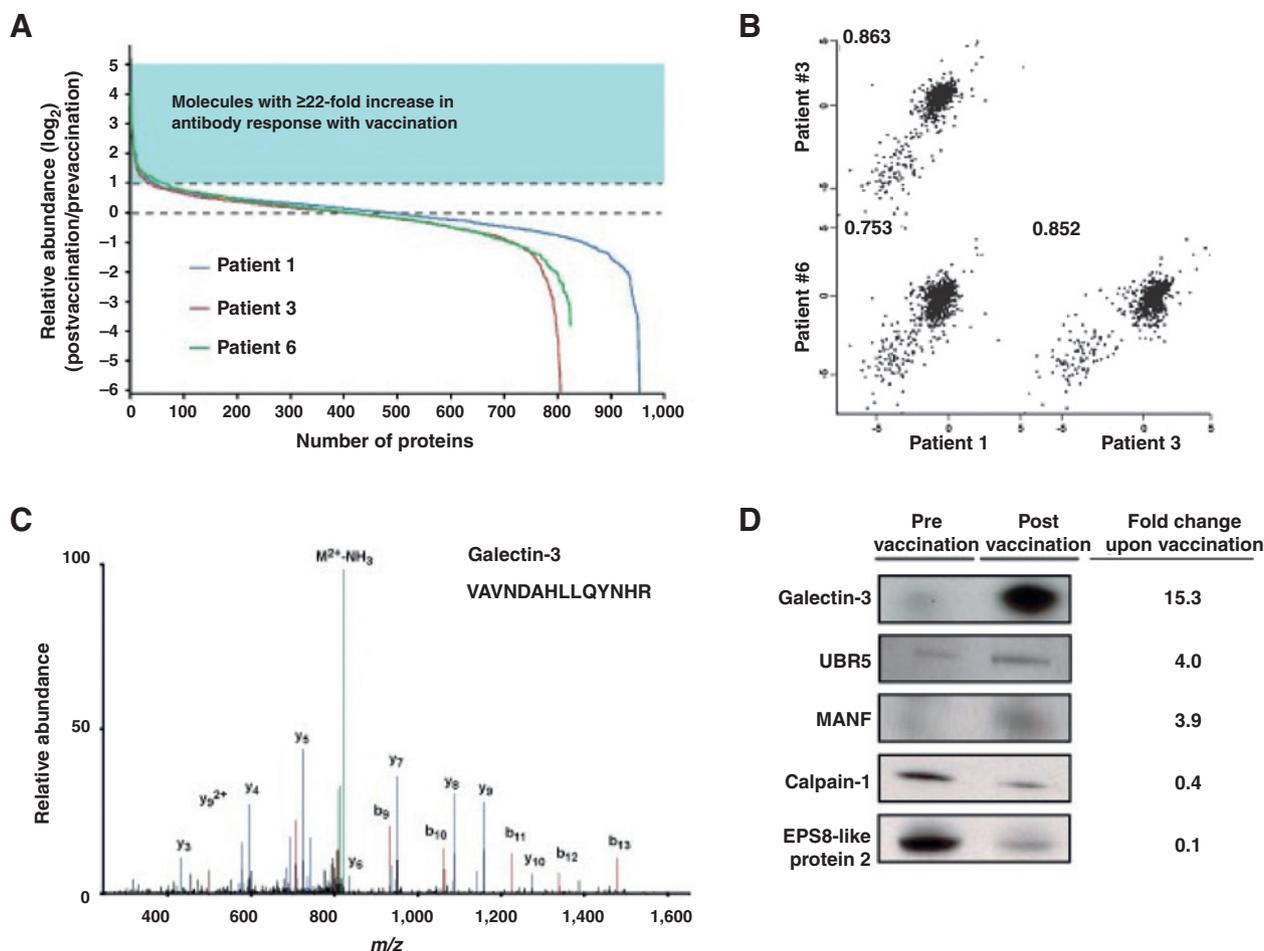
Identification of proteins by the SASI approach

