Antibody Microarray Interrogation of Tissue and Plasma for the Improved Early Detection of Pancreas Cancer

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Abstract

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Pancreas cancer, most frequently pancreatic ductal adenocarcinoma (PDA), is the tenth most common cancer but is the fourth leading cause of cancer deaths in the United States, with a five-year survival rate of just 6%. This is due to its asymptomatic nature and resistance to current treatment regimens. The majority of patients present with unresectable disease upon diagnosis of PDA, and, even with chemotherapy, have a median survival of only 6-11.1 months. Those who are diagnosed with resectable disease and undergo surgery, the only current potential curative option, have improved five-year survival rates but will also still eventually succumb to recurrent

metastatic disease. Thus, efforts are being directed towards both developing better early detection strategies and improving treatments of advanced disease. The focus of this doctoral dissertation was to find biomarkers, or measurable biological characteristics, that can be used for early detection of pancreas cancer. I utilized our laboratory's experience in the design and fabrication of antibody microarrays to create a pancreas cancer-specific platform consisting of >4000 unique features to identify protein biomarkers. We have also used a variation on our platform to identify and preliminarily validate markers in pre-diagnostic triple negative breast cancer plasma samples. In efforts to find proteins that could serve as true early detection biomarker of PDA, I took a dual approach, interrogating the primary tumor tissue and plasma proteome of a highly faithful mouse model of pancreas cancer as well as mining human plasma samples collected from a cohort of women before they were diagnosed with and eventually succumbed from PDA. Using this approach, I identified and validated a panel of 7 tissue markers in the mouse model, which were also able to distinguish early stages of pancreas cancer from chronic pancreatitis. This included STK4, a novel biomarker of PDA that is up regulated at preinvasive disease stages and that I show is also up regulated in human PDA. Furthermore, the dynamic expression pattern of STK4 at early disease stages suggests it may functionally contribute to pancreas cancer progression. Plasma biomarkers discovered in the mouse model were cross-referenced with biomarkers identified in pre-diagnostic human plasma samples to further validate and better define their utility as markers for human PDA. These parallel array experiments yielded a panel of 3 proteins, ERBB2, TNC and ESR1, all of which have been previously identified as markers of PDA in samples collected at diagnosis, and that thus warrant further validation in patients at high risk of developing pancreas cancer. Collectively, these antibody microarray experiments identified promising plasma and tissue markers that call for

further follow up as potential diagnostics and could help us gain insight into the molecular processes underlying disease progression.

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CHAPTER 1

INTRODUCTION

The insidious nature of pancreatic ductal adenocarcinoma

Cancers of the pancreas, 90% of which are pancreatic ductal adenocarcinomas (PDA) (1), are the 10th most common cancer type but the 4th leading cause of cancer death in the United States. This is largely due to the dismal 5-year survival rate of ~6% for patients with PDA, the worst survival rate of all solid tumors (Cancer Facts and Figures 2013, American Cancer Society). The poor prognosis that PDA portends occurs because it is effectively asymptomatic at early stages and therefore almost uniformly detected at advanced (i.e. locally unresectable or widely metastatic) disease stages. Screening by continued monitoring of an organ, such as screening for colorectal cancer by colonoscopy, cannot be performed with the pancreas. The location of the pancreas and pathological origins of PDA—terminal ductules are the site where the most common PDA precursor pancreatic intraepithelial neoplasia (PanIN) lesions arise (2)—combined with its low incidence collude to hinder efforts to detect disease at its early stages.

The lack of effective screening and absence of overt symptoms until advanced disease stages results in the majority of patients (>80%) presenting with locally unresectable or metastatic PDA at diagnosis. Frustratingly, PDA remains resistant to current treatment regimens. Until most recently, the standard of care was the deoxycytidine analog gemcitabine, which affords a trifling median survival of ~6 months (3). Data pooled from a number of randomized trials examining a total of 2200 patients showed that just 10% of patients receiving gemcitabine show an objective response (4). In addition, a study analysis of data collected in the

National Cancer Database from 1985-95 saw no significant differences in survival between patients with unresectable tumors receiving chemotherapy, radiation therapy or both and those that did not (5).

Many clinical trials seeking to improve upon these disheartening statistics have been undertaken. These have included combination chemotherapies or gemcitabine treatment in combination with targeted therapies and the pitfalls of these efforts are described in a number of reviews documenting clinical trials and pancreas cancer (4, 6, 7). Thus far, the combination chemotherapy of oxaliplatin, irinotecan, fluorouracil, and leucovorin (FOLFIRINOX) has shown the greatest efficacy, improving median survival to 11.1 months (7, 8). Patients either receive this highly cytotoxic regimen or a combination of nab-paclitaxel and gemcitabine, which affords an improved median survival over gemcitabine alone of 8.5 months (9).

Improving treatment of advanced PDA therefore remains a great challenge in the clinic, and is being tirelessly pursued. As a result, surgical resection remains the only curative option. Yet, of the ~15% of patients that undergo tumor resection following diagnosis, almost all still eventually succumb to invasive or metastatic disease, with 5-year survival rates for these patients across studies in the range of 20% (1, 5, 10, 11). These results bare comparison to the 5-year survival rates in patients with localized disease of other cancers. When looking at statistics between 2003 and 2009 for breast, colon and rectal, lung and bronchial, prostate and ovarian cancers as well as melanoma, for localized disease, 5-year survival rates are all greater than 90% except for lung and bronchial cancers, with ~54% of patients surviving 5 years or more (seer.cancer.gov/statfacts/html, October 2013). As staging of all cancers is based on the TNM classification system developed by the American Joint Committee on Cancer (AJCC), this and

the frequency of metastatic disease recurrence suggest that microscopic PDA lesions (< 2 cm) are already highly malignant and have micro metastatic potential (12).

The decreased death rates for these other cancers have occurred due to both improvements in treatment and in early detection. Testing asymptomatic individuals over 50 annually for the presence of occult blood in feces—a noninvasive test—with resultant colonoscopy or flexible sigmoidoscopy has improved 13-year cumulative mortality from colorectal cancer by 33% (13). Incorporating data regarding screening, adjuvant therapy and death rates from breast cancer from 1975-2000, the overall reductions in death rates due to screening by mammography are estimated to be between 28% and 65% (14). The observed improvements in survival times with resection and the positive impact of negative resection margins on survival times (15) and, conversely, the poor prognoses with larger tumor sizes and positive lymph nodes (16), suggest that more effective early detection strategies will also improve outcomes for PDA.

Screening for PDA remains challenging given its low incidence and location of the pancreas. Pancreatic lesions are currently screened for using the radiographic imaging modalities magnetic resonance cholangiopancreatography (MRCP), positron emission tomography (PET), computed tomography (CT) or a combination of these. However, these screening tools can only detect tumors larger than 0.5 – 1 cm (17). The retroperitoneal location of the pancreas generally limits biopsying until after a pancreatic lesion is suspected. Biopsying by endoscopic ultrasound fine needle aspiration (EUS-FNA) is most common, and while generally safe, it does have associated complications (pancreatitis, bleeding, abdominal pain) in a minority (~3%) of cases (18). From an early detection standpoint, while EUS-FNA can have

sensitivities as high as 93%, it still cannot necessarily differentiate pancreatitis from malignant lesions nor can it properly identify precancerous lesions (17).

A noninvasive test, such as one looking for a marker in body fluids that could complement current imaging modalities, is therefore an ideal alternative for identifying early markers of PDA. Such a test is more difficult to develop and implement than colonoscopy, where diagnosis can occur visually, thus carrying the added benefit of identifying premalignant polyps and leading to their timely removal (7). For prostate cancer, blood-based screening for the carbohydrate antigen PSA has yielded controversial results: it is still debated whether it has truly decreased mortalities or whether it has simply led to overdiagnosis and overtreatment (19). CA19-9, another carbohydrate antigen, is the only current marker for pancreas cancer, discovered using monoclonal antibodies raised against the sera of colorectal patients (20). Like PSA, it unfortunately has little diagnostic value, with elevated levels associated with other cancers and benign diseases (21) and sensitivities and specificities ranging from 70-90% and 68-91%, respectively (22).

However, with the technological advancements over the past two to three decades, we are poised to improve early detection and treatment of PDA. The advent of molecular cloning in the 1980's and 1990's led to the establishment of a genetic and histological progression model for PDA (23) (see below), which has laid a framework in which pancreas cancer can be further studied; for early detection studies, the precursor lesions leading to pancreas cancer provide this setting. Subsequent technological advances in genomics and proteomics have sought to identify mutations, gene expression changes, changes in proteins and processes required for pancreas cancer progression. The progress made here is illustrated by the consolidation of these data into a compendium of biomarkers or signatures of PDA and its precursors (24), serving as a valuable

resource to corroborate results and help contextualize novel findings. In turn, the development of mouse models based on the characterization of PDA have allowed hypotheses to be tested regarding genetic contributions, processes contributing to disease formation and progression and regarding treatment strategies for advanced disease stages (25, 26).

Within this setting, I initiated proteomic studies to further identify biomarkers of PDA that would contribute to improving its early detection. Studies were conducted in parallel using human plasma and plasma serially collected from a highly faithful mouse model of PDA. In addition, further harnessing the power of mouse models, I also sought to identify tissue biomarkers of disease, as further characterization of stages of PDA could provide relevant information regarding resectability of patient tumors as well as help differentiate PDA from other diseases such as chronic pancreatitis.

I utilized a large-format antibody microarray platform with >4000 antibodies some of which were selected based on their previously reported roles as early biomarkers of PDA. Antibody microarrays serve as promising proteomic discovery tools, and the fact that they provide a directed approach using antibodies that can have up to picomolar affinities for their cognate antigens make them ideal platforms for the identification of low abundant plasma biomarkers (27). As discovery tools, antibody microarrays are limited by the number of analytes. To enrich our potential for discovery, our customized platform included over 4000 unique analytes, consisting of both recombinant and full-length antibodies (the latter being the focus of the studies discussed below), and included comprehensive coverage of proteins from all cellular compartments and involved in myriad biological processes.

In these studies, we were fortunate to gain access to pre-diagnostic plasma samples made available by the Women's Health Initiative (WHI). Sample sets from prospective studies are ideal for early detection of PDA as diagnostic plasma, regardless of stage, is generally associated with poor disease outcomes. The utility of biomarkers discovered in diagnostic plasma can also be further assessed and validated in pre-diagnostic sample sets. Thus, pairing our antibody microarray platform with the WHI sample set, I was in a good position to both discover new early plasma protein markers of PDA as well as further validate markers identified by others at earlier stages of disease.

The use of our antibody microarray platform with a highly faithful mouse model allowed me to run parallel studies using plasma representing preinvasive and invasive disease stages, while also looking for (overlapping) markers in the tissue. Thus, I could track tissue and plasma proteins within a practical length of time, from definitively early stages of disease until mice succumbed to invasive or metastatic disease. In addition, novel markers discovered in tissue could also be pursued to provide further insight into initial stages of disease and potential vulnerabilities of PDA.

Collectively, the goal of the mouse to human biomarker studies was to identify putative early detection markers of PDA that could be further contextualized in the mouse. The results of these efforts led to some important discoveries, with future directions discussed. Importantly, our pre-diagnostic plasma interrogation yielded markers identified by others in diagnostic samples. They thus warrant follow up, with the greatest application being to assess their potential as biomarkers in individuals that have a familial history or genetic predispositions that increase their risk of developing pancreas cancer. A panel of markers discovered in the mouse

tissue studies have been further validated and warrant follow up, both from a biological perspective and in human tissue as putative signatures of disease stage.

