Spatiotemporal Proteomic Analyses during Pancreas Cancer Progression Identifies Serine/Threonine Stress Kinase 4 (STK4) as a Novel Candidate Biomarker for Early Stage Disease*

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Pancreas cancer, or pancreatic ductal adenocarcinoma, is the deadliest of solid tumors, with a five-year survival rate of <5%. Detection of resectable disease improves survival rates, but access to tissue and other biospecimens that could be used to develop early detection markers is confounded by the insidious nature of pancreas cancer. Mouse models that accurately recapitulate the human condition allow disease tracking from inception to invasion and can therefore be useful for studying early disease stages in which surgical resection is possible. Using a highly faithful mouse model of pancreas cancer in conjunction with a high-density antibody microarray containing ~2500 antibodies, we interrogated the pancreatic tissue proteome at preinvasive and invasive stages of disease. The goal was to discover early stage tissue markers of pancreas cancer and follow them through histologically defined stages of disease using cohorts of mice lacking overt clinical signs and symptoms and those with end-stage metastatic disease, respectively. A panel of seven up-regulated proteins distinguishing pancreas cancer from normal pancreas was validated, and their levels were assessed in tissues collected at preinvasive, early invasive, and moribund stages of disease. Six of the seven markers also differentiated pancreas cancer from an experimental model of chronic pancreatitis. The levels of serine/threonine stress kinase 4 (STK4) increased between preinvasive and invasive stages, suggesting its potential as a tissue biomarker, and perhaps its involvement in progression from precursor pancreatic intraepithelial neoplasia to pancreatic ductal adenocarcinoma. Immunohistochemistry of STK4 at different stages of disease revealed a dynamic expression pattern further implicating it in early tumorigenic events. Immunohistochemistry of a panel of human pancreas cancers confirmed that STK4 levels were increased in tumor epithelia relative to normal tissue. Overall, this integrated approach yielded several tissue markers that could serve as signatures of disease stage, including early (resectable), and therefore clinically meaningful, stages. Molecular & Cellular Proteomics 13: 10.1074/mcp.M113.036517, 3484–3496, 2014.

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In 2013 the American Cancer Society estimated there were 45,220 new cases of cancer of the pancreas, the most common form of which is pancreatic ductal adenocarcinoma (PDA),1 with an estimated 38,430 deaths (1). The incidence of PDA increased on average by 1.5% per year from 2004 to 2008, a trend that is expected to continue. Because of the frequently asymptomatic character of PDA at early stages, diagnosis generally occurs only after locally advanced or metastatic spread, and the majority of patients therefore present with unresectable disease. This fact, combined with the notable resistance of PDA to chemotherapy, usually yields a very poor prognosis, with less than 20% survival past 12 months after diagnosis (2).

Surgical resection with curative intent improves five-year survival rates from less than 5% to ~20% (3), yet resection is possible in only 10% to 15% of patients because of the low probability of identifying resectable disease in asymptomatic patients. A combination of tissue resection coupled with adjuvant therapy may improve outcomes. Although several biomarker candidates have been identified, none have met all the criteria necessary for clinical usefulness (4). In addition, there is a need for a standard histological grading system with clear definitions of preclinical disease. Therefore, the goal of this study was to discover early stage tissue markers that could serve as signatures of disease stage, including early (resectable), and therefore clinically meaningful, stages.

1 The abbreviations used are: PDA, pancreatic ductal adenocarcinoma; PanIN, pancreatic intraepithelial neoplasia; ADM, acinar to ductal metaplasia; p19ARF, ARF tumor suppressor; BSG, basigin; DSC2, desmocolin 2; FN1, fibronectin 1; RFC4, replication factor C (activator 1) 4; SMAD2, SMAD family member 2; STK4, serine/threonine stress kinase 4; KPC, KrasLSL-G12D/D1; Trp53LSL-R172H/H20648, and Sunil R. Hingorani‡‡/H11001; p48Cre, or KrasLSL-G12D/D1; Trp53LSL-R172H/H20648, and Sunil R. Hingorani‡‡/H11001; Pdx1-Cre; CP, chronic pancreatitis; IHC, immunohistochemistry; TMA, tissue microarray.
patients (4). Accurate diagnosis of PDA can be further con-
founded by difficulties in distinguishing it from chronic pancreatitis (CP) and the associated fibrosis; conversely, pancreatitis can also be initiated by a tumor causing ductal obstruction (5). A non-invasive blood-based test would be ideal for early detection and diagnosis, but no validated markers currently exist with sufficient sensitivity and spec-
ificity to improve clinical decision making. Early stage tissue markers could complement current imaging (MRI, computed tomography, endoscopic ultrasound) and CA19.9 plasma screening modalities (6) and improve the identification of resectable disease, as well as provide further insight into pancreas cancer etiology.

Mouse models that clinically, histologically, and molecularly recapitulate human PDA have been instrumental in elucidating the molecular mechanisms underlying PDA etiology and progression (7–11). Studies in these systems have shown that pancreas-specific expression of mutant Kras can initiate pre-
cursor pancreatic intraepithelial neoplasms (PanINs) that pro-
grress to invasive adenocarcinoma (7) and that the course, rate of progression, and metastatic capability of PDA are affected by the additional loss and/or mutation of key tumor suppres-
sor genes (e.g. Dpc4/Smad4, Cdkn2a/ink4a, and Trp53), as well as the relative timing of these events (8, 10, 11). Accurate mouse models can also complement static genomic and proteomic studies of human samples because they enable spatiotemporal monitoring of markers from disease initiation to metastasis. Although many static analyses of putative biomarkers have been performed on resected tissues (12, 13), fewer studies have taken advantage of genetically engineered mouse models of PDA that progress from well-characterized precursor lesions to invasive and metastatic disease to dis-
cover protein biomarkers and chronicle their distribution and expression during disease progression (14, 15).