The pre- and postvaccination serum samples from 3 group A patients (GVAX responders) were used for the SASI screen. Patients 1, 3, and 6 had a prolonged DFS status (65, 61, and 43 months, respectively) and were alive at the end of the study. We identified 1,280 unique proteins (Fig. 2A–C) with a range of change from 16-fold increase to a 10-fold decrease in postvaccination serum. Of the identified proteins, 799 (62%) were found in at least two samples and 472 (37%) were found in all three samples (Supplementary Fig. S1). Annexin A2 protein, previously identified as a biologically relevant PDA target (11), showed a median 1.3-fold increase and a maximum fold change of 1.6. Thus, we narrowed our list (Supplementary Fig. S2) from 1,280 proteins to 31 proteins (Supplementary Table S1) by employing Annexin A2-based thresholds and other rigorous selection criteria. Of the 31 proteins, three proteins, galectin-3, Annexin A2, and pyruvate kinase were identified previously by a 2-DE proteomic approach (8, 11) and are currently under investigation for their

role in PDA pathogenesis and progression. Concordance of these two methods in identifying these three proteins (Table 1) further validated the capability of the SASI screen to uncover biologically relevant PDA targets. Another identified protein was HLA class I histocompatibility antigen (HLA; Table 1). Antibody response to allogeneic non-self HLA expressed by the Panc 10.05 cell line used in the GVAX therapy is expected and provided another internal positive control, validating the SASI approach once again. The remaining 27 proteins provided us with a list of targets that warranted further study.

PSMC5, MYPT1, and TFRC are antibody targets of the immune response against PDA

We pursued eight (Table 1) of the 27 short-listed proteins for validation partially based upon previous PDA serial analysis of gene expression (SAGE) data (17) and reagent availability. Using purified recombinant FLAG-tagged proteins expressed in HEK293 cells (obtained from Origene) for Western blot analysis, we examined the antibody responses before and after the third vaccination of 7 of 12 group A patients with favorable DFS for eight proteins (Table 1). Anti-FLAG antibody served as a positive

**Figure 2.**

Protein identification and validation using the SASI approach. A, distribution of relative abundance of proteins precipitated with antibodies. Three biologic experiments (denoted as patients 1, 2, and 3) were performed, each quantitatively identifying about 800 to 900 proteins differentially precipitated between prevaccination and postvaccination. Only a small fraction of proteins were observed to induce a highly differential antibody response after vaccination, indicating that most of the antibodies may still target similar epitopes regardless of vaccine. B, Pearson correlation. Pair-wise comparison of proteins observed in common showed relatively high Pearson correlation coefficients were 0.75 (patient 1 vs patient 6), 0.85 (patient 3 vs patient 6), and 0.86 (patient 1 vs patient 6), respectively. C, a representative MS/MS spectrum. A peptide (VAVNDAHLLQYNHR) derived by trypsin protease from galectin-3 was identified. The spectral assignment is displayed. D, confirmation of the quantitative mass spectrometry (MS) results. Five molecules detected by the quantitative MS were used for the Western blot analysis. Pre- and postvaccination antibodies of patient 6 (also used for SASI screen) with favorable outcome (DFS > 3 years) were used for immunoprecipitation. The precipitate was separated by SDS-PAGE followed by Western blot analysis using antibodies against the indicated proteins. The fold change as detected by mass spectrometry in the SASI screen is shown to the right of each blot and correlates well with the qualitative results in the Western blot analysis, confirming that the MS data are all in keeping with the orthogonal method.

control to confirm the presence of the protein in the blot. Five of eight tested proteins (PSMC5, MYPT1, TFRC, HDGFRP2, and RDH11) showed an increased antibody response postvaccination in five or more patients studied. Therefore, these proteins were chosen for further Western blot analysis.