The results of these studies raise further questions and provide some insight into the complexity of biomarker research, especially in the context of such a heterogeneous disease as pancreas cancer. As more patient samples are analyzed and more hypotheses are tested in mouse models of pancreas cancer, biomarkers of PDA such as those discovered here will gain further utility, with the ultimate goal that they can be effectively applied to earlier diagnosis of this insidious disease. Thus, the advancements described above have poised us to both detect and treat pancreas cancer better, such that we can finally see an increase in survival times and rates for this uniformly fatal disease.

BACKGROUND

The establishment of a genetic and histological progression model for PDA

In a seminal paper in 1990, Fearon and Vogelstein proposed a genetic and histological progression model for colorectal cancer (28). This was based on linking mutations identified in adenomas and colorectal tumors with clinical features, such stage and tumor size. For example, the identification of *KRAS* mutations in ~50% of tumors and adenomas greater than 1cm in size suggested that oncogenic *ras* may serve as an initiating event in a subset of tumors, or serve to expand a smaller adenoma into a larger, more dysplastic one. Similarly, allelic loss of a portion of chromosome 17p, containing the p53 gene, was observed in 75% of carcinomas but not in adenomas, suggesting loss of this gene occurs later in disease progression. These observations led the authors to suggest that at least 4-5 genetic alterations are required for the formation and

of malignant tumors, and a sequence of genetic events, with mutations in the tumor suppressor gene *APC* as the tumor initiating event, were linked to progression from normal colonic epithelium through early, intermediate and late adenomas to carcinoma and metastases (28).

A cancer progression model outlines the major genetic events that underlie the clonal evolution of a tumor and puts these in their biological context. It defines what precancerous events lead to the development of a malignancy, and thereby provide a means for detecting and targeting disease at early stages (2). It differentiates between and provides markers for (genetic, functional, histological) various stages of disease. It is also essential to establish a general consensus with defined nomenclature to contextualize future results.

As the pancreas cannot be biopsied by non-invasive procedures, the establishment of a progression model for PDA was delayed relative to that of colorectal, prostate and breast cancer. Unlike the colorectal cancer progression model, which was developed based on the characterization of tissue taken at all stages of disease progression, the PDA progression model was developed from extensive histological assessment of *resected* pancreas cancer tissue, of adjacent normal tissue and from pancreatic sections from patients who succumbed from other cancers.

One of the earliest comprehensive studies of resected tumor tissue was conducted by Cubilla and Fitzgerald in 1976. They compared the histology of over 300 pancreatic sections from pancreas cancer surgically resected or collected at autopsy and sections from patients without clinical disease. They observed that sections from pancreas cancer patients displayed extensive ductal papillary hyperplasia at much higher frequencies than were seen in normal

pancreatic tissue. This was accompanied by marked cytological atypia and carcinoma in situ, neither of which were evident in normal tissue (29).

In a subsequent study, Pour, Sayed and Sayed histologically assessed 83 pancreata collected at autopsy from military veterans, of which 2 had clinically known pancreas cancer and three exhibited metastatic disease. They noted that alterations were largely confined to the ducts and ductules in the head of the pancreas and uncovered two additional early cancers and seven cases of carcinoma in situ, 3 of which had pancreatic cancer and 4 with cancer in other organs. Importantly, they saw replacement of acinar cells by mucous pseudoductular cells and ductal hyperplasia in 32 (39%) patients. Notably, the most severe forms of hyperplasia, namely ductular hyperplasia, were associated with carcinoma in situ and pancreas cancer, with histology similar to that observed in invasive carcinomas. They concluded that ductular cells appeared to have potential to become cancerous (30).

These studies and those of other groups identified similar increases in hyperplasia in patients with pancreas cancer, yet they were conducted on static tissue representing end-stage disease. Thus, the progressive nature of these lesions had to be inferred. Fortunately, additional reports showed a small number of patients who had undergone partial pancreatectomies and whose resected tissue showed evidence of these ductal lesions, presented with PDA years after their surgeries, thus supporting that these ductal lesions can progress to invasive disease (2, 31).

It was the advent of molecular genetic technologies that helped to firmly establish that the characterized ductular hyperplastic lesions were precursors, or, in other words, clonally progressed to invasive carcinoma. Studies of resected tumor tissue identified high frequencies of mutations in *KRAS*, primarily at codon 12, point mutations in *TP53* with accompanying loss of

heterozygosity (LOH), and inactivating mutations accompanied by LOH or homozygous deletions of *CDKN2A* and *SMAD4* (32-34). As described in the seminal papers outlining the progression model for PDA, subsequent studies showed that the frequencies of these mutations increased along the continuum of increasing cellular and architectural atypia in the ductular lesions described above (2, 23).

Thus, a genetic and histological progression model for PDA, with the most common precursor preceding the development of PDA termed pancreatic intraepithelial neoplasia (PanIN), was developed and accepted in 1999 (Figure 1). By definition, PanIN develop in the terminal ductules of the pancreas, with grading of lesions from PanIN-1 to PanIN-3 based on the degree of nuclear and architectural atypia. This progression is also accompanied by increasing levels of proliferation, from a 0.41% proliferative index in normal ducts to 22% and 37% of PanIN-3 and invasive PDA lesions staining positive for Ki-67, respectively (35). PanIN-1A lesions are characterized by changes from a cuboidal to columnar morphology in the ductal epithelium. PanIN-1B lesions acquire a micropapillary morphology extending into the ductal lumen. Progression to PanIN-2 is defined by an increasing degree of cytological and architectural atypia, and nuclear pleomorphism. PanIN-3, or carcinoma in situ, displays more atypia, complete loss of cell polarity, nucleoli become more prominent with increased mitoses and, in some cases, budding off of ductal structures into the luminal space.

Mutations in *KRAS*, specifically in codon 12, and in some cases at codons 13 and 61, are found in ~90% of all PDA tumors, the highest mutation frequency of any *RAS* gene for any cancer (36-39). Subsequent studies revealed that these *KRAS* mutations occurred in early, low-grade lesions (39, 40), leading to the postulation that oncogenic *KRAS* initiates neoplastic events in the pancreatic ducts. Overexpression of the epidermal growth factor receptor (EGFR) protein

ERBB2 or HER2/NEU, has also been observed in early PanIN lesions, and is ultimately evident in ~90% of PDA cases. Mutations in the tumor suppressor genes *CDKN2A* (95%), *TP53* (75%) and *SMAD4* (55%) have been identified in PanIN-2 and PanIN-3 lesions (2, 33) and are therefore thought to play a role in promoting tumor invasion and metastasis. Loss of *CDKN2A* and *SMAD4* expression is achieved by homozygous deletion of both alleles, inactivation of one allele coupled with loss of heterozygosity (LOH) of the remaining allele or, in the case of *CDKN2A*, promoter hypermethylation. Conversely, mutations in *TP53* manifest exclusively as missense mutations combined with LOH of the remaining allele (41).

This framework set the stage for early detection studies of PDA. It also provided targets for directed therapies, such as farnesyl-transferase inhibitors targeting KRAS, with the potential of targeting even early preinvasive lesions. However, as noted above in the study by Pour, Sayed and Sayed, hyperplastic lesions are also found in the general population. A study by Andea, et al. discovered low grade PanIN-1 lesions in a significant proportion of normal pancreata and even higher grade lesions are present in pancreata from patients with benign diseases such as chronic pancreatitis (42). In addition to improving early detection of such an asymptomatic and aggressive disease, precursor lesions with malignant potential must also be properly identified. This is exceedingly difficult when PanIN lesions are microscopic and initiate in the terminal ductules.

The surge in publications further characterizing PanIN lesions, other precursor lesions and invasive PDA at the genetic and proteomic levels, and beyond to the metabolomic and epigenomic levels, represent the need to more effectively detect PDA and understand at the molecular (and therefore targetable) level what drives its progression. Collectively, these can be thought of as biomarker studies. Our contribution was to identify early detection markers of

PDA that could further help our understanding of early stages of disease with the hope that markers could also be applied in a clinical setting. A background on biomarkers, the challenges of identifying early detection markers for PDA and the current state of research in this field are discussed in the next section.

BIOMARKERS AND PDA

Background on biomarkers

In efforts to further characterize PDA to identify disease at earlier stages and establish more effective treatments to better combat advanced disease, many studies have focused on identifying pancreas cancer biomarkers. By definition, a biomarker is a "characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic endpoint (43, 44)." This definition therefore encompasses many different biological characteristics in myriad contexts, from genetic and epigenetic markers, to changes in protein and metabolite levels, to physiologically measured parameters such as increased blood pressure.

As the utility of biomarkers lies in their ability to differentiate between two biological states (45), it follows that cancer biomarkers seek to distinguish cancerous biological samples—urine, feces, plasma, serum, ascites, sputum, tissue samples—from undiseased samples. Cancer biomarkers can further be subdivided into three classes: diagnostic, prognostic and predictive (44). Examples of each type of biomarker respectively include measuring prostate-specific antigen (PSA) levels to diagnose prostate cancer, a 21-gene signature used to assess probability of distance recurrence in patients with estrogen-receptor and lymph node positive breast cancer

receiving tamoxifen therapy (46), and determining the mutational status of *BRAF* to predict response to the drug vemurafenib in patients with metastatic melanoma (47).

For pancreas cancer, just as the low incidence of disease, its resistance to current treatments of disease, its asymptomatic nature and the location of the pancreas conspire to make it virtually 100% fatal, these characteristics have also hindered efforts for identifying biomarkers, especially early detection biomarkers. This has not stopped a vast number of researchers from interrogating various 'omes,' including the genome, proteome, metabolome and miRNA-ome of pancreas cancer cell lines, cyst and ascites fluid, urine, plasma and sera, and resected tumor tissue (see gene of interest in pancreaticcancerdatabased.org).

The compendia of pancreas cancer markers organized by Harsha et al., with a total of 2,516 differentially expressed genes from 2,325 papers, serves as a good resource to cross-reference markers discovered by different groups using different methodologies (24). Given that none of these markers, including the 1,094 markers identified in precursor lesions, have been effectively translated to the clinic to improve survival rates, it also serves as a sobering reminder of the complexity of pancreas cancer biomarker research, of the lack of translation of discovered markers across various technological platforms, and of the additional research needed in this field.

The genetic complexity of PDA, which is exemplified by a recent study where deep sequencing was conducted on 24 resected PDA tumors, provides one reason why biomarkers have not yet translated well to the clinic. In cataloguing mutations, deletions, and amplifications, and pairing them with gene expression analysis, the authors identified a set of 12 core pathways that are genetically altered in PDA. Yet the average tumor had 63 genetic alterations, and, when

comparing the genomic landscape between individual tumors, the specific genes altered were generally different across tumors, encouraging therapeutics that target processes rather than individual genes (48). Furthermore, the complexity of the genomic landscape persists with clonal evolution of metastases. In a study of metastases in ten patients, rearrangements were found in all metastatic lesions, in some but all metastases or unique to individual metastases, suggestive of clonal evolution and genetic heterogeneity of cells with metastatic capability (49).

To help elucidate the potential application of some of the markers listed in the pancreas cancer compendium, data needs to be integrated to further contextualize putative markers. Such integration is harder with the low incidence of PDA but could lead to better treatment responses in subsets of patients. For example, if patients enrolled in a clinical trial with a particular tumor mutation signature respond favorably to treatment X, then these mutations may serve as predictive biomarkers. A recent study of tumor tissue from autopsy patients showed a negative correlation between the number of driver mutations in a tumor and overall survival: if mutations in *KRAS*, *CDKN2A*, *DPC4* and *TP53* were all present versus mutations in just one or two them, then overall survival was lower and metastatic burden post-resection was increased (50). Thus, cataloguing these mutations in resected tumors could serve as a set of prognostic markers, which in turn would help make decisions regarding post-operative therapy.