Proteomic technologies enable the molecular characterization of biological samples at the structural and functional level (16), and multiplex antibody microarray platforms allow the characterization of multiple analytes simultaneously. Antibody microarrays have the added benefits of requiring minimal sample volumes and the ability to identify their cognate analytes with pico- to femtomolar sensitivities (17, 18). Given their low cost, immunoassay-based diagnostics are thought to be the most applicable proteomic technology in a clinical setting (19, 20).

The KPC mouse model of PDA employs conditional pancreas-specific, endogenous expression of KrasG12D and Trp53R172H and spontaneously develops precursor PanINs with 100% penetrance that progress to invasive and metastatic PDA (8). We thus found this an ideal model to use in conjunction with a novel, high-dimensional, pancreas-cancer-
tailored antibody microarray to assay for proteomic changes during disease progression (18, 21, 22). The microarray con-
sisted of ~2500 antibodies and represents, to our knowledge, the largest antibody array platform produced. Our goal was to follow the evolving primary tumor proteome from PanIN to invasive PDA. Using this approach, we identified and vali-
dated a panel of tissue markers differentiating early stages of tumorigenesis from both the normal organ and chronic pancreatitis. We identified the serine/threonine kinase STK4 as a novel early marker of PDA progression and confirmed its expression in human PDA.

EXPERIMENTAL PROCEDURES

Animal Husbandry and Cerulein Administration—KPC mice on a mixed SV129/CS7BL/6 background were generated as previously described (8). To model chronic pancreatitis, six 2-month-old wild-
type animals received daily intraperitoneal injections of cerulein (5 μg) for 23 consecutive days. Full necropsies and tissue sampling were conducted within six hours of the final injection. All mouse procedures were conducted in accordance with the Fred Hutchinson Cancer Research Center IACUC guidelines.

Antibody Microarray Experiments (Printing and Hybridization)—Antibody microarray slides were printed and tissue lysates were incubated on arrays as previously described (21), with minor modifications. Briefly, antibodies were printed in triplicate on Nexterion slide H hydrogel slides (Schott, Mainz, Germany) in 48 blocks with a 16 × 16 block format for a total of 4096 unique features. Antibodies were typically printed at a final concentration of 350 μg/ml. Proteins in tissue lysates (200 μg of total protein) were labeled with Cy5 (cases and controls) or Cy3 (reference) (GE Health Biosciences, Pittsburgh, PA) following a “case/control versus reference” procedure to remove dye bias from the analysis. Arrays were incubated with labeled lysates for 1.5 h, washed to remove background, and then scanned and analyzed using an Axon Genepix 4200A scanner (Molecular Devices, LLC, Sunnyvale, CA).

Array Analysis and Statistics—For each antibody feature, the difference in case and control signal (red channel) relative to the reference (green channel), known as the M value, was calculated as \( \log_{2}(R/G) \), where \( R \) is red corrected and \( G \) is green corrected using the normexp background correction method (23). Saturated array spots were flagged, and triplicate antibodies with coefficients of variation greater than 10% were removed prior to array normalization.

Following localized background correction, print tip loess intra-array normalization was performed. Inter-array green channel quantile adjustment was then applied to normalize the reference (green) signal. The median intensity of triplicate spots was determined for each antibody feature, and the control signal was subsequently standard-
dized to have a mean signal of zero with a standard deviation of 1. Linear regression was then employed to assess the difference between case and control signal for each antibody, adjusting for the day each array was hybridized. The p values calculated via linear regression represent the ability of candidate markers to distinguish KPC from control pancreata. Candidate protein markers were then ranked based on the coefficient, also known as the odds ratio, which is a log2-based measurement of signal intensity. Markers with positive coefficients are greater in cases versus controls, and negative coef-
cients represent the converse. All normalization procedures and analyses were conducted using the R statistical computing software program incorporating the “limma” package for microarray read-in and normalization (24). A summary of these steps is supplied in the supplemental material.

Tissue and Cell Lysates—Tissue samples from the head of the pancreas were collected from KPC, control, and reference (wild-type) animals at 2- and 4-month time points and frozen in liquid nitrogen. Frozen tissues were weighed and lysed in a 10:1 ratio of lysis buffer to tissue weight using 1% Nonidet P-40, 0.25% deoxycholate, 0.25% octyl-β-d-glucopyranoside, and 0.25% amidosulfobetaine-14 sup-

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implemented with phosphatase inhibitors, protease inhibitor mixture, and PMSF. For immunoblotting experiments, tissue lysates were prepared using a 10:1 ratio of volume to tissue weight of 0.5% TritonX-100, 0.5% deoxycholate in Tris-buffered saline plus the above-mentioned protease and phosphatase inhibitors.