Next, we expanded our analysis and evaluated the prevalence of recognition of the five proteins by antibody responses pre- and postvaccination from all 12 group A patients who responded to the vaccine. We also compared sera from these patients with sera from 12 of the 21 group B patients who did not respond to the vaccine. The selection of these 12 patients among the total of 21 in the group with DFS < 3 years was based on the number of vaccinations received. The 12 selected patients received at least three vaccinations, thereby allowing us to best compare the antibody responses before and after the third vaccination in both the responders and the nonresponders.

When we examined the 12 group A patients, PSMC5 elicited an increased antibody response in 8 patients and a decrease in one (Table 2; Supplementary Fig. S3A). When these results were compared with those from 12 group B patients (DFS < 3 years) where 2 patients had an increased antibody response and 2 patients had a decreased response, this difference was statistically significant ($P < 0.05$). For the 12 group A patients' MYPT1, an increased antibody response was observed in 9 patients and a decrease in 1 patient (Table 2 and Supplementary Fig. S3B) compared with 12 group B patients (DFS < 3 years) where 5 patients had an increased antibody response and 4 patients had a decreased antibody response ($P = 0.3$). TFRC elicited an increased antibody response in 8 of the 12 patients and a decrease in 2 patients (Table 2 and Supplementary Fig. S3C) compared with group B patients (DFS < 3 years) where 2 of the 12 patients had an increased antibody response and 2 patients

Table 1. List of proteins of interest identified by the SASI approach

Protein	Gene symbol	Average fold change (postvaccination/prevaccination)	Patients with increased antibody response (total of 7 patients)
Galectin 3 ^a	LGALS3	11.0	N/A
MHC class I antigen A	HLA-A	8.2	N/A
26S proteasome, regulatory subunit 8	PSMC5	4.6	6/7
HDGF-2	HDGFRP2	3.2	5/7
Prohibitin-2	PHB2	2.4	4/7
Retinol dehydrogenase 11	RDH11	2.0	5/7
MYPT1	PPP1R12A	1.7	6/7
Transferrin receptor	TFRC	1.7	6/7
Pyruvate kinase ^a	PKM2	1.7	N/A
Pyroline-5-carboxylate reductase 1	PYCR1	1.6	4/7
2,4-dienoyl-CoA reductase	DECR1	1.4	4/7
Annexin A2 ^a	ANXA2	1.4	N/A

NOTE: The average fold change is determined by SILAC. The table also shows a summary of Western blot analysis before and after the third vaccination antibody response of 7 (of the 12) group A patients with favorable DFS for eight proteins. PSMC5, MYPT1, TFRC, RDH11, and HDGFRP2 were selected for further evaluation because they had an increased antibody response in at least 5 out of the 7 patients tested.

Abbreviation: N/A, not applicable.

^aThe proteins identified by previous approach.

had a decreased response; this difference was statistically significant ($P < 0.05$).

RDH11 and HDGFRP2 were two proteins identified by SASI that did not show any major difference in antibody response between the responders and nonresponders. These proteins were therefore excluded from further analysis.

Increased PSMC5, MYPT1, and TFRC tissue expression correlates with PDA development

Antibody responses can be induced against oncoproteins due to changes in their expression levels, localization, or posttranslational modifications (18–21). Available serial analysis of gene expression (SAGE) data suggests that PSMC5, MYPT1, and TFRC may be overexpressed in PDA compared with normal pancreas (17). Therefore, we were interested in establishing the expression levels and tissue location of PSMC5 (Fig. 3A and B), MYPT1 (Fig. 3A and C), and TFRC (Fig. 3A and D) in PDA samples. We analyzed paired PDA tissue samples from 45 of the 60 patients enrolled in the phase II study (J9988, all that were available; ref. 6). The PSMC5 protein is a part of the 26S proteasome typically located in the cytoplasm. We found 94% of PDA express cytoplasmic PSMC5 in malignant compared with 24% nonmalignant ductal epithelium ($P < 0.01$). There was significantly ($P < 0.01$) more nuclear PSMC5 in malignant (72%) cells compared with nonneoplastic ductal epithelium (8%), with an intermediate amount of expression in preneoplastic pancreatic intraepithelial neoplasia lesions. MYPT1, part of the Rho kinase pathway, showed significant expression in cytoplasm of the PDA (95%) compared with normal ductal epithelium (8%; $P < 0.01$). MYPT1