The relevance of putative early detection markers cannot be assessed as well in autopsy patients or in resected tumor tissue as these patients, representing a paltry 15% of all PDA patients (5), will still eventually succumb to disease. Retrospective studies do help identify markers that could be useful as 'stagers' of disease or as markers regarding whether a suspicious lesion should be resected—*GNAS* mutations as markers of more aggressive cystic precursor lesions termed intraductal papillary mucinous neoplasms (IPMN) that progress to PDA is one

example (51)—but they cannot determine the utility of a marker in preventing or prolonging disease. These markers need to be applied up front in conjunction with current screening methods such as the CA19-9 radioimmunoassay or with imaging modalities. A marker of T1N0M0 disease, which does afford improved survival times, would be useful. It is therefore of especial importance that different groups using multiple platforms identify the same biomarkers, and that these markers become evident at early (i.e. PanIN or IPMN), and also remain across all, disease stages.

Biomarkers, early detection and PDA

As >90% of all PDA tumors harbor activating mutations in *KRAS* (32, 48), detection of mutant KRAS in plasma or other non-invasive samples would serve as a logical early detection test. However, no antibodies have been developed to effectively distinguish mutant from WT forms of this protein (52). Furthermore, while efforts have been ongoing to detect *KRAS* in biological fluids, this requires sensitivities that current sequencing technologies have not yet achieved, resulting in about a 50% success rate of detecting mutant *KRAS* in the plasma or serum of patients with PDA containing this mutation (53).

The only current FDA-approved biomarker for PDA is the carbohydrate antigen CA19-9. First discovered in antigen binding inhibition studies using serum from colorectal and pancreas cancer patients (20), a radioimmunoassay is performed on patients with pancreatic cancer to assay levels of this carbohydrate antigen. CA19-9 is expressed at low levels (< 40 U/ml) and then becomes elevated in the sera of some PDA patients. Yet, it has limited diagnostic value since it is also elevated in the sera of patients with cholangiocarcinoma, colorectal cancer, and hebatobiliary disease, and in benign conditions including pancreatitis, biliary obstruction,

cirrhosis and thyroiditis (21, 54, 55). In addition, patients with Lewis-null blood types fail to produce CA19-9 such that this blood-based test has no utility in about 5-10% of the general population (44, 54). With a positive predictive value of 0.9% in asymptomatic individuals (55), it is not surprising that there are large-scale efforts to discover better diagnostics for PDA.

Thus, in further characterizing and seeking new and more clinically relevant biomarkers of disease, researchers have focused on identification of early markers in plasma, ascites, pancreatic juices, urine and resected tumor tissue. This has led to an extensive characterization of PDA at the genomic, proteomic, glycomic and metabolomic level. Notably, some biomarkers shown to be elevated in resected tumor tissue have corresponding increases in plasma or sera. Studies by various research groups suggest that MUC1, and variations on its glyocsylated forms, is a promising early detection biomarker. Shown to be elevated in the sera of pancreas cancer patients, specific glyocsylated forms of MUC1 appear unique to PDA, they arise early in progression of precursor pancreatic intraepithelial neoplasia lesions (at PanIN-1 stages) and remain elevated throughout disease progression (56). More recent evidence suggests that MUC1 also has prognostic significance, further implicating it as a marker of disease progression (16). Adding to the complexity is the apparent changes in MUC1 glycosylation patterns with PDA progression (57).

As the lengthy list in Harsha, et al.'s compendium shows, the list of biomarkers is extensive and continues to grow. Similar to MUC1, survivin transcript and protein levels increase with advancement from PanIN-1 lesions to PDA with a shift to predominantly nuclear expression in high-grade lesions (58). Additional early detection markers include Plectin-1, showing increasing expression from early PanIN to invasive PDA, with only 0-3.85% of PanIN-1/2 lesions expressing versus 60% of PanIN-3 lesions expressing this cytolinker protein (59).

The authors additionally showed increased Plectin-1 in metastatic deposits and that using Plectin-1-targeted peptides as contrast agents served to identify primary tumors and metastases by computed tomography (CT) in orthotopic mouse models of PDA. Neutrophil gelatinase-associated lipocalin (NGAL) is another putative early detection marker of PDA, with elevated expression seen in PanIN-1 lesions, in moderately, but not poorly differentiated, PDA relative to levels in chronic pancreatitis, and increased levels also observed in the sera of PDA patients (60).

Periostin (POSTN) was also shown to be elevated at the mRNA level in PDA as well as staining tissue and showing elevated sera levels (61). Other markers include claudin 18 (at both the mRNA and protein level) (62, 63), which differentiates PDA from normal and reactive pancreatic ducts, the blood-based markers TIMP1, CEA, OPG and ICAM-1, identified using multiplex bead-based assays (64). Stable isotope labeling of amino acinds (SILAC) of pancreatic cancer cells followed by LC-MS/MS on conditioned media (the secretome) was led to the discovery of the novel PDA biomarkers CD9, perlecan and fibronectin receptor (65), although their expression was not specifically corroborated in precursor PanIN lesions.

While biomarker discovery has been successful in further characterization of PDA, placing the myriad biomarkers discovered into their biological and clinical context (i.e. validation and implementation) remains a great challenge. Given the high rate of benign pancreatic cysts in the general population (66), identification of GNAS mutations differentiating malignant from benign cysts in cyst fluid shows promise as an important diagnostic and prognostic biomarker for malignant precursor intraductal papillary mucinous cystic neoplasms (51). Many prognostic biomarkers such as MUC1 and MSLN, shown to contribute to clinical parameters predicting survival following surgical resection, also warrant further follow up; with

improvements in endoscopic ultrasound and other imaging modalities as well screening of high-risk patient cohorts, these studies seem achievable with larger patient numbers.

Validation and clinical utilization of early detection markers is perhaps most difficult. Histological assessment in a large cohort of patients revealed the presence of precursor PanIN-1 lesions, and the presence of PanIN-2 lesions (20%) and PanIN-3 lesions at a low frequency (4%), in the general population and in patients with CP, respectively (42). While the authors also showed a progressive increase in PanIN frequency from normal pancreata (28%) to chronic pancreatitis (63%) and ductal adenocarcinoma (82%), these data illustrate that "benign" or indolent PanIN lesions need to also be differentiated from malignant lesions, further complicating the biomarker discovery process. Furthermore, markers of early lesions in resected tumor tissue only represent 10-15% of the population of patients with PDA, and therefore may not be markers applied to all patients. Similarly, as samples collected at diagnosis (tumor, plasma, etc.) generally represent clinically advanced disease generally resistant to treatment, markers using such samples may not represent those that could be applied as early diagnostics to the general population of asymptomatic individuals. Finally, as pancreas cancer only occurs in ~40,000 people each year, thus representing the tenth most common cancer in the US, applying biomarkers that achieve the same sensitivities and specificities as in small, often biased patient cohorts (for example, patients enrolling in particular population screening studies are often healthier than the general population) to the general population, will also be a challenge. There is approximately a 1 in 10,000 chance that a patient in the general population will have pancreas cancer. As a result, with a pretest probability of 0.0001, the chance that a marker with 90% sensitivity and specificity will identify PDA in the general population is 0.0089% (7). In

examining a cohort of more than 20,000 asymptomatic patients, Kim et al. similarly found that CA19-9 had positive and negative predictive values both less than 1% (67).

The focus of our research was to also to identify biomarkers of PDA, thereby contributing to the enrichment of and further validating some of the current compendium of biomarkers. In order in an attempt to more effectively identify early detection markers of disease, and to potentially further characterize PDA etiology and progression, I took advantage of human plasma samples collected in prospective studies (pre-diagnostic biomarker identification) and available genetically engineered mouse models of PDA (early detection and characterization of progression from PanIN to PDA with 100% latency). Using a multiplex proteomic assay, I interrogated both sample sets, which included both plasma and tissue collected from a highly faithful mouse model of PDA. Before discussing the details and results of our approach, background on proteomic technologies and mouse models of pancreas cancer will be briefly discussed.

APPROACHES TO PROTEOMIC BIOMARKER RESEARCH

While biomarkers encompass anything measurable in a biological sample, thus including genomic expression and epigenomic analyses, RNA sequencing and miRNA quantification as well as DNA sequencing, since our studies focused on measuring the changes at the protein and posttranslational level, the focus of this section will be on introducing and describing various proteomic technologies.

Srivastava and Srivastava note that the word proteomics derives from the Greek word "'proteus,' meaning an ancient Greek god… a symbol of the original matter from which we are

created (43)." Thus, proteomics encompasses the study of life at the functional level, and, implicit in its definition, does so by studying the global changes in the levels of proteins. Proteomic technologies can be subdivided into quantitative two-dimensional gel electrophoresis (2DE) and mass spectrometry-based approaches, and semi-quantitative immuno-based approaches such as bead-based immuno-assays and antibody microarrays.

As the older 2DE technology, which identifies proteins based on their mass (MW) and ionic (isoelectric point) properties (68), exhibits a poor dynamic range, most current proteomic studies pair protein fractionation methodologies with shotgun mass spectrometry-based approaches, or utilize multiplex immuno- or peptide-based approaches (for identifying auto-antibodies, for example). Various fractionation technologies include 2DE, liquid chromatography (LC) or reverse phase LC (RP LC), strong cation exchange (SCX)(68) as well as the more recently published 3D IPAS (separation based on charge, hydrophobicity and mass) (69) .

Mass spectrometry-based approaches provide unbiased, global measurements of proteins in biological samples. Generally, a shotgun or bottom up, approach is employed whereby proteins in a sample are fragmented into their peptides, volatized and mass to charge ratios (m/z) and intensities (spectral counts) determined for unique peptides. This is followed by the reconstruction and identification of proteins by comparing individual peaks in each mass spectrometry run (m/z ratios within a given MW range) to a database of peptides and their masses (70). An IPAS mass spectrometry-based approach was employed using serum from a genetically engineered mouse model (GEMM) of PDA incorporating conditional endogenous heterozygous expression of mutant *Kras* and loss of the *Ink4a/p19Arf* locus to identify early detection biomarkers of disease. In turn, some of these markers were identified in the sera and

resected tumor tissue of PDA patients (71). A more recent LC-MS/MS analysis of laser-captured microdissected PanIN and IPMN lesions in GEMM's of PDA also identified mature serum albumin (ALB1) and thymosin β-4 (TMSB4X) as early lesion markers that were also observed in human PDA tumor tissue (72). These studies not only highlight the utility of mass spectrometry-based proteomic approaches for PDA biomarker discovery but also provide evidence that proteomic studies in mouse models are translatable to the human condition.

Koomen et al. do note some limitations to mass spectrometry-based interrogation of plasma. The use of tryptic digestion in tandem MS/MS eliminates information about the sequences of proteins or peptides occurring naturally in plasma, for example. Furthermore, in proof of principle LC-MALDI-MS/MS experiments, they were unable to identify low abundant proteins but identified almost 100 cleavage products from fibrinogen (73). This highlights the importance of fractionation experiments to increase the dynamic range of protein identification that is not otherwise inherent using MS technologies.

Complementary immuno-based approaches including multiplex luminex beads and antibody microarray technologies provide targeted assessment of the levels of proteins in biological samples. While stable isotope labeling can yield quantitative measurements using MS technologies, antibody microarray platforms allow semi-quantitative comparisons of the levels of proteins between two biological samples. Printing of antibodies on microarray slides enables accurate characterization of the identities of proteins (for example, phosphorylation-specific antibodies) using small sample volumes (74). For plasma biomarker studies, no fractionation experiments affecting protein solubilities need be performed and the high affinity of antibodies for their cognate antigens still allow detection of low abundance proteins (27).