**Immunohistochemistry**—Four-micrometer sections of formalin-fixed paraffin-embedded tissues were deparaffinized with xylene, sequentially rehydrated, and subjected to antigen retrieval in Trilogy pH 8.0 Buffer (Cell Marque, Rocklin, CA) in a pressure cooker for 7 min. All antibody incubations were performed at room temperature on a Dako Autostainer Plus (Agilent Technologies, Santa Clara, CA). Slides were counterstained with hematoxylin (Agilent) for 2 min and then coverslipped. For STK4 analyses, sections were incubated with a primary rabbit monoclonal antibody (1:200). The epitope for the STK4 antibody is located in the first 100 amino acids of human STK4, which shares 100% homology with the murine ortholog. All images were taken with a Nikon DS-V1 brightfield camera using NIS Elements 3.2 Basic Research Image software (Nikon Instruments Inc., Melville, NY). Regions of normal parenchyma (n = 14) and areas of acinar to ductal metaplasia (ADM) (n = 14), PanIN-1 (n = 17), PanIN-2/3 (n = 19), glandular PDA (n = 8), and chronic pancreatitis (n = 11) were first identified via H&E staining, and STK4 immunohistochemistry (IHC) intensity was subsequently assessed in serial sections using a 0–3 scoring system (0 = negative, 1+ = weak, 2+ = moderate, 3+ = strong) in a blinded fashion by two investigators independently (S.R.H. and J.L.S.). Welch’s t test was used to determine statistically significant differences in intensity.

**Immunoblotting**—Protein concentrations were estimated using the BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). Pancreatic protein lysates (30 µg) from KPC, control, and cerulein-treated animals were boiled for 3 to 5 min in Laemmli sample buffer plus 3% β-mercaptoethanol prior to analysis via SDS-PAGE. Lysates were separated on NuPAGE Novex 4–12% Bis-Tris gels (Invitrogen, Grand Island, NY) and transferred to nitrocellulose. The blots were subsequently blocked in 3% non-fat dry milk/PBS and incubated with the following antibodies: STK4, FN1 (Abcam, Cambridge, MA; catalog nos. ab51134 and ab2413), DSC2, BSG, RFC4 (SDIX Technologies, Newark, DE; catalog nos. 2247.00.02, 2602.00.02, and 3824.00.02), SMAD2 (ABM Inc., Richmond, BC, Canada; catalog no. Y021322), p19ARF (Santa Cruz Technologies, Inc., Dallas, TX; catalog no. sc-32749), and α tubulin (Cell Signaling Technology, Beverly, MA; catalog no. 2125). To confirm the specificity of the STK4 antibody, we performed RNA interference experiments to stably knock down STK4 and observed specific loss of the presumptive STK4 band of the correct molecular weight (supplemental Fig. S1). Goat anti-rabbit IRDye 800 (Rockland, Gilbertsville, PA; catalog no. 611-132-002), goat anti-rat IRDye 800, and donkey anti-rabbit IRDye 680 (Li-Cor Biosciences, Lincoln, NE; catalog nos. 926–32219 and 926–68023) were used as secondary antibodies. All immunoblots were scanned and densitometry measurements conducted using a Li-Cor Odyssey Infrared Imager (Li-Cor Biosciences, Lincoln, NE). Two-tailed unpaired t tests were used to compare band intensities between KPC and control or CP lysates using GraphPad Prism 5.0.

**Rapid Autopsy Program and Human Tissue Microarrays**—Pancreatic tumors and other tissue specimens were obtained with consent and institutional review board approval from surgically resected samples or from decedents through the Rapid Autopsy Program at the University of Nebraska Medical Center. To minimize tissue degradation, organs were harvested within three hours post-mortem, and the specimens were either flash-frozen in liquid nitrogen or fixed in formalin (25). Tissue microarrays (TMAs) were made from paraffin blocks of formalin-fixed tissue from rapid autopsies and control specimens of pancreas from non-cancerous transplant donors using cores 2.0 to 2.5 mm in size that were then cut into 4-µm sections and mounted on charged slides. IHC for STK4 was performed, and expression levels were evaluated based on the area and intensity of staining in PDA. As would be expected for late-stage PDA, significant portions of the cores consisted of stroma, which were largely negative for STK4. Scoring was confined to epithelial tumor cells identified histologically. Specifically, the percentage of PDA cells expressing STK4 was multiplied by the intensity of staining, using scores of 0, 1+, 2+, and 3+ for non-existent, weak, moderate, and intense staining, respectively. This approach would yield a maximum score of 300 (3+ staining in 100% of cells). Scoring was performed independently and in a blinded fashion by two investigators (S.R.H. and J.L.S.), and the two scores were averaged. An unpaired, two-tailed Welch’s t test was used to determine statistical differences in STK4 levels between PDA and normal cores.

**RESULTS**

**Antibody Microarray Discovery of Novel and Previously Identified Preinvasive and Invasive PDA Markers**—Previously we fabricated and applied successive iterations of array platforms to discover and validate ovarian (18, 21) and breast cancer serum and plasma markers (22), as well as to identify plasma autoantigen–autoantibody complexes (26). Using this experience together with our knowledge of pancreas cancer pathogenesis, we created a customized pancreas-cancer-specific antibody microarray platform. We selected antibodies against putative blood and tissue biomarkers described in the pancreas cancer biomarker compendium (27), with an emphasis on those reported as markers of PanIN-3 (carcinoma in situ) and candidates with quantitative data suggesting they were most highly expressed in PDA. The total of ~2500 unique antibody features included many antibodies against proteins in signaling pathways implicated in PDA pathogenesis (e.g., TGFβ signaling, Wnt signaling, extracellular matrix remodeling) and also included coverage for proteins with a broad array of functions ranging from mismatch repair proteins to glycolytic enzymes to cytokines and their receptors for the discovery of novel markers. This pancreas-cancer-tailored microarray therefore represents a comprehensive platform for proteomic interrogation.