expression was also observed in cancer that was invading pancreatic nerves and regional lymph nodes. Membranous MYPT1 (45%) was only observed in PDA and not in normal ductal epithelium ($P < 0.01$). Similarly, 89% of the PDA expressed cytoplasmic and 21% membranous TFRC ($P < 0.01$), whereas none of the normal ducts showed any cytoplasmic or membranous TFRC expression, consistent with previous reports (22). The fact that TFRC is a well-studied marker in PDA and other cancers further supports that the SASI approach is well suited in identifying biologically relevant oncoproteins.

To validate our staining results, we used PDA tissue from a neoadjuvant GVAX study (J0810), in which patients received a single GVAX vaccination 2 weeks prior to surgery. The expression of PSMC5 (Supplementary Fig. S4A and S4B), MYPT1 (Supplementary Fig. S4A and S4C), and TFRC (Supplementary Fig. S4A and S4D) largely mirrored those samples we found in the nonvaccinated patients (J9988 study). It is important to note that the expression data from the two studies differed in the amount of nuclear PSMC5 (J9988 study 72% vs. J0810 study 27%) and membrane TFRC (J9988 study 21% vs. J0810 study 45%) staining of the cancer cells. It is not clear whether these differences represent variation in the patient populations in each of the GVAX study or performance of the antibody.

Differential expression of nuclear PSMC5 and cytoplasmic and membrane MYPT1: a new diagnostic tool

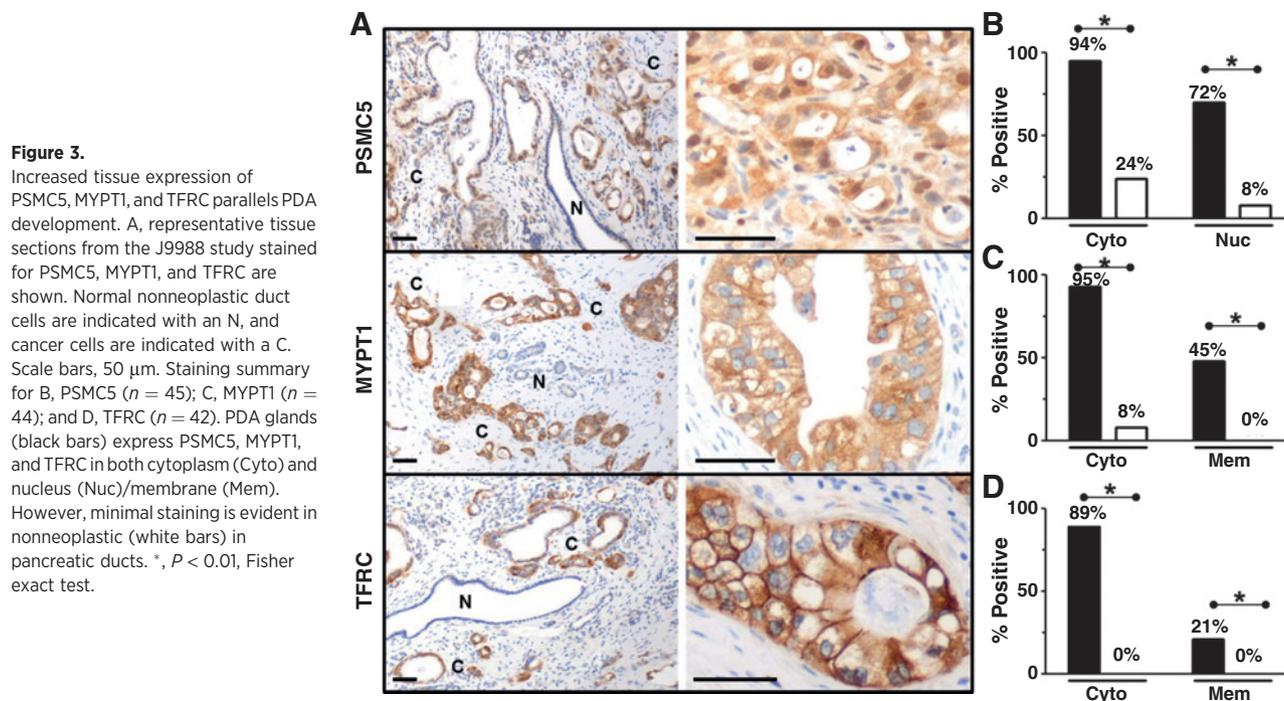
As this is the first report that PSMC5 and MYPT1 are malignancy-associated markers, we further evaluated their prevalence of expression in a panel of cancers. TMAs of bile duct, lung,