A study using 129 single-chain antibody variable region fragments (scFv) specific for 60 cytokines and other proteins involved in immune regulation identified a signature of 29 analytes that was able to differentiate sera from PDA patients with short-term (<12 months) survival times from those with longer (>24 months) survival times (75). A more recent study by the same group using a similar recombinant antibody platform with 121 unique features targeting 57 proteins identified a 25-protein signature diagnostic signature for PDA with and AUC of 0.88, although the sensitivities and specificities of 73% and 75%, respectively, mean that this signature could not be effectively applied as screening test (76). The above-mentioned study that identified the marker panels TIMP1, ICAM1 and OPG, and CEA, TIMP-1 and CA19-9 as promising early detection markers that improved upon the diagnostic utility of CA19-9 alone, used antibodies conjugated to luminex beads (64). Antibody array analysis of urine from a small cohort PDA patients targeting 60 different proteins showed that 2 proteins and 17 proteins, respectively, differentiated female and male PDA patients from their same sex healthy controls (77).

I used a self-fabricated antibody microarray platform as I interrogated both plasma and tissue samples for biomarker discovery. While it has little diagnostic capability, CA19-9 was discovered by an immune-based approach, and it is generally thought that mass spectrometry-based technologies would not have been able to discover this serum antigen due to their lower dynamic range. An antibody microarray approach was also logical given our previous experience using arrays to discover ovarian and breast cancer serum plasma biomarkers, our access to a robotic printer and the myriad antibodies that we have accrued through the successive iterations of array platforms.

My initial experience with our array platform came with contributing to prior studies conducted on pre-diagnostic triple negative breast cancer plasma samples. In these, I contributed to the array printing procedure, preparing plasma samples for interrogation on our arrays, and became familiar with elements of the array incubation protocol, thus providing essential background on the array protocol and preparing me for any pitfalls in subsequent array studies. The results of this breast cancer microarray study were published (78) and are included in Chapter 2.

In turn, I applied this experience towards leading the pancreas cancer antibody array studies, the main focus of my dissertation. For these studies, I selected additional antibodies to create an array platform customized for PDA biomarker discovery (discussed below). This platform was additionally of much larger scale than the other platforms used in the other PDA array studies described above. As one of the caveats of all antibody-based platforms is cross-reactivity (79), I conducted immunoblotting and immunohistochemistry validation experiments on candidates from our tissue array studies and additional array experiments on independent plasma samples to further validate candidates identified in our pre-diagnostic plasma biomarker studies. In chapter 3, the mouse models of PDA and their current contributions to our understanding of pancreas cancer are introduced, and the model that I employed in these studies is highlighted. This then segues into the array studies I conducted on pancreatic tissue using this highly faithful mouse model of PDA to discover and validate putative early detection tissue markers of pancreas cancer.

CHAPTER 1 FIGURE

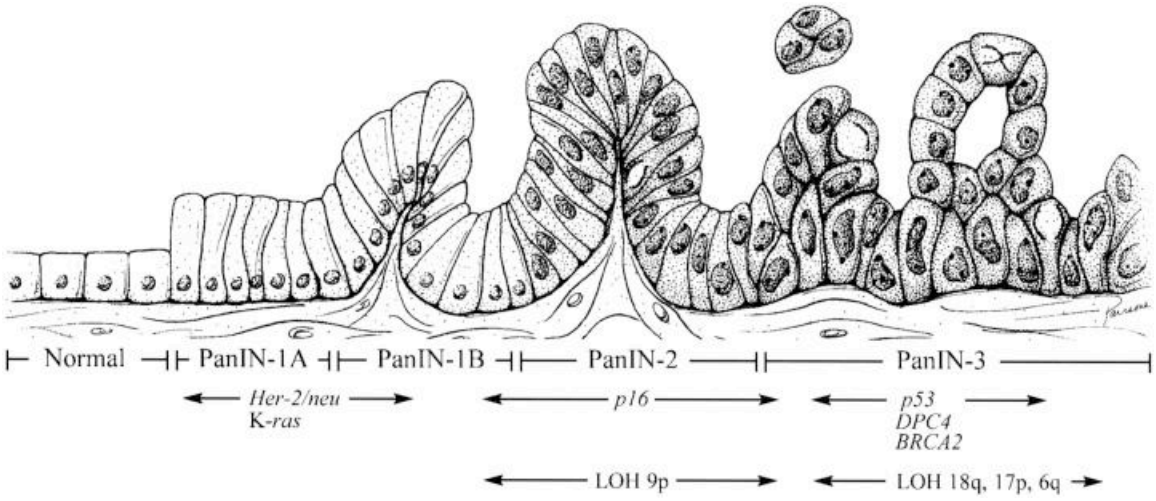

Figure 1. A histological and genetic progression model for pancreatic ductal adenocarcinoma (PDA). A series of precursor lesions, termed pancreatic intraepithelial neoplasms (PanIN), originate in the terminal ductules and progress to PDA. Increasing cytological and nuclear atypia is observed with progression from PanIN-1 to PanIN-3, or carcinoma in situ. Careful mutational analysis of hyperplastic ducts associated with PDA identified underlying mutations with high frequencies in the proto-oncogene *KRAS*, the tumor suppressor genes *CDKN2A* (*p16*), *TP53* and *DPC4* and the mismatch repair gene *BRCA2* as well as over expression of the receptor tyrosine kinase HER/Neu (ERBB2). The identification of these mutations at various stages of nuclear and cytological atypia (i.e. PanIN) helped confirm that precursor lesions clonally expand and precede invasive PDA. For example, activating *KRAS* mutations, found in >90% of PDA tumors, can be identified as early as PanIN-1 lesions, and are thus thought to be the initiating event for PDA. This was later confirmed in a GEMM model developed based on the PDA progression model (12). Image taken from (2).

CHAPTER 2

Discovery and preliminary confirmation of novel early detection biomarkers for triple-negative breast cancer using preclinical plasma samples from the Women's Health Initiative observational study

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ABSTRACT

Triple-negative breast cancer is a particularly aggressive and lethal breast cancer subtype that is more likely to be interval-detected rather than screen-detected. The purpose of this study is to discover and initially validate novel early detection biomarkers for triple-negative breast cancer using preclinical samples. Plasma samples collected up to 17 months prior to diagnosis from 28 triple-negative cases and 28 matched controls from the Women's Health Initiative Observational Study were equally divided into a training set and a test set and interrogated using a customized antibody array. Data were available on 889 antibodies, and in the training set statistically significant differences in case vs. control signals were observed for 93 (10.5%) antibodies at $p<0.05$. Of these 93 candidates, 29 were confirmed in the test set at $p<0.05$. Areas under the curve for these candidates ranged from 0.58 to 0.79. With specificity set at 98%, sensitivity ranged from 4% to 68% with 20 candidates having a sensitivity ≥20% and 6 having a sensitivity ≥40%. In an analysis of KEGG gene sets, the pyrimidine metabolism gene set was upregulated in cases compared to controls ($p=0.004$ in the testing set) and the JAK/Stat signaling pathway gene set was downregulated ($p=0.003$ in the testing set). Numerous potential early detection biomarkers specific to triple-negative breast cancer in multiple pathways were identified. Further research is required to follow-up on promising candidates in larger sample sizes and to better understand their potential biological importance as our understanding of the etiology of triple-negative breast cancer continues to grow.

INTRODUCTION

Annual or biennial mammography is effective at detecting breast cancer early and has been shown in multiple randomized trials to reduce mortality rates (80). However, its effectiveness varies by breast cancer subtype. With respect to hormone receptor status, it has been shown that interval-detected cancers are 1.8 to 2.6-fold more likely to be estrogen receptor (ER) negative compared to screen-detected tumors (81, 82). Improving the early detection of ER- cancers is of great clinical importance because these tumors are more likely to present at an advanced stage, and a higher stage carries a higher risk of breast cancer mortality (83).

One approach to developing new tools for detecting cancer early is through the identification and validation of blood based cancer specific biomarkers. In applying this approach to breast cancer, one potential challenge is its considerable heterogeneity. The characterization of distinct molecular subtypes of breast cancer based on patterns of gene expression has shifted how we approach this complex disease (84, 85). The unique molecular signatures of the different subtypes suggest that they likely have unique etiologies, and a growing number of studies indicate that several well-established breast cancer risk factors differ markedly in their associations with the various molecular subtypes (86-92). The most common subtypes are ER+ (comprising the luminal A and luminal B subtypes), while one of the most aggressive and difficult to treat subtypes is triple-negative (TN) breast cancer. These tumors lack ER, progesterone receptor (PR), and HER2-neu (HER2) expression and the majority of them have the so-called basal-like phenotype (93, 94). Beyond their molecular differences, this subtyping is also of considerable clinical relevance given the differences in survival rates of luminal A and TN cancers: while luminal A tumors have a ~90% 5-year survival rate, the reported 5-year survival rate for TN breast cancers ranges from 35-80% (93, 95-97). Thus, given

the molecular, clinical and epidemiological differences from ER+ cancers, one might reasonably hypothesize that there may be unique early detection biomarkers specific to TN breast cancer, and that biomarkers for this subtype may be more readily discovered given the highly aggressive nature of these tumors. One challenge to the discovery of useful biomarkers for TN disease is the procurement of sufficient samples collected prior to disease diagnosis. Large cohort studies that have collected biospecimens and have good follow-up are excellent potential sources.

The purpose of this study was to discover and initially validate novel biomarkers for the early detection of TN breast cancer using a novel high-density antibody array and plasma samples collected prior to diagnosis among women enrolled in the Women's Health Initiative (WHI) observational study. The antibody microarray contains approximately 1000 antibodies to many important signaling proteins important in inflammatory, immune response, proliferation, and insulin signaling pathways. Content includes many cytokines, adipokines and other growth factors, and is enriched for antibodies to secreted and/or membrane proteins. This includes proteins in pathways known to be deregulated in breast cancer including those involved in apoptosis, angiogenesis, T-cell activation/infiltration, inflammation/prostaglandins, insulin, and insulin resistance signaling. Since antibodies are nature's best affinity capture reagents, they are perfectly suited for characterizing complex proteomes such as human plasma due to their high affinity and specificity and when used in a high dimensional format can give a rather comprehensive view of the plasma proteome. We have previously shown that this approach has excellent concordance with ELISA assays for specific proteins, and has yielded new biomarkers of ovarian cancer that have been confirmed by alternate methods (98-101).

MATERIALS AND METHODS

Study design:

We conducted a nested case-control study of breast cancer within the Women's Health Initiative (WHI) Observational Study (OS), a prospective cohort of 93,676 post-menopausal women enrolled from 1993 to 1998 in the United States. Detailed descriptions of the design and methods of the WHI OS have been previously published (102, 103). Our nested case-control study included 28 ER-/PR-/HER2- breast cancer cases and 28 controls without a prior history of any type of cancer, individually matched 1:1 to cases on age at enrollment (±1 year), race/ethnicity (white, black, Hispanic, Asian/Pacific Islander, or other), blood draw date (±1 year), and clinical center of enrollment. Cases were included in this study if they had an available study blood specimen drawn within 17 months prior to their breast cancer diagnosis. Information on ER, PR, and HER2 status was obtained from medical records and centrally adjudicated by WHI staff. The 28 matched sets were divided equally and randomly into a training set, used for discovery, and an independent testing set, used for confirmation.