To discover new and further validate previously reported tissue markers of pancreas cancer and compare their levels during disease progression, pancreata were harvested from 2- and 4-month-old KPC mice and from age-matched controls (n = 5 at each time point). Based on previously published results, the 2-month time point was selected as representative of early stage disease, as it displays predominately normal pancreatic parenchyma, whereas large regions of preinvasive and invasive PDA predominate at 4 months (8). None of the animals from either time point displayed overt clinical signs of disease (e.g., cachexia, lethargy, jaundice, ascites) at the time of sacrifice, nor did they manifest macroscopic or microscopic metastases at necropsy (i.e., equivalent to pathologic Stage 2 or less in humans). As expected and previously described (8), pancreata from 4-month-old KPC animals were more nodular and fibrotic than those from 2-month-old animals (supplemental Fig. S2A). A larger proportion of 2-month
pancreatic parenchyma was still histologically normal. Detailed histological assessment of two distinct levels (＞100 μm apart) of resected pancreata revealed that four of the five 4-month-old KPC animals had progressed to invasive PDA, and the other had progressed to carcinoma in situ, or PanIN-3. In the younger cohort, two animals only had small regions of focally invasive adenocarcinoma, and the remainder progressed only as far as PanIN-2 (supplemental Fig. S2B).

Bulk tissue lysates were prepared from KPC and control animals, and the proteins were fluorescently labeled and incubated on the antibody microarray platform to identify candidates distinguishing PDA from normal pancreas (Fig. 1A, schematic). After array scanning, data extraction, and normalization, linear regression was used to rank candidate markers by $p$ value based on their ability to differentiate bulk KPC tissue from normal pancreata (expressed as a coefficient incorporating red/green fluorescence intensity ratio). In all, 71 proteins distinguished KPC from normal pancreatic tissue ($p$ value < 0.05); of these, 30 exhibited increased expression and 41 showed decreased expression in KPC pancreata (Fig. 1B and Table I). Twenty-nine of these markers also exhibited significant changes in levels in KPC tissue between the two time points (denoted as “stage” coefficients and $p$ values), potentially chronicling disease progression (Table I). Cross-referencing our list of 71 markers to the Pancreas Cancer Biomarker compendium (27) and Pancreatic Cancer Database, we found that 29 represented novel candidate markers of PDA (the remaining 42 have been previously implicated in pancreas cancer in at least one study (supplemental Table S1)).

**Immunoblotting Validates a Panel of Markers for Early Stages of PDA**—To validate candidate markers discovered with our array platform, we assessed protein levels in KPC and control pancreatic tissue lysates by immunoblotting. We focused our validation studies on markers with increased expression in KPC lysates, concentrating on 16 highly up-regulated proteins that also included a balance of novel and previously reported candidate markers for PDA. Seven of these 16 putative markers yielded bands of the predicted molecular size and were significantly elevated in KPC relative to control tissue, and these were evaluated further. Of the remaining nine, antibodies against eight failed to detect any bands or displayed multiple weak bands that were not interpretable (specifically, immunoblots for four potential markers showed bands in positive control cell lysates but failed to show corresponding bands in tissue lysates, three showed no bands in either cell or tissue lysates, and one showed multiple weak bands in tissue lysates and no bands in control cell lysates), and the ninth did not match the predicted molecular weight. These results might reflect differences in antibody performance under array and immunoblotting conditions.

The seven confirmed candidates included the novel potential PDA markers DSC2, STK4, and RFC4, as well as previously identified markers p19ARF, FN1, SMAD2, and BSG (Fig. 2). In comparisons of band intensities in the immunoblots, six of these seven proteins distinguished cancer from control at the 4-month time point, and five of the seven—p19ARF, BSG, FN1, RFC4, and SMAD2—distinguished cancer from normal tissue with significance even at 2 months and therefore represent potential markers of preinvasive disease. We note some degree of heterogeneity in control samples (for example, for STK4 and DSC2 at the 2-month time point) that could result from the inclusion of lymph nodes (see supplemental Fig. S3A) and portions of the main pancreatic duct within the...
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### TABLE I