Table 2. Correlation of increased antibody response to PSMC5, MYPT1, and TFRC postvaccination with response to vaccine

		Total	Number of patients		P^a
			Increase	Decrease	
PSMC5	Responders	12	8/12 (67%)	1/12 (8%)	<0.05
	Nonresponders	12	2/12 (17%)	2/12 (17%)	
MYPT1	Responders	12	9/12 (75%)	1/12 (8%)	=0.3
	Nonresponders	12	5/12 (42%)	4/12 (33%)	
TFRC	Responders	12	8/12 (67%)	1/12 (8%)	<0.05
	Nonresponders	12	2/12 (17%)	2/12 (17%)	

NOTE: Responders are patients with DFS > 3 years (group A), whereas nonresponders are patients with DFS < 3 years (group B).

^aFisher exact test.



liver, colon, and breast cancers were evaluated for MYPT1 (Fig. 4A) and PSMC5 (Fig. 4B) expression. We found significantly greater cytoplasmic MYPT1 expression in pancreatic cancer (93%; $P < 0.01$) compared with biliary (54%), colon (32%), breast (19%), lung (5%), and liver (0%) cancers. Membranous MYPT1 expression was also expressed significantly more in pancreatic cancers (28%) than all the other cancer types examined. Cytoplasmic PSMC5 expression was seen at relatively higher frequency in all cancer types examined except biliary (22%) cancers. Nuclear PSMC5 expression was high in pancreatic cancers (70%), with breast cancers (35%) being the

next most frequent cancer examined. Significantly more cytoplasmic MYPT1 staining was seen in ER⁺ (39%) compared with HER2⁺ (13%) and basal triple-negative (6%) breast cancer subtypes, whereas cytoplasmic PSMC5 was common in all types of breast cancer: basal (triple-negative, 94%), HER2⁺ (82%), and ER⁺ (88%; Supplementary Fig. S5A–S5D). Although these proteins are expressed in other cancer types, we found greater expression of cytoplasmic and membranous MYPT1 by pancreatic cancers/PDAs when compared with the other tumor types ($P < 0.01$). In addition, nuclear PSMC5 expression seems to be PDA-specific ($P < 0.01$). Thus, by