Laboratory methods:

These preclinical samples were evaluated on a customized antibody array populated with 977 full length antibodies to many secreted, integral membrane, cytoplasmic and nuclear proteins involved in a diverse array of signaling pathways. Detailed descriptions of our protocols for array fabrication, plasma treatment, plasma labeling, incubation of plasma with arrays, array scanning and statistical analyses have been previously reported (98, 100). Triplicate features of each antibody were printed on Nexterion Slide H hydrogel-coated glass slides (Schott, Elmsford, NY) using a Genetix Q-array 2 microarray printer (San Jose, CA) and blocked with 0.3%

ethanolamine, 0.05 M sodium borate pH 8.0. Albumin and IgG were depleted from 100 µl plasma using a ProteoPrep Immunoaffinity Albumin and IgG Depletion Kit (Sigma Chemical, St. Louis, MO) per the manufacturer's directions. The depleted plasma was concentrated to its original volume using Amicon Ultra 10k MWCO centrifugal filters (Millipore, Billerica, MA), measured for total protein concentration by BCA assay (Pierce Biotechnology, Rockford, IL), and labeled with the amine reactive dyes Cy3- and Cy5-maleimide (GE Amersham, Piscataway, NJ) according to the manufacturer's instructions. Unincorporated dye was removed using Amicon Ultra 10k MWCO centrifugal filters. For this study, 500 µg case and control plasma were labeled with Cy5, and separately incubated for 90 minutes with Cy3-labeled reference plasma (a common pool of plasma comprised of samples collected from 7 women aged 45-72 years was used as a reference for all samples) in approximately 100 µl total volume (kept from drying using LifterSlips, Fisher Scientific, Pittsburgh, PA). After washing slides were scanned in a GenePix 4000B microarray scanner and data extracted using GenePix Pro 6.0 software (Molecular Devices, Sunnyvale, CA).

Statistical analysis

For each antibody, fold change of signal (red channel) compared to reference (green channel), the M value, was calculated as $\log2(R_c/G_c)$; where R_c is red corrected and G_c is green corrected (using the normexp background correction method developed by Smyth) (104). Technical sources of variation were normalized using loess procedures developed for microarrays, including within-array print-tip loess and between-arrays quartile normalization. Following normalization, triplicate features were summarized using their median. M-values were further normalized using linear regression to remove the systematic bias due to

experimental factors such as printing and hybridization day. After this normalization data were available on a total of 889 antibodies. All statistical analyses were conducted on M values, and analyses of the training set data and testing set data were performed independently.

Values were standardized such that the mean value and standard deviation of the cancer free control group were set to zero and one, respectively. Multivariate linear regression was used to compute log2 odds ratios (OR), p-values, and 95% confidence intervals (CI) for case versus control comparisons. All ORs were adjusted for age, race/ethnicity, body mass index, menopausal hormone therapy use, and array hybridization day. We also calculated the area under the curve (AUC) and the sensitivity at 98% specificity as metrics of the extent to which individual markers could discriminate between cases and controls. AUC estimates were two-sided, such that an AUC>0.5 indicates that the marker is higher in cases compared to controls and an AUC<0.5 indicates that it is lower among cases compared to controls.

We conducted Gene Set Enrichment Analyses (GSEA) based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) gene sets that are available from the Molecular Signatures Database (MSigDB) (http://www.broadinstitute.org/gsea/msigdb/index.jsp). The 889 antibodies available for analysis correspond to 732 unique genes. Of the 186 KEGG gene sets available from MSigDB, our arrays included at least 5 proteins corresponding to gene members in 91 of these gene sets. With respect to the GO gene sets, of the 1454 available GO gene sets, our arrays included at least 5 gene members in 535 of these gene sets. We then tested if the proteins corresponding to groups of genes in a given gene set had a higher statistical ranking than the proteins not in this gene set based on Wilcoxon testing in the training set. Gene sets that were statistically significantly

different in cases compared to controls were then evaluated in the testing set using the same approach.

RESULTS

The cases and controls in our training and test sets were generally balanced with respect to age and race/ethnicity (Table 1). Somewhat higher proportions of cases compared to controls were overweight or obese (body mass index ≥25.0 kg/m^2) in both the training and testing sets. The same proportion of controls and cases in both training and testing sets were not currently using menopausal hormone therapy, though there was some variation in the proportions using unopposed estrogen versus combined estrogen and progestin regimens.

In the training set, of the 889 antibodies assessed, 93 (10.5%) showed statistically significant differences in signal between cases and controls at $p<0.05$; in the testing set, 146 (16.4%) were statistically different. Of the 93 candidates identified in the training set, 29 were validated in the test set at $p<0.05$ (Table 2). Twenty-eight of these 29 candidates were higher in cases compared to controls and the AUCs for these individual candidates ranged from 0.58 to 0.79. With sensitivity set at 98%, across these 29 candidates specificity ranged from 4% to 68%. As a comparison, a prostate specific antigen (PSA) value of ≥4.1 has an estimated 20.5% sensitivity and 93.8% specificity for detecting prostate cancer (105). Here, 20 of the 29 candidates had a sensitivity of ≥20% and 6 had a sensitivity of ≥40% including NADH dehydrogenase 1a subcomplex 10 (NDUFA10, 68%), protein-tyrosine phosphatase, mitochondrial 1 (PTPMT1, 52%), integrin beta-1 (ITGB1, 48%), mast/stem cell growth factor receptor (KIT, 46%), DNA-directed RNA polymerase (POLR2L, 43%), and ephrin-A5 (EFNA5, 41%), again with specificity set at 98%. We also applied a more stringent multiple testing correction procedure to

our analysis. Ten candidates from the training set had a p-value of <0.01 and independently five of these ten discriminated TN cases from controls at a nominal p<0.005 in the testing set (DUSP9, EED, EFNA5, ITGB1, and PTPMT1), so that these five have a Bonferroni-corrected p-value of <0.05.

An additional eleven candidates had a p-value of <0.1 in the training set and a p-value of <0.05 in the test set, and 12 had a p-value of <0.05 in the training set and a p-value of <0.1 in the test set (Table 3). Among these twenty-three candidates, 2 had sensitivities of ≥40% at 98% specificity, toll-like receptor 2 (TLR2, 52%) and anoctamin 1 (ANO1, 43%).

For our gene set analysis, a total of 91 KEGG gene sets met our inclusion criteria of ≥40% sensitivity at 98% specificity. Of these gene sets, seven had a p-value <0.05 in our training set, of which 2 had p-values <0.05 in our testing set (Table 4). These were pyrimidine metabolism, which was upregulated in cases compared to controls (p-value=0.0004 in the training set and p-value=0.004 in the testing set) and the JAK/Stat signaling pathway, which was downregulated in cases compared to controls (p-value=0.03 in the training set and p-value=0.003 in the testing set). A total of 535 GO gene sets met our inclusion criteria, 62 of which had a p-value <0.05 in our training set. Of these sixty-two, 17 had a p-value <0.05 in the testing set (Table 4). These included several gene sets with overlapping membership related to cellular and RNA metabolism, regulation of DNA transcription, and interleukin activity.

DISCUSSION

In this biomarker discovery and preliminary confirmation study utilizing preclinical plasma samples, we identified several novel putative early detection biomarkers for triple-negative breast cancer. Interpreting the results of a study such as this is challenging given that

our understanding of the molecular characteristics of TN breast cancer is evolving (106). Numerous functional groups of genes and proteins have been identified to be overexpressed in TN breast cancer including activators of signaling pathways that are deregulated in cancer, cell growth related genes involved in proliferation and cell cycle control, tyrosine kinase receptors that participate in transcription and signal transduction, as well as extracellular matrix receptors and other genes involved in the structure and anchoring of basal epithelial cells (107). Consequently there are multiple individual and groups of plasma proteins that could feasibly differentiate TN breast cancer cases and cancer-free controls preclinically. As might be expected for biomarkers released into the plasma, we observed that a higher percentage of significantly changed proteins had at least some portion exposed extracellularly compared to the list of antibodies as a whole (46% vs. 37%). Of the 29 proteins with $p<0.05$ in both the training and test sets, seven are membrane proteins with extracellular protein domains, four are secreted, and three are involved in protein export to the plasma membrane (assignments based on Ingenuity Pathway Analysis).

In order to evaluate sets or families of proteins that were changed in cases compared to controls, we performed gene set enrichment analyses (GSEA). Based on this approach, cytokine signaling, and specifically the JAK-STAT signaling pathway, were of interest. With respect to individual cytokines, CCL27 and CCL28 were present at higher levels among cases compared to controls with reasonable AUC values (0.76 and 0.67, respectively) and sensitivities at the designated 98% specificity cut-off (25% and 29%, respectively). Among the other individual biomarkers identified, ephrin A5 (41% sensitivity) has previously been observed in serum (108) and has been described as a potential cancer therapeutic target (109). SRP54 (41% sensitivity) is also found in plasma (110) and has been shown to be upregulated in breast cancer

(http://www.itb.cnr.it/breastcancer/). FAS was observed with two different antibodies (one performing with 36% sensitivity and the other with 25% sensitivity), and FAS/FAS-ligand expression are significant predictors of skeletal spread in primary breast cancers (111, 112). Cleaved extracellular domains of integral membrane proteins could also be compelling biomarkers. Integrin β1 and integrin β-like 1 proteins (48% and 29% sensitivity at 98% specificity) are interesting candidates due to the role of the β-1 integrins in cell growth control and breast tumor induction (113). Finally, several statistically significant nuclear and cytoplasmic proteins were observed in the training and test sets. Most of these are known to be expressed in breast tissue and several have been shown to be overexpressed in cancer including DUSP9, TSPO, BRCA1, HEXIM1, POLR2L, UPP1, XBP1, and RNF113A. Many previous biomarker studies have also observed cytoplasmic and nuclear proteins in blood (110) and suggest higher proliferation/apoptosis/necrosis of tumor tissue may be the cause. Not yet mentioned are some of the top ranked candidates based on sensitivity at 98% specificity, including NDUFA10 (68% sensitivity), PTPMT1 (52%), and KIT (46%). Of these three, KIT has the highest direct relevance to cancer as it regulates cell survival and proliferation. NDUFA10 has both dehydrogenase and oxidoreductase activity and is involved in electron transfer from NADPH to the respiratory chain. PTPM1 is a protein phosphatase that is important for ATP production.

A limitation of the laboratory approach used is that we were only able to evaluate biomarkers for which there were antibodies included on the array. Thus, we did not perform a fully comprehensive assessment of the plasma proteome so the potential of biomarkers not included on the array could not be assessed. This in particular limited our gene set analyses as the gene sets we could assess were limited by the candidates included on the array. The array

itself also has certain limitations. Although the array yielded data on 889 putative proteins, it uses only a single antibody to capture the antigen. Consequently, there is neither enzymic amplification nor the specificity inherent in sandwich ELISA assays which require antigens to bind to two different antibodies at different epitopes. However, we have optimized dye labeling and plasma processing methods to concentrate and label the less abundant plasma proteins to levels several-fold higher than in native plasma thereby increasing sensitivity to the point that we could readily detect pg increases in IL1b (98). Furthermore, this methodology is inherently "discovery" in nature and will require further follow-up and validation of promising biomarkers in independent sample sets. As mentioned previously, the interpretation of our results is also hampered by the limited, though evolving, knowledge regarding the biological underpinnings of triple-negative breast cancer as there is also emerging evidence that there are multiple subtypes of TN disease (106, 114).

This study suggests that there may be unique early detection biomarkers specific to triple-negative breast cancer. These candidates warrant additional follow-up in larger studies to further characterize their potential clinical utility.