| Gene name | Protein name | Coefficient | p value | Stage coefficient | Stage p value |
|-----------|--------------|-------------|---------|-------------------|---------------|
| KLK6      | Kallikrein-related peptidase 6 | 4.40 | 0.0010 | 3.04 | 0.0143 |
| TP53      | Tumor protein 53 | −1.11 | 0.0029 | −1.45 | 0.0005 |
| TNC       | Tenascin C | 1.67 | 0.0036 | −0.33 | 0.5150 |
| CDH       | Cadherin (pan) | −3.27 | 0.0042 | −1.14 | 0.2343 |
| LMTK2     | Lemur tyrosine kinase 2 | 1.88 | 0.0044 | −1.08 | 0.0754 |
| FN1       | Fibronectin 1 | 2.15 | 0.0045 | 2.61 | 0.0010 |
| HOXA5     | Homeobox A5 | −1.47 | 0.0048 | 0.32 | 0.4854 |
| BRAF      | v-raf murine sarcoma viral oncogene homolog B1 | −1.63 | 0.0067 | −1.83 | 0.0034 |
| BRCA2     | Breast cancer 2, early onset | 3.24 | 0.0080 | 4.67 | 0.0005 |
| RBM15     | RNA binding motif protein 15 | −1.74 | 0.0083 | 0.20 | 0.7336 |
| STAT3     | Signal transducer and activator of transcription 3 (pY705) | −2.18 | 0.0086 | −1.72 | 0.0305 |
| STK4      | Serine/threonine kinase 4 | 5.98 | 0.0101 | 5.73 | 0.0139 |
| FABP3     | Fatty acid binding protein 3 | 1.98 | 0.0115 | 1.25 | 0.0824 |
| TP53      | Tumor protein 53 | 1.33 | 0.0120 | −0.17 | 0.7118 |
| HOXD13    | Homeobox D13 | −1.06 | 0.0122 | −1.07 | 0.0125 |
| STTN1     | Statmin | −1.52 | 0.0136 | 0.40 | 0.4425 |
| CDKNA2    | p19 ARF (mouse-specific) | 0.88 | 0.0151 | −0.68 | 0.0477 |
| USP3      | Ubiquitin specific peptidase 3 | 2.12 | 0.0152 | −0.02 | 0.9793 |
| IL18      | Interleukin 18 (interferon-γ-inducing factor) | −1.06 | 0.0178 | 0.01 | 0.9776 |
| Smad2     | SMAD family member 2 | 1.87 | 0.0188 | −0.46 | 0.4753 |
| BAD       | BCL2-associated agonist of cell death | −0.74 | 0.0206 | 1.02 | 0.0035 |
| MGMT      | O-6-methylguanine-DNA methyltransferase | −1.83 | 0.0214 | 1.43 | 0.0657 |
| MEK1      | Mitogen-activated protein kinase kinase 1 (pT291) | −1.06 | 0.0234 | 2.55 | 0.0009 |
| DSC2      | Desmocollin 2 | 3.04 | 0.0236 | 1.66 | 0.1936 |
| GRB2      | Growth factor receptor-bound protein 2 | −2.68 | 0.0243 | −2.30 | 0.0725 |
| PTPRB     | Protein tyrosine phosphatase, receptor type, B | −0.66 | 0.0273 | 1.82 | 0.0000 |
| NECAB3    | N-terminal EF-hand calcium binding protein 3 | 1.67 | 0.0294 | −2.59 | 0.0043 |
| CAV1      | Caveolin 1 (pY14) | 1.77 | 0.0296 | 0.42 | 0.5757 |
| IGF2      | Insulin-like growth factor 2 (somatomedin A) | −1.10 | 0.0302 | 0.80 | 0.1030 |
| RPS6KB1   | Ribosomal protein S6 kinase, 70 kDa, polypeptide 1 | −1.41 | 0.0306 | −2.68 | 0.0004 |
| TGFB1     | Transforming growth factor, β1 receptor 1 | −1.00 | 0.0310 | 1.90 | 0.0009 |
| DDH2      | DDH2 domain containing 2 | −1.19 | 0.0312 | 0.14 | 0.7792 |
| AMBP      | α-1-microglobulin/bikunin precursor | 1.64 | 0.0322 | 1.20 | 0.1080 |
| DOK1      | Docking protein 1, 62 kDa (downstream of tyrosine kinase 1) | −1.95 | 0.0331 | −3.19 | 0.0014 |
| MEF2A     | Myocyte enhancer factor 2A | −1.09 | 0.0347 | −2.28 | 0.0003 |
| TACSTD1   | Epithelial cell adhesion molecule | −1.78 | 0.0347 | −0.30 | 0.7232 |
| MES2      | Mes2 | 2.50 | 0.0351 | 1.82 | 0.1156 |
| CTNNB1    | Catenin (cadherin-associated protein), β1, 88 kDa | −1.09 | 0.0358 | −0.55 | 0.2743 |
| MMP7      | Matrix metalloproteinase 7 (matripsylin, uterine) | 0.99 | 0.0358 | 0.52 | 0.2490 |
| STAT1     | Signal transducer and activator of transcription 1, 91 kDa | −0.68 | 0.0359 | −1.49 | 0.0011 |
| FLT1      | FMS-related tyrosine kinase 1 (VEGFR) | −0.77 | 0.0360 | 1.29 | 0.0023 |
| CSF3R     | Colony stimulating factor 3 receptor (granulocyte) | −1.28 | 0.0363 | −0.33 | 0.5562 |
| MITF      | Microphthalmia-associated transcription factor | −1.05 | 0.0364 | 0.80 | 0.0993 |
| MAMDC4    | MAM domain containing 4 | 2.01 | 0.0369 | 0.32 | 0.7209 |
| FES       | Feline sarcoma oncogene | 1.30 | 0.0376 | 1.52 | 0.0181 |
| BIRC5     | Baculoviral IAP repeat containing 5 (survivin) | 1.45 | 0.0376 | 0.63 | 0.3099 |
| MAPKAPK2  | Mitogen-activated protein kinase-activated protein kinase 2 | −2.08 | 0.0376 | −2.04 | 0.0435 |
| RFC4      | Replication factor C (activator 1) 4, 37 kDa | 1.68 | 0.0377 | 1.47 | 0.0646 |
| BSG       | Basigin (Ok blood group) | 0.95 | 0.0392 | −0.55 | 0.2051 |
| IRS2      | Insulin receptor substrate 2 | −2.30 | 0.0392 | 1.11 | 0.2884 |
| KLK14     | Kallikrein-related peptidase 14 | 1.70 | 0.0400 | 2.09 | 0.0138 |
| KRT18     | Keratin 18 | −2.59 | 0.0400 | −3.43 | 0.0091 |
| MDM2      | Mdm2, p53 E3 ubiquitin protein ligase homolog (mouse) | −1.29 | 0.0400 | 0.14 | 0.8217 |
| CLDN18    | Claudin 18 | 3.52 | 0.0405 | 1.85 | 0.2507 |
| NLRP7     | NLR family, pyrin domain containing 7 | −2.61 | 0.0412 | 0.96 | 0.4229 |
| PRDX2     | Peroxiredoxin 2 | 0.61 | 0.0413 | −2.16 | 0.0000 |
| HRAS      | v-Ha-ras Harvey rat sarcoma viral oncogene homolog | 0.70 | 0.0415 | 1.01 | 0.0064 |
| MSI2      | Musashi homolog 2 (Drosophila) | 1.33 | 0.0422 | −0.08 | 0.8889 |
Antibody microarray experiments comparing bulk pancreatic lysates from 2- and 4-month KPC animals with age-matched control pancreatic lysates were conducted. A total of 71 candidate markers with \( p \) values < 0.05 were identified via logistic regression analysis. A red/green semi-quantitative coefficient is also shown, with a positive coefficient denoting greater protein levels in KPC versus control tissue; a negative coefficient means levels were reduced in KPC tissue. Markers are listed based on ascending \( p \) values. A “stage” coefficient and \( p \) value for each marker was also computed and shows whether the marker increased or decreased with statistical significance in KPC tissue between the 2- and 4-month time points.