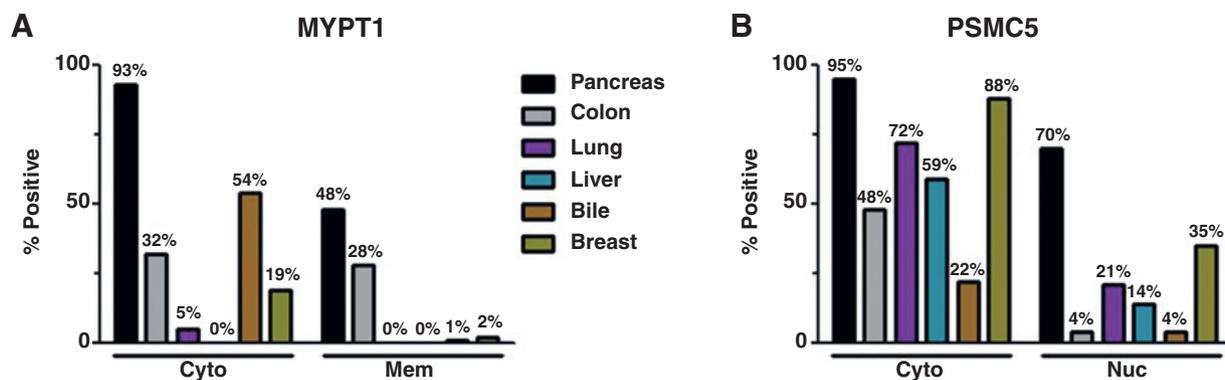


Figure 4. Evaluating MYPT1 and PSMC5 expression in biliary, lung, liver, colon, and breast cancers using tissue microarrays. A, comparison of cytoplasmic and membranous MYPT1 staining in PDA ($n = 44$) versus biliary ($n = 90$), lung ($n = 91$), liver ($n = 36$), colon ($n = 72$), and breast ($n = 54$) cancers. Both cytoplasmic (Cyto) and membranous (Mem) expression of MYPT1 is PDA-specific ($P < 0.01$). B, comparison of cytoplasmic and nuclear PSMC5 staining in PDA ($n = 45$) versus biliary ($n = 82$), lung ($n = 83$), liver ($n = 36$), colon ($n = 57$), and breast cancers ($n = 54$). Nuclear expression (Nuc) of PSMC5 is PDA-specific ($P < 0.01$). However, cytoplasmic expression of PSMC5 is significantly expressed in PDA compared with 72% of lung, 59% of liver, and 48% colon cancers, but not in 88% of breast cancer.

utilizing differences in nuclear PSMC5, and cytoplasmic and membranous MYPT1 staining among various cancers, we can develop a differential diagnosis panel model for PDA that can be further validated in the clinic.

Discussion

We have developed a reliable quantitative proteomics approach we call "SASI" to identify and categorize proteins that are potential therapeutic targets in pancreatic cancers. The SASI screen identified more than 2,500 proteins, including those that are recognized by vaccine-induced differentially expressed antibodies found only in patients responding to therapy. This approach also identified new PDA-associated proteins that can be used to differentiate PDA from premalignant lesions and from other non-PDA cancers.

We validated the design and application of this SASI approach in three distinct ways. First, SILAC ratios were mirrored by Western blot analyses for the corresponding proteins. Second, SILAC identified postvaccine-induced antibody responses to allogeneic HLA molecules, an expected finding (the vaccine comprises allogeneic whole tumor cells), allowing the allogeneic response to serve as a natural positive control. Third, three proteins (Annexin a2, pyruvate kinase, and galectin 3) that were previously identified using a less specific 2D approach were also identified by SASI (8, 11). All three proteins are being explored for biologic relevance in PDA. As an example, antibody responses to Annexin A2 correlate with improved overall survival following GVAX therapy. In addition, we recently reported that annexin A2 induces epithelial–mesenchymal transition thereby facilitating metastases in a mouse model of PDA, and Annexin A2 antibody therapy reduces the incidence of metastases (11).

Our results imply that the vaccine-induced antibody response to PSMC5, MYPT1, and TFRC may be a marker of clinical benefit. However, further studies with more patient samples are necessary to validate our findings. An increased antibody response post-vaccination correlates to a longer and favorable DFS, whereas a decreased response postvaccination correlates to a shorter and unfavorable DFS. The data also suggest that these proteins are antigenic targets of vaccine-induced humoral responses in PDA patients. Most significantly, the antibody responses detected against these proteins in patients with DFS > 3 years suggest an antitumor potential of targeting these proteins.