CHAPTER 2 TABLES

Table 1: Descriptive characteristics of triple-negative cases and cancer free controls in the training and testing sets

	Training set				Testing set			
	Cases		Controls		Cases		Controls	
Characteristic	n	%	n	%	n	%	n	%
Age								
50-59	5	35.7	5	35.7	5	35.7	5	35.7
60-69	7	50.0	7	50.0	7	50.0	7	50.0
70-79	2	14.3	2	14.3	2	14.3	2	14.3
Race/ethnicity								
Non-Hispanic white	11	78.6	11	78.6	12	85.7	12	85.7
African American	2	14.3	2	14.3	2	14.3	2	14.3
Hispanic white	1	7.1	1	7.1				
Body mass index, kg/m^2								
<25.0 (normal)	4	28.6	7	50.0	5	35.7	7	50.0
25.0-29.9 (overweight)	4	28.6	5	35.7	4	28.6	4	28.6
≥30.0 (obese)	6	42.9	2	14.3	5	35.7	3	21.4
Current use of menopausal hormone therapy								
Non user	8	57.1	8	57.1	8	57.1	8	57.1
Current unopposed estrogen user	3	21.4	5	35.7	4	28.6	3	21.4
Current estrogen+progestin user	3	21.4	1	7.1	2	14.3	3	21.4

Table 2: Log2 ratios and p-values of top ranked antibodies with p-values <0.05 in both the training and test sets

Antibody name	Gene name	Training set log2 ratio	Training set p-value	Test set log2 ratio	Test set p-value	AUC	Sensitivity at 98% specificity
P-value <0.05 in both the training set and the test set							
NADH dehydrogenase 1a subcomplex, 10	NDUFA10	1.78	0.005	1.43	0.018	0.79	68%
Protein-tyrosine phosphatase, mitochondrial 1	PTPMT1	1.85	0.000	1.62	0.001	0.72	52%
Integrin beta-1	ITGB1	1.33	0.007	2.22	0.001	0.76	48%
Mast/stem cell growth factor receptor	KIT	0.65	0.049	2.24	0.001	0.75	46%
DNA-directed RNA polymerase L, 7.6 kDa	POLR2L	1.16	0.044	1.00	0.049	0.69	43%
Ephrin-A5	EFNA5	1.09	0.009	1.46	0.002	0.70	41%
Regulator of G-protein signaling 5	RGS5	0.80	0.019	1.34	0.007	0.64	39%
Stomatin-like protein 2	STOML2	2.07	0.018	1.96	0.009	0.66	36%
TNFR superfamily member 6 (Ab1)	FAS	1.69	0.005	1.78	0.006	0.62	36%
Signal transducer and activator of transcription 6	STAT6	2.05	0.013	1.69	0.003	0.69	33%
Cysteine-rich protein 2	CSRP2	1.28	0.010	1.76	0.020	0.60	31%
Single-stranded DNA binding protein 1	SSBP1	0.97	0.049	1.63	0.005	0.62	31%
Embryonic ectoderm development protein	EED	1.96	0.002	1.59	0.007	0.71	31%
Small inducible cytokine A28	CCL28	1.31	0.006	1.27	0.012	0.67	29%
Signal recognition particle 54 kDa protein	SRP54	1.11	0.024	1.19	0.017	0.55	29%
p16	CDKN2A	1.26	0.034	2.00	0.026	0.58	27%
Small inducible cytokine A27	CCL27	1.35	0.020	1.63	0.015	0.76	25%
Antigen 85-A	fbpA	1.30	0.029	1.41	0.008	0.70	25%
Mitogen-activated protein kinase kinase 1	MAP2K1	1.38	0.023	1.49	0.009	0.63	22%
Breast and ovarian cancer susceptibility protein 1	BRCA1	1.63	0.005	1.30	0.013	0.67	20%
Endoglin	ENG	1.79	0.006	1.34	0.007	0.69	19%
Exportin-T	XPOT	1.66	0.049	1.27	0.047	0.67	19%
DiGeorge syndrome critical region gene 6	DGCR6	0.98	0.032	1.70	0.008	0.66	19%
Propionyl-CoA carboxylase alpha chain m	PCCA	1.58	0.048	1.35	0.013	0.60	19%
Adaptor-related protein complex 3, beta 2 subunit	AP3B2	1.98	0.012	1.12	0.011	0.68	19%
Dual specificity protein phosphatase homolog 9	DUSP9	1.45	0.003	2.57	0.005	0.63	18%
Peripheral-type benzodiazepine receptor	TSPO	1.19	0.047	1.46	0.004	0.53	8%
TNF receptor-associated factor 4	TRAF4	-1.97	0.016	-2.49	0.036	0.41	4%

Table 3: Log2 ratios and p-values of top ranked antibodies with p-values <0.1 in the training and test sets

Antibody name	Gene name	Training set log2 ratio	Training set p-value	Test set log2 ratio	Test set p-value	AUC	Sensitivity at 98% specificity
P-value <0.1 in the training set and and <0.05 in the test set							
Uridine phosphorylase 1	UPP1	1.32	0.075	1.33	0.045	0.67	33%
YEATS domain-containing protein 4	YEATS4	1.60	0.065	0.66	0.050	0.68	32%
Tyrosine-protein phosphatase, receptor type, E	PTPRE	1.19	0.069	1.59	0.009	0.74	31%
TNFR superfamily member 6 (Ab2)	FAS	1.25	0.053	1.43	0.009	0.66	25%
Hexamethylene bis-acetamide inducible 1	HEXIM1	0.91	0.050	1.20	0.044	0.67	22%
Guanine nucleotide-binding protein beta	GNB4	1.33	0.074	1.99	0.031	0.62	21%
Src-like-adaptor	SLA	1.22	0.063	1.17	0.043	0.67	20%
Apoptotic protease-activating factor 1	APAF1	1.17	0.062	1.74	0.014	0.63	14%
Vacuolar ATP synthase subunit G 1	ATP6V1G1	1.75	0.086	0.88	0.012	0.65	11%
X box-binding protein 1	XBP1	1.16	0.075	1.20	0.021	0.71	8%
PTP, non-receptor type 11	PTPN11	-0.62	0.066	-1.39	0.048	0.30	4%
P-value <0.05 in the training set and and <0.1 in the test set							
Toll-like receptor 2	TLR2	2.10	0.001	1.07	0.087	0.71	52%
Anoctamin 1	ANO1	1.49	0.006	1.52	0.062	0.69	43%
Hydroxysteroid (17-beta) dehydrogenase 10	HSD17B10	1.67	0.013	1.27	0.082	0.60	30%
Integrin, beta-like 1	ITGBL1	1.91	0.004	1.40	0.059	0.60	29%
G patch domain and KOW motifs protein	GPKOW	1.66	0.001	1.06	0.071	0.74	22%
Myosin Va	MYO5A	2.46	0.023	1.41	0.075	0.76	22%
RING finger protein 113A	RNF113A	0.78	0.023	1.22	0.076	0.64	18%
nNOS	NOS1	-0.72	0.006	-1.98	0.063	0.41	8%
p300	EP300	-1.62	0.005	-1.41	0.097	0.36	7%
CD45	PTPRC	-1.82	0.019	-1.41	0.090	0.38	4%
Patched	PTCHD1	-1.35	0.020	-2.13	0.064	0.40	4%
c-Myc	MYC	-1.45	0.011	-1.88	0.063	0.46	0%

Table 4: KEGG and GO gene sets with p-values <0.05 in both the training and testing sets

Gene set name	Number of genes in set	Number of unique genes observed	Training set AUC	Training set p-value	Testing set AUC	Testing set p-value
KEGG sets						
Pyrimidine metabolism	98	6	0.89	0.0004	0.82	0.0040
JAK/Stat signaling pathway	155	48	0.42	0.0308	0.39	0.0033
GO sets						
Behavior	149	19	0.62	0.0496	0.69	0.0016
RNA biosynthetic process	626	66	0.44	0.0484	0.40	0.0017
Transcription DNA dependent	624	66	0.44	0.0484	0.40	0.0017
RNA metabolic process	811	69	0.43	0.0353	0.40	0.0029
Regulation of RNA metabolic process	458	48	0.42	0.0321	0.40	0.0090
Regulation of transcription DNA dependent	453	48	0.42	0.0321	0.40	0.0090
Stress activated protein kinase signaling pathway	49	14	0.64	0.0433	0.66	0.0159
Hematopoietin interferon class D 200 domain cytokine receptor activity	32	7	0.20	0.0070	0.24	0.0163
Cell proliferation	501	76	0.44	0.0336	0.43	0.0180
Regulation of nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	602	68	0.43	0.0278	0.43	0.0251
Chemoking receptor binding	43	7	0.76	0.0160	0.74	0.0281
G protein coupled receptor binding	54	7	0.76	0.0160	0.74	0.0281
Transmembrane receptor activity	411	45	0.40	0.0087	0.42	0.0315
Regulation of cellular metabolic process	768	98	0.43	0.0196	0.44	0.0327
Interleukin binding	24	5	0.23	0.0386	0.23	0.0343
Interleukin receptor activity	19	5	0.23	0.0386	0.23	0.0343
Regulation of metabolic process	780	101	0.44	0.0206	0.44	0.0343

CHAPTER 3

MOUSE MODELS OF PANCREAS CANCER

Mouse models have contributed greatly to our understanding of the processes governing mammalian developmental and disease processes. Genetic manipulation of individual and combinations of genes has helped to identify gene function(s), and have led to dissection of signaling pathways, for example through identifying epistatic interactions. The significant contributions that GEMM have made to our understanding of biology is highlighted by the 2007 Nobel Prize in Medicine being awarded to Dr.'s Capecchi, Evans and Smithies for their work in gene targeting, which led to the development of knockout and transgenic mice, and, with the discovery and elaboration of conditional Cre-lox recombinant technologies, the subsequent advent of models driving tissue-specific expression from endogenous promoters. One of the first transgenic models created drove expression of oncogenic c-*HRAS* expression in the acinar compartment of the pancreas using the rat elastase promoter, resulting in formation of neoplasia in newborn animals, and extensive acinar anomalies (hyperplasia and anaplastic lesions) in mice that survived to adulthood (115).

Only with the relatively recent establishment of the genetic and histological progression model for PDA, have faithful GEMM of PDA emerged. These have been instrumental in helping to test hypotheses created with identification of specific mutations in resected and autopsy tissue from human PDA patients. Mouse models used for cancer research include xenograft and orthotopic transplantation models, and transgenic and knockin-mice. Cells are injected either subcutaneously or into the specific organ of interest into immuno-compromised or

immuno-deficient mice, in xenograft and orthotopic models, respectively. These models afford the ability to study human cancer cells in vivo, as well as lead to the selection of metastatic cells, but with the caveat that important contributing effects of the tumor microenvironment are excluded (116).

Transgenic mouse models express non-physiological levels of oncogenes and dominant negative tumor suppressor genes because ectopic promoter and enhancer elements are used. Transgenic technologies can sometimes result in the concatamerization of the transgene of interest, leading to super physiological expression. Endogenous GEMM that utilize 'lox-stop-lox' cassettes drive conditional expression of mutant genes or loss of tumor suppressor genes ins specific organs from their endogenous loci and have the added benefit that they enable heterozygous expression of oncogenes, for example, as occurs with somatic mutations in humans (116).