**Fig. 2. Immunoblot validation of candidate biomarkers.** Antibody microarray analyses confirmed that STK4, RFC4, BSG, p19ARF (A, B), FN1, DSC2, and SMAD2 (C, D) distinguish KPC from control tissue with statistical significance. Protein levels from immunoblotting experiments (A, C) were quantified using densitometry and normalized to \( \alpha \)-tubulin levels from two independent experiments of at least five KPC and four control lysates for each time point (all samples for direct comparisons were run on the same gels). The bracketed doublet bands were quantified for SMAD2. Statistically significant differences in protein levels between KPC and control tissue at individual time points was computed using an unpaired two-tailed \( t \) test (\( ^* \) \( p \) value < 0.05; \( ^{**} \) \( p \) value < 0.01; \( ^{***} \) \( p \) value < 0.001) (B, D). Mean values for individual samples from replicate experiments from KPC and control lysates for each time point are plotted; the mean across all samples within each time point is also indicated. Increases in expression of RFC4, FN1, and STK4 between 2- and 4-month KPC time points were also statistically significant (RFC4 \( p \) value = 0.008, FN1 \( p \) value = 0.04, STK4 \( p \) value = 0.04).
bulk tissue lysates. However, even with this heterogeneity, levels of these proteins were still greater in KPC than in control lysates.

**Distinguishing Early Stages of PDA from Pancreatitis**—Differentiating pancreas cancer from or identifying it amid CP can be challenging clinically, and many putative biomarkers that successfully distinguish pancreas cancer from normal tissue fail to differentiate it from CP (28). To model CP, we used a protocol similar to that reported by Strobel et al. (29), administering daily intraperitoneal injections of the cholecystokinin analog cerulein for just over 3 weeks. Necropsies were conducted within 6 h of the final injection, and bulk tissue lysates were prepared from harvested pancreata. Histologically, all mice displayed increased acinar cell damage associated with an inflammatory infiltrate, edema, ectatic structures, and fibrosis (supplemental Fig. S2C).

Levels of six of the seven-marker panel were significantly greater in KPC than CP tissue; only SMAD2 levels were unchanged in the two conditions (Fig. 3A). Increased levels of DSC2, FN1, RFC4, and STK4 were observed in 4-month KPC lysates compared with CP, and p19ARF and BSG differentiated 2-month KPC tissue from CP with significance. DSC2 levels differentiating 2-month KPC and levels of BSG and p19ARF differentiating 4-month KPC from cerulein-treated pancreata also approached significance (p values = 0.09, 0.08, and 0.08, respectively) (Fig. 3B).

**Dynamic Changes in Marker Expression between Invasive and Metastatic Disease Stages**—Each marker in the panel of seven proteins was able to distinguish 2- and 4-month KPC tissue from control specimens, and all except SMAD2 also differentiated KPC from CP tissue at one of the two time points. To assess how these markers change among preinvasive, invasive, and terminal disease stages, we next examined their expression levels in an independent cohort of moribund KPC animals with widely metastatic disease. As expected, many markers, including p19ARF, FN1, RFC4, and SMAD2, increased in lysates from moribund compared with 4-month KPC pancreata (Figs. 4A and 4B). All but one of the moribund animals had a high metastatic disease burden. The increased expression of DSC2 between 4-month and moribund stages was not statistically significant (p value < 0.07).

Levels of STK4 and BSG increased between 2 and 4 months of disease and then plateaued (Figs. 4C and 4D), implying a potential role for these proteins in the transition from PanIN to PDA. To further validate one potentially novel marker, STK4, we performed IHC on tissues from indepen-
dent cohorts of control, 2-month-old, 4-month-old, and moribund KPC animals, as well as cerulein-treated animals.

**STK4 Expression Increases during PanIN-to-PDA Progression**—In normal pancreatic parenchyma, STK4 displayed a punctate cytoplasmic expression pattern in both acini and ductules (Fig. 5A). STK4 signal intensity increased considerably in regions of damaged acinar structures undergoing dropout and metaplasia (also known as acinar to ductal metaplasia) (Fig. 5B). However, this seemed unique to damaged acini in mutant mice: STK4 expression did not increase in areas of extensive acinar damage or fibrosis in the CP pancreata (Fig. 5C). Strong STK4 expression was observed in lymphocytes in the normal pancreas; in the peripancreatic lymph nodes of CP, 2-month, and moribund KPC tissues; and in Peyer’s patches in the duodenum (not shown).