We also noted a reduced antibody response to many proteins in patients that had vaccine-induced antibody responses. It is not clear if responders had serologic tolerance or if developing antibodies to a small number of proteins is critical to the response. These questions are under investigation.

It is interesting to note that the SASI approach identified PDA-associated proteins located in a number of sites within PDA cells. Our expression studies revealed that cytoplasmic and nuclear PSMC5, and cytoplasmic and membranous MYPT1 and TFRC, are preferentially expressed in PDA compared with normal pancreas tissue. Thus, this SASI approach is sensitive enough to identify differentially expressed proteins between malignant and nonmalignant cells. However, the mechanism by which antibody responses can be induced to nuclear and cytoplasmic proteins in this case is unclear. It is possible that GVAX-induced lymphoid aggregates (B cells and T cells observed to be infiltrating PDAs; ref. 12), facilitate B-cell responses to lysed tumor cells. Finally, TFRC is differentially expressed in PDA and other cancers (21),

providing additional support for the SASI approach in identifying differentially expressed proteins.

TMA analyses revealed that expression of nuclear PSMC5, and cytoplasmic and membranous MYPT1, is highly specific for PDA compared with other cancers. The other cancer types analyzed that also stained positive for these markers are gastrointestinal-derived adenocarcinomas with similar morphologies. Colon cancer is one of the most common adenocarcinomas. The presentation of biliary cancer can overlap with PDA. Thus, by combining PSMC5 and MYPT1 staining, we can develop a differential diagnosis method. We have shown that PDA stains strongly for both PSMC5 and MYPT1. From the other tumors studied, only colon cancer stains highly for both markers. However, the nuclear staining seen in PDA is not as substantial in colon cancers. Thus, we can distinguish one cancer from the other using these two markers.

In summary, the SASI approach can be used to identify proteins that have a differential sera–antibody response among different patients or pre- and posttreatment from the same patient. Specifically, this approach can identify prognostic/predictive biomarkers of response to targeted therapies. In addition, SASI can identify protein targets of antibody responses associated with improved survival in patients with cancer. PDA-specific staining of cytoplasmic and membranous MYPT1 and nuclear PSMC5 can be coupled to the current PDA diagnosis protocol to improve specificity and sensitivity in successfully diagnosing PDA. Finally, these markers also hold potential to serve as novel therapeutic targets for PDA treatment. Future studies will include the testing of mAb therapies targeting these proteins for treating PDA.

Disclosure of Potential Conflicts of Interest

E.M. Jaffee reports receiving commercial research grants from Roche and Bristol-Myers Squibb and has provided expert testimony (potential royalties) for Aduro. R.A. Anders reports receiving commercial research support from Bristol-Myers Squibb and Five Prime and is a consultant/advisory board member for Adaptive Biotech. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: D.T. Jhaveri, M.-S. Kim, L. Huang, D.A. Laheru, A. Pandey

Development of methodology: D.T. Jhaveri, M.-S. Kim, L. Huang, R. Sharma, R.A. Anders

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D.T. Jhaveri, M.-S. Kim, E.D. Thompson, L. Zheng, D.T. Le, R.A. Anders

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D.T. Jhaveri, M.-S. Kim, E.D. Thompson, A.P. Klein, L. Zheng, D.A. Laheru, A. Pandey, R.A. Anders

Writing, review, and/or revision of the manuscript: D.T. Jhaveri, M.-S. Kim, L. Huang, R. Sharma, A.P. Klein, D.T. Le, D.A. Laheru, A. Pandey, E.M. Jaffee, R.A. Anders

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D.T. Jhaveri, R.A. Anders

Study supervision: D.T. Jhaveri, A. Pandey, E.M. Jaffee, R.A. Anders

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Darshil T. Jhaveri, Min-Sik Kim, Elizabeth D. Thompson, et al.

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