The pathological differences that can result from non-physiologic and physiologic levels of expression of oncogenes and dominant negative or gain-of-function tumor suppressor genes, is exemplified by comparing the phenotypes between transgenic mice that express mutant *Kras* from CK19-and elastase promoters with those expressing conditional heterozygous mutant *Kras* from its endogenous promoter. CK19-Kras mice display lymphocytic infiltration, occasional hyperplasia and gastric mucous neck cell hyperplasia (117), and Ela-*Kras* mice exhibit developmental defects, with those surviving to adulthood displaying some hyperplastic ducts but a lack of progression to invasive disease (118). The *KC* mouse model of PDA is one of the earlier mouse models employing the lox-stop-lox (LSL) Cre technology (12). Conditional endogenous expression of mutant *Kras* using *Pdx-1* or *Ptf1a* as promoters driving *Cre* expression, and hence $Kras^{G12D/+}$ expression in all cell types in the pancreas, conversely, leads to

the development of PanIN lesions that progress to invasive and metastatic disease and mice. This *KC* mouse model clinically, histologically and molecularly phenocopies the human condition and is considered the first GEMM to successfully resemble human PDA (26). Thus, the underlying method used to generate mouse models greatly impacts the resultant phenotype. The *KC* model and iterations incorporating additional mutations and temporal control of mutant gene expression are therefore most widely used for studying PDA.

Building upon "lox-stop-lox"-Cre (LSL) technologies, inducible systems using tetracycline, for example, afford temporal control over the expression of mutant oncogenes. Such systems have helped to highlight the contribution of pancreatic injury to PanIN formation when oncogenic *Kras* is turned on in the adult pancreas (119), as well as the requirement for *KrasG12D* in maintenance of PDA following disease establishment (120). Additional studies incorporating GFP into Cre-lox systems has enabled the tracking of mutant *Kras* expressing cells and has provided insight into how mutant p53 contributes to disease progression (121) and the strong metastatic potential of tumor cells at early disease stages (122).

Thus, there is now an extensive repertoire of mouse models of PDA, generally incorporating mutant *Kras* in combination with the loss of tumor suppressor genes or expression of dominant negative mutations. Some models progress through precursor mucinous cystic neoplasms (123) and intraductal papillary mucinous neoplasms (124), some through classical PanIN precursor lesions; some models show accelerated disease and others present with more indolent disease. GEMM of PDA have been instrumental in corroborating the contributions of genetic alterations such as mutant *Kras* to disease initiation and progression, the processes underlying acinar to ductal metaplasia (125-127), and have greatly helped in our understanding of the role of inflammation in disease progression (128). They have enabled the dissection of

important signaling pathways in PDA progression and have also been used to test novel imaging modalities (17). They have more recently been used as preclinical models for testing therapeutics and have also shown the importance of the tumor microenvironment in preventing access of chemotherapeutics (129, 130). Furthermore, they add a spatiotemporal component to studying disease that studies on resected tumor tissue cannot provide. Given the paucity of resected tumor tissue from PDA patients, GEMM will continue to feature extensively in PDA research: they will continue to allow identification of novel genes that contribute to disease progression, to find tumor vulnerabilities and test therapies, and to determine molecular drivers of metastasis.

From a biomarker perspective, GEMM are useful for the discovery and validation of detection markers and for tracking disease from initiation to end stages. I used the *KPC* mouse model, incorporating conditional heterozygous expression of $Kras^{G12D}$ and $Trp53^{R172H}$ from their endogenous promoters using an LSL-Cre system in our biomarker research (Figure 1). These mice faithfully recapitulate human PDA at the clinical, histological, and molecular level. *KPC* mice progress to PDA through classic PanIN precursors, have a short median lifespan (~5 months) that make spatiotemporal tracking of disease feasible in a short period of time, have metastases at frequencies and sites mirroring what is observed in human PDA and, importantly, have 100% disease penetrance (131). Thus, this model enables the identification of markers of PanIN lesions that *will* progress to PDA rather than markers of indolent or benign PanIN lesions.

KPC mice display some disease heterogeneity, with mice living from just 2 months to as long as 12 months. I would argue that this heterogeneity makes biomarker research in this model more translatable to human PDA: biomarkers discovered must therefore be present despite this heterogeneity, making them more applicable to the general population in which patients present with heterogeneous forms of PDA. Markers identified additionally have more prognostic and

mechanistic (e.g. metastasis-specific markers) relevance in the setting of molecular and pathological heterogeneity.

As elaborated below, I used *KPC* mice sacrificed at different stages of disease, thereby identifying early disease markers and tracking them from early to metastatic disease stages in the primary tumor. Thus, in conjunction with a large format antibody microarray platform developed specifically for pancreas cancer biomarker discovery, I believe this is a powerful approach for biomarker discovery and validation in a preclinical model that has been shown to have direct application to human PDA (132).

Proteomic tracking in vivo during pancreas cancer progression identifies STK4 as a novel early detection tissue biomarker

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SUMMARY

Pancreas cancer, or pancreatic ductal adenocarcinoma (PDA), is the deadliest of solid tumors with a 5-year survival rate of <5%. Detection of disease when resection is possible has been shown to improve survival rates but access to tissue and samples to identify early detection markers is confounded by the insidious nature of pancreas cancer. Mouse models that recapitulate the human condition can therefore be useful for early detection studies, allowing tracking of disease from inception to the establishment of metastases. Using a highly faithful mouse model of pancreas cancer in conjunction with a high-density antibody microarray containing ~2500 full-length antibodies, we interrogated the tissue proteome at preinvasive and invasive stages of disease. The goal was to discover early detection markers of pancreas cancer and monitor them throughout disease progression. A panel of 7 up-regulated proteins distinguishing pancreas cancer from normal pancreas was validated and their levels assessed in tissue collected at preinvasive, early invasive and moribund disease stages. Six of the 7 markers also differentiated pancreas cancer tissue from an experimental model of chronic pancreatitis. The levels of serine/threonine stress kinase 4 (STK4) increased between preinvasive and invasive stages, suggesting a potential role for it as a marker of and perhaps also involved in the progression from pancreatic intraepithelial neoplasias (PanIN) to PDA. Immunohistochemistry (IHC) of STK4 at different stages of pancreas cancer identified a dynamic expression pattern further implicating it in early tumorigenic events. IHC of a panel of human pancreas cancer cores confirmed that STK4 levels are also increased in human PDA tumor epithelia compared to normal tissue. Overall, this approach yielded multiple markers that could serve as signatures of disease stage, including at early, resectable, and therefore clinically meaningful, stages of disease.

INTRODUCTION

In 2012 the American Cancer Society estimated there were 43,920 new cases of cancers of the pancreas, the most common form of which is pancreatic ductal adenocarcinoma (PDA), with an estimated 37,930 deaths (Cancer Facts and Figures 2012). Unfortunately, the incidence of PDA increased on average by 1.5% per year from 2004-2008, with death rates increasing concomitantly by 0.4%. The poor prognosis of PDA is due to both its typically asymptomatic nature and resistance to most treatment regimens: the majority of patients still present with unresectable disease at diagnosis and less than 20% of these patients survive past twelve months (3).

Surgical resection for curative intent has been shown to improve five-year survival rates from less than 5% to approximately 20% (1). Identification of early stage (I/II) tumors upon surgical resection also portends more favorable prognoses (10). Yet resection is possible in only 10-15% of patients (5). Accurate diagnosis of PDA can be further confounded by difficulty distinguishing it from chronic pancreatitis (CP), whose high degree of fibrosis can alter pancreatic morphology to resemble PDA; conversely CP can also be initiated by a tumor causing ductal obstruction (133). While a non-invasive blood-based test would be ideal for early detection and diagnosis, no validated markers currently exist with sufficient sensitivity and specificity to improve clinical decision-making. Thus, tissue markers could serve to confirm disease stage and assess prognosis, both valuable for making proper diagnoses and informed decisions regarding surgical resection and use of neoadjuvant/adjuvant therapy. Identification of early stage tissue markers may also provide further insight into pancreatic cancer etiology.

Mouse models that clinically, histologically and molecularly recapitulate human PDA (12, 123, 131, 134, 135) have been instrumental in elucidating the molecular mechanisms underlying PDA etiology and progression. For example, these studies have shown that pancreas-specific expression of mutant *Kras* can initiate PanIN lesions that progress to invasive adenocarcinoma (12), and that the disease course, rates and metastatic capabilities of PDA are affected by the additional loss and/or mutation of key tumor suppressor genes (*Dpc4*/*Smad4*, *Cdnk2a*/*ink4a* and *Trp53*) and the timing of their loss (123, 131, 135). Similarly, useful mouse models can complement static genomic and proteomic biomarker studies of human samples because they enable spatiotemporal monitoring of markers and processes from initiation of disease to metastatic stages. While many studies have used genetics to identify proteins important for PDA tumorigenesis, and looked at biomarker expression levels and frequencies in various precursor lesions in resected tumor tissue (56, 59), fewer studies have used genetically engineered mouse models of PDA to *discover* protein biomarkers and follow their changes in distribution and expression *during* disease progression (71, 136).

We elected to use the well established mouse model of PDA that employs conditional pancreas-specific endogenous expression of $Kras^{G12D/+}$ and $Trp53^{R172H/+}$ (131), and hereafter referred to as the *KPC* mouse model—in conjunction with a novel high dimensional, pancreas-cancer tailored antibody microarray to assay for proteomic changes during disease progression (78, 98, 100). This microarray consisted of ~2500 full length antibodies and represents, to our knowledge, the largest available array platform. Our goal was to 'follow' the evolving primary tumor proteome by identifying specific protein markers in early pancreatic intraepithelial neoplasms (PanIN), the most common precursors to PDA, and tracking them spatiotemporally as disease progressed. Using this proteomic approach, we identified and validated a panel of

markers differentiating early stages of PDA tumorigenesis from both the normal organ and from a model of chronic pancreatitis. In so monitoring protein expression changes among preinvasive, invasive and metastatic disease stages, 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/C57BL/6 background were generated as previously described (131). To model chronic pancreatitis, six 2-month wild type animals were subjected to intraperitoneal injections of 100 μl of 50 μg/ml cerulein for 23 consecutive days. Full necropsies and tissue sampling were conducted within 6 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 incubated on arrays as previously described with minor modifications (100). Briefly, antibodies were printed in triplicate on Nexterion slide H hydrogel slides (Schott, Germany) in a 16 x 16 block format for a total of 4096 unique features. Antibodies were typically printed at a final concentration of 350 μg/ml (unless their stock concentrations were lower). Proteins in tissue lysates (200 μg 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.

Labeled lysates were incubated on arrays for 1.5 hours and, following a series of washes to remove background, the arrays were scanned and analyzed using an Axon Genepix 4200A scanner (Molecular Devices, LLC, Sunnyvale, CA).

Array analysis and statistics

Saturated array spots were flagged and triplicate antibodies with coefficients of variation >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. An average of the median intensity of triplicate spots for each antibody feature was calculated and control signal was subsequently standardized to have a mean signal of zero with a standard deviation of 1. Logistic regression analysis using the unpaired model: 'case~control + stage,' where 'stage,' representing preinvasive and invasive tissue samples, is included as a variable in the model, was used to calculate p-values for each unique antibody feature. Candidate protein markers were then ranked based on their coefficient (a positive coefficient meaning greater in cancer than controls, a negative value meaning lower in cancer versus controls) and p-values. 'Stage' p-values and coefficients comparing signal between the 2- and 4-month *KPC* lysates were also calculated to identify changes in marker levels between the two time points. All normalization procedures and analyses were conducted using R statistical computing software program.

Tissue and cell lysis

Sections of tumor tissue 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% NP-40, 0.25% deoxycholate, 0.25% octyl-β-d-glucopyranoside and 0.25% amidosulfobetaine-14 supplemented with phosphatase inhibitors, protease inhibitor cocktail 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; cell lysates were prepared using 200 μl of detergent for 60-mm dishes and 400 μl for 100-mm dishes.