Though weak punctate expression was observed in acinar cells, STK4 expression in PanIN-2 and PanIN-3 lesions was dramatically increased (Fig. 5E) and remained elevated in invasive PDA (Fig. 5F), consistent with the results obtained from immunoblots. STK4 expression was specific to PanIN and invasive epithelial cells, as the stromal infiltrate lacked expression (apart from the subset of immune cells mentioned above). Whereas normal pancreatic parenchyma weakly expressed STK4 in control samples, lymph nodes showed strong STK4 expression (supplemental Fig. S3A), potentially explaining why one 2-month control and one CP lysate showed significant STK4 in immunoblots. Levels of STK4 in PanIN-2/3 and PDA, but not in ADM, were significantly increased relative to normal parenchyma (PanIN-2/3 versus normal, p value < 0.0001; PDA versus normal, p value < 0.01;
To determine whether STK4 is expressed in human PDA—To determine whether STK4 is expressed in human PDA, we performed IHC on a TMA containing 25 PDA cases and 8 non-diseased pancreas transplant donor controls. Consistent with our observations in murine PDA, the stroma was negative for STK4 with the exception of infiltrating lymphocytes. Staining intensity was scored for epithelial tumor cells (see “Experimental Procedures”) identified via H&E staining. Of the 25 PDA cases in the TMA, 23 were evaluable. Of these, 22 showed predominantly nuclear STK4 expression in tumor epithelial cells, frequently accompanied by weak to moderate cytoplasmic staining (Figs. 6A–6C). STK4 expression ranged from weak (1+) in <1% of cells (a score of 1) to strong (3+) in 70% of cells (a score of 210), with IHC scores ranging between 1 and 210 (mean of 67); four cases showed weak staining in 0% to 20% of tumor epithelia (composite scores 1–15), nine showed weak to strong STK4 in 20% to 50% of epithelia (scores 17–60), and nine showed moderate to strong staining in 40% to 85% (scores 70–210).

The composite scoring for control tissues (19 cores from eight different individuals) ranged from 7 to 40 (mean of 20).

ADM versus normal, p value < 0.14). Furthermore, STK4 levels in PanIN-2/3, PDA, and ADM were also significantly greater than in CP tissue (PanIN-2/3 and PDA versus CP, p value < 0.0001; ADM versus CP, p value < 0.02), PanIN-2/3, PDA, and ADM also had significantly increased levels of STK4 relative to PanIN-1 (PanIN2/3 versus PanIN-1, p value < 0.0001; PDA versus PanIN-1, p value < 0.0001; ADM versus PanIN-1, p value < 0.01). These differences might be underestimated, as two animals had more sarcomatoid morphology in their primary tumors, and STK4 levels were lower in these regions than in well-differentiated PDA (supplemental Fig. S3B).

Matched liver and lung metastases from the moribund cohort of KPC animals showed cytoplasmic STK4 expression confined to metastatic epithelia comparable to levels observed in primary tumors (Figs. 5G and 5H). STK4 expression was also influenced by differentiation state, as regions of both primary tumors and metastases with sarcomatoid morphology displayed lower STK4 levels than more glandular areas (supplemental Figs. S3B and S3C). These IHC results further support the quantitative results from our immunoblotting experiments in a separate cohort of animals: STK4 increases during PanIN-to-PDA progression and peaks during the carcinoma in situ (PanIN-3) and invasive stages of disease.

STK4 Is Expressed in Human PDA—
Weak cytoplasmic and weak to moderate nuclear STK4 reactivity was seen in only a low percentage of acinar and ductal cells (Figs. 6D–6F), with a maximum of 20% of cells in any control sample showing any expression. More specifically, four controls showed negligible to weak staining in 0% to 8% of the pancreatic parenchyma, three showed weak to moderate staining in <20%, and one showed moderate STK4 in 20% of the tissue assessed. Importantly, areas that showed these heterogeneous staining patterns were generally confined to the periphery of individual cores.

An unpaired, two-tailed Welch’s t test comparing the IHC scores between human PDA and normal pancreas tissue showed that STK4 expression was significantly increased in PDA (p value < 0.003). Our data thus clearly indicate that STK4 expression is increased in both human and murine PDA. We do note, however, that cellular localizations did appear to be distinct, with human PDA showing predominantly nuclear staining and murine PDA more cytoplasmic expression. Although the functional relevance of this localization is not clear, we found that these results were recapitulated in human and murine PDA cell lines. We performed immunofluorescence analyses of STK4 in primary cells isolated from murine PDA, normal human ductal pancreatic epithelia, and human PDA cell lines (CFPAC shown; Panc1 and MiaPaCa gave similar results (not shown)). Human PDA cell lines displayed predominantly nuclear STK4 expression with some cytoplasmic staining; human ductal pancreatic epithelia and KPC PDA cells showed more cytoplasmic localization (supplemental Fig. S4).

DISCUSSION

Pancreas cancer portends the worst prognosis of solid tumors, and its incidence in North America is rising. Although the underlying genetics of PDA are well characterized, this knowledge has not yet translated to improved early detection tests, diagnostic markers, or successful targeted therapies. The plasma marker for PDA currently approved by the U.S. Food and Drug Administration, CA19–9, is of limited diagnostenic value and is not recommended for use in screening or in routine assessments of disease recurrence or response to therapy without accompanying imaging studies or biopsies (30). Although many extensive “omic” studies have been performed to identify “early detection” markers, many of these have understandably had to rely on samples collected concurrently with or following PDA diagnosis. Samples collected prior to diagnosis are scarce, and even patients that manifest clinically resectable tumors likely have micrometastatic disease, raising the bar for meaningful early detection. In an effort to focus on very early stages of PDA where intervention could be more effective, we used a highly faithful mouse model of PDA as a platform to interrogate tissues from the earliest onset of preinvasive (PanIN) disease formation to advanced disease stages. Importantly, in the KPC mouse model, faithful recapitulation of disease occurs along the PanIN-to-PDA trajectory in all animals (8). Together with a high-dimensional pancreas-cancer-tailored antibody microarray, we identified and validated candidate tissue markers of early disease stages, determined how their expression changed with histologically defined disease progression, and further assessed the spatiotemporal characteristics of one particularly interesting candidate, serine/threonine stress kinase 4. The characteristics of STK4 expression during disease progression are intriguing and might suggest a potential functional role in pancreas cancer progression. Thus, this integrated, cross-species approach also has promise for providing insight into possible roles of individual candidate markers in disease etiology and pathogenesis.

Of the 71 putative tissue markers discovered in our array experiments, 42 have been previously implicated in one or more studies of pancreas cancer (see supplemental Table S1), confirming the fidelity of our approach. These markers include up-regulation of claudin 18 (31), matrix metalloproteinase 7 (32), and the secreted kallikrein-related peptidase 6 (33, 34), as well as markers previously reported as downregulated, including E-cadherin and the homeobox transcription factor HOXD13 (35). The panel of seven proteins whose increased expression we validated and monitored during PDA progression included some novel (DSC2, RFC4, and STK4) and some previously identified (p19ARF, FN1, BSG, and SMAD2) putative markers. Using the KPC mouse model, we were able to identify some markers (FN1, BSG, and SMAD2) at earlier stages of disease than previously reported. Four proteins (BSG, SMAD2, p19ARF, and RFC4) distinguished preinvasive disease in 2-month-old KPC tissue from normal controls, whereas six of the seven (BSG, SMAD2, p19ARF, RFC4, FN1, and STK4) differentiated 4-month-old KPC tissue from normal. The majority of markers also distinguished invasive PDA (4-month KPC) from CP, with p19ARF and BSG each able to do so at the 2-month disease stage.

The panel of seven proteins described here might therefore warrant further scrutiny in human PDA. Current imaging modalities used for screening asymptomatic individuals cannot differentiate PDA from CP, nor can they identify microscopic PanIN (36–38). Thus, assessing the levels of these candidates early stage disease markers in fine needle biopsies could help to confirm the presence of malignant lesions earlier than conventional tests. Patients at high risk for PDA, in particular, might especially benefit from such evaluations.

Characterization of the marker panel in preinvasive, invasive, and metastatic disease stages revealed that levels of STK4 and BSG increased between the 2-month (preinvasive) and 4-month (invasive without metastasis) KPC cohorts and then plateaued. Whereas BSG has been previously implicated in pancreas cancer progression, promoting secretion of matrix metalloproteinases from supporting stroma (39), STK4 is a novel putative biomarker for pancreas cancer. STK4 (also known as MST1) is a serine/threonine kinase in the Hippo...
signaling pathway that has been implicated in myriad processes (40). In KPC PDA, STK4 expression increased in regions of acinar damage and ADM and then decreased in true PanIN-1/A/B lesions. Increased STK4 was not observed in areas of injury in CP. STK4 levels subsequently increased in PanIN-2 and PanIN-3 lesions in KPC mice and remained elevated in invasive and metastatic disease. Importantly, when we set the cutoff at the lowest levels of STK4 in KPC lysates, STK4 had 100% sensitivity and 80% specificity in distinguishing early invasive murine PDA (4-month time point) from CP.

STK4 is also expressed at higher levels in the epithelia of primary human PDA collected at autopsy than in normal pancreas. In human PDA, STK4 was found in both cytoplasmic and nuclear compartments, although nuclear expression predominated. Nuclear and cytoplasmic STK4 expression was also observed in cancer samples analyzed in the ProteinAtlas project (41, 42), with antibodies distinct from ours and further confirmed by immunofluorescence on human PDA cell lines. The levels and localization both might figure in determining the functional status of this enzyme, as mammalian Hippo kinases promote apoptosis upon nuclear translocation (43), and loss of this function might portend a more aggressive disease.

The role of mammalian Hippo kinases in cancer is not yet well delineated. Although classically thought to exert proapoptotic and antiproliferative effects (40), recent studies suggest that hippo kinases have functions that vary in a tissue-specific and context-dependent manner (44–46). It is also not known whether oncogenic Kras or mutant Tp53 modulates Hippo signaling. Whereas both Kras and Trp53 were always mutated in the mice used here, the underlying genetics in the human samples we examined are unknown. Such differences could also account for the variation in STK4 localization in human and murine PDA. Nevertheless, and perhaps most important, STK4 served as a marker of disease in both murine and human tissue. Further studies are required to determine whether there are potential functional implications of elevated and compartment-specific STK4, particularly the increased levels observed at early stages of disease. Characterization of other Hippo pathway members (for example, yes-associated protein 1) at earlier disease stages could help address whether and at what level Hippo pathway signaling is disrupted in PDA. RNA interference studies in vitro and studies in genetically engineered mouse models incorporating knockout of STK3/4 (45, 46) might also help elucidate the functional implications of STK4 signaling in PDA.

In summary, our results support the concept that the assessment of markers throughout the course of disease progression in faithful genetically engineered mouse models can further inform static studies of resected human tumors. Such analyses could help focus efforts to the most clinically relevant tissue markers and help place candidate markers into their appropriate pathophysiological context. These tissue markers also potentially could be used for disease detection in high-risk patients and, if ultimately shown to be directly related to disease, could perhaps inform clinical decision making.

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