Immunoblotting and immunohistochemistry

Protein concentrations were estimated using the BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). Thirty μg of pancreatic protein lysates from *KPC*, controls and cerulein-treated animals were boiled for 3-5 minutes in Laemmli sample buffer plus 3% β-mercaptoethanol prior to analysis by SDS-PAGE. Lysates were separated on NuPAGE Novex 4-12% Bis-Tris gels (Life Technologies, Grand Island, NY), and transferred to nitrocellulose using 20x NuPAGE transfer buffer (Life Technologies) supplemented with 10% methanol at 105V. The blots were subsequently blocked in 3% non-fat dry milk/PBS and incubated with the following: STK4 and FN1 (Abcam, Cambridge, MA), DSC2, BSG, RFC4 (SDIX Technologies, Newark, DE), SMAD2 (ABM Inc., Richmond, BC, Canada) p19ARF (Santa Cruz Technologies, Inc., Dallas, TX) and alpha tubulin (Cell Signaling Technology, Beverly, MA). Fluorescein donkey anti-rabbit antibody IRdye 800 (Rockland, Gilbertsville, PA), goat anti-rat IRdye 800 and goat anti-rabbit IRdye 680 (Li-Cor Biosciences, Lincoln, NE) 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). 2-tailed unpaired t-tests were used to compare band intensities between *KPC* and control/CP lysates using GraphPad Prism 5.0.

For immunohistochemistry (IHC), four-micron sections of formalin-fixed paraffin-embedded tissues were deparaffinized with xylene and sequentially rehydrated followed by antigen retrieval in Trilogy pH 8.0 Buffer (Cell Marque, Rocklin, CA) in a pressure cooker for 7 minutes. 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 minutes and then cover slips were added. For STK4 analyses, serial 5 μm sections were stained with hematoxylin and eosin or STK4 antibody (1:200). All images were taken with a Nikon DS-Vi1 brightfield camera using NIS Elements 3.2 Basic Research Image software (Nikon Instruments Inc., Melville, NY).

Table of antibodies used in tissue array validation studies

Antibody	Source	Use
STK4	Abcam	IB, IHC
FN1	Abcam	IB, IHC
DSC2	SDIX	IB
BSG	SDIX	IB
RFC4	SDIX	IB
SMAD2	ABM	IB
p19ARF	Santa Cruz	IB
alpha tubulin	Cell Signaling	IB (loading ctrl)

IB = immunoblotting
IHC = immunohistochemistry

Rapid Autopsy program and human tissue microarrays

Pancreatic tumors and other tissue specimens were obtained with consent and IRB approval from surgically resected samples or from decedents through the Rapid Autopsy Program at the University of Nebraska Medical Center. To ensure minimal degradation of tissue, organs were harvested within three hours post mortem and the specimens flash frozen in liquid nitrogen or

placed in formalin for immediate fixation (57). Tissue microarrays (TMAs) were made from paraffin blocks of formalin fixed tissue from rapid autopsies and control specimens of uninvolved pancreas from non-cancerous donors using 2.0 - 2.5 mm cores, which were cut into 4 micron sections and mounted on charged slides. STK4 expression was evaluated and scored based on percentages of stained cells and their staining intensities. The number of stained cells was multipled by their staining intensity, assigned a score of 1, 2, or 3 for weak, moderate and intense staining, respectively; this approach would yield a maximum score of 300. Tumor epithelial cells were scored in PDA cores and the percentage of total cells in normal cores. Scoring was independently verified in blinded fashion (S.R.H. and J.L.S.). Infiltrating immune cells and endothelial tissue within the tumor were considered part of the stroma. 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

Proteomic technologies enable the molecular characterization of biological samples at the structural and functional level (43), and multiplex antibody microarray platforms allow the characterization of multiple analytes simultaneously. They have an added benefit of requiring minimal sample volumes while still identifying their cognate analytes with pico- to femtomolar sensitivities (77, 98). Given their low cost, immuno-based proteomic technologies are thought to be the most applicable proteomic technology in a clinical setting (27, 137).

We have extensive experience with array fabrication and biomarker discovery, having created successive iterations of platforms to discover and validate ovarian and breast cancer serum and plasma markers (78, 98, 100) as well as to identify plasma autoantigen-autoantibody complexes (138). Using this experience together with our knowledge of pancreas cancer pathogenesis, we created a customized pancreas cancer-specific antibody microarray platform, carefully selecting antibodies against markers. We selected antibodies against putative PDA biomarkers included in the pancreas cancer biomarker compendium (24), with an emphasis on selecting candidates reported as PanIN-3 markers and candidates with quantitative data suggesting they were most highly expressed in PDA. The total of ~2500 unique antibody features thus included many antibodies against proteins involved in pathways implicated in PDA (TGFβ signaling, Wnt signaling, extracellular matrix remodeling, for example) as well as having coverage for proteins with a broad array of functions, from mismatch repair proteins to glycolytic enzymes to cytokines and their receptors for the discovery of novel markers. This pancreas cancer-specific microarray therefore represents a comprehensive platform for proteomic interrogation.

To discover new and further validate previously reported tissue markers of pancreas cancer and to track their expression changes during disease progression, pancreata were harvested from 2- and 4-month old *KPC* mice, respectively, as well as age-matched controls (n=5 *KPC* and n = 4 controls at each time point). None of the animals from either time point displayed overt clinical signs of disease (e.g. cachexia, lack of movement, jaundice or malignant ascites) at the time of sacrifice, and no animals manifested any histologically identifiable metastases. As expected, pancreata from 4-month *KPC* animals were more nodular and fibrotic appearing than those from 2-month animals (supplementary figure 1A). Histology also

confirmed that a larger proportion of 2-month pancreatic parenchyma was still normal, and that both 2-month and 4-month *KPC* mice presented with PanIN lesions and, when present, invasive PDA as characterized previously (131). Histological assessment of two distinct regions of hematoxylin and eosin sections of resected pancreas revealed that four of the five 4-month *KPC* animals had progressed to focally invasive PDA (the other progressed to PanIN-3, or carcinoma in situ); two animals in the younger cohort had small regions of focal adenocarcinoma and the remainder only showed PanIN-2 lesions (supplementary figure 1B).

Bulk tissue lysates were prepared from *KPC* and control animals, their proteins were fluorescently labeled and they were incubated on our antibody microarray platform to identify candidate proteins distinguishing PDA from normal pancreas (Figure 1A, schematic). After array scanning, data extraction and normalization, logistic regression was used to rank candidate markers by p-value and their ability to differentiate bulk *KPC* tissue from normal pancreata (expressed as a coefficient incorporating red/green fluorescence intensity ratio). Altogether, seventy-one proteins distinguished 2-month (preinvasive) and 4-month (invasive) *KPC* tumors from normal pancreatic tissue (p-value <0.05), 30 of which exhibited increased expression and 41 decreased expression in *KPC* tissue (Figure 1B and Table 1). 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 1). Twenty-nine of the 71 markers represent novel candidate biomarkers (the remaining 42 have been previously implicated in pancreas cancer (Supplementary Table 1)).

Array marker validation by immunoblotting identifies a panel of markers for early stages of PDA

To validate candidate markers discovered using our array platform, we assessed protein levels in both 2-month and 4-month *KPC* and control pancreatic tissue by immunoblotting lysates, focusing on 16 highly up regulated markers. Seven markers showed significantly elevated levels in *KPC* compared to control tissue. Of the remaining nine, eight failed to show detectable bands and one displayed bands at the incorrect molecular weight. The seven validated candidates included the novel potential PDA markers desmocolin 2 (DSC2), serine-threonine stress-related kinase 4 (STK4), and replication factor C (activator 1) 4 (RFC4), as well as previously identified markers p19ARF, fibronectin 1 (FN1), SMAD family member 2 (SMAD2), and basigin (BSG) (Figure 2). While six of these 7 markers distinguished case from control at the 4-month time point, 4 of the 7—p19ARF, BSG, FN1 and SMAD2—distinguished cancer from normal tissue with significance even at 2 months, i.e., they were biomarkers of preinvasive PDA. DSC2 and RFC4 also distinguished 2-month *KPC* from controls with borderline p-values <0.07. Increased levels of p19ARF were only statistically significant when comparing 2-month case and controls, not for the 4-month time point.

Marker panel distinguishes early stages of PDA from pancreatitis

Differentiating pancreas cancer from chronic pancreatitis can be challenging clinically, and many putative biomarkers that successfully distinguish pancreas cancer from normal tissue fail to differentiate it from chronic pancreatitis (139). To model chronic pancreatitis, we used a protocol similar to that reported by Strobel et al., administering daily intraperitoneal injections of the cholecystokinin analog, cerulein, in a cohort of 6 wild type mice for just over 3 weeks (140). Necropsies were conducted within 6 hours 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 (supplementary figure 1C).

In comparing levels of the panel of 7 markers between *KPC* and chronic pancreatitis (CP) tissue, BSG, DSC2, FN1, RFC4, STK4 and p19ARF were appreciably greater in *KPC* tissue; SMAD2 was the only marker whose levels were similar between *KPC* and CP tissue (Figure 3A,B). Increased levels of BSG, DSC2, FN1, RFC4 and STK4 were observed in 4-month *KPC* lysates compared with CP levels while p19ARF differentiated 2-month *KPC* tissue from CP with significance. Increased levels of DSC2 and BSG differentiating 2-month *KPC* from cerulein-treated pancreata also approached significance (p-values = 0.059 and 0.053, respectively) (Figure 3C, D).

Assessing changes in protein levels of the marker panel between invasive and metastatic disease stages

Each marker in the panel of seven proteins was able to distinguish 2- or 4-month *KPC* tissue from control specimens and all except SMAD2 also differentiated *KPC* from chronic pancreatitis tissue at one of the two time points. Thus, they represent potential early detection markers. To assess how these markers change between preinvasive, invasive and terminal disease stages we next examined their expression levels in a cohort of moribund *KPC* animals. FN1, p19ARF, RFC4 and DSC2 all displayed appreciable increases in expression between 4-month *KPC* tissue lysates and in primary tumor tissue taken from the 8 moribund *KPC* animals (Figure 4A,B), of which all but one animal succumbed to a high metastatic disease burden. The increase in expression from invasive to terminal or metastatic disease stages was most apparent

for RFC4, FN1 and p19 ARF. While an increase in expression for DSC2 was noticeable between 4-months and moribund stages, it was not statistically significant (p-value <0.07).

Levels of STK4 and BSG remained relatively unchanged between 4-month and moribund *KPC* tissue (Figure 4C, D), whereas they increased between 2- and 4-month tissue lysates (p-value = 0.05 and p-value <0.05, respectively) (Figure 2). The increase in BSG and STK4 expression between preinvasive and invasive disease stages and the lack of further increase thereafter, may imply a potential role for these proteins in the transition from PanIN to PDA. To further validate STK4, we conducted IHC on tissues from independent cohorts of control, 2-month, 4-month, and moribund *KPC* animals, as well as our cerulein-treated cohort.

STK4 exhibits a biphasic expression pattern during PanIN-to-PDA progression

In normal pancreatic parenchyma, STK4 displayed a punctate cytoplasmic expression pattern both in acini and ductules (Figure 5A). STK4 signal intensity increased considerably in regions of damaged acinar structures undergoing dropout and metaplasia, also known as acinar to ductal metaplasia or ADM (Figure 5B). However, this seemed unique to damaged acini in the *KPC* background: STK4 expression did not increase in areas of extensive acinar damage or fibrosis in the pancreata of the CP cohort (Figure 5C). STK4 expression was also seen in lymphocytic cells in normal pancreata, in the peripancreatic lymph nodes of CP, 2-month and moribund *KPC* tissue, and in Peyer's patches in the duodenum (not shown).

Interestingly, PanIN-1A and PanIN-1B lesions showed lower STK4 staining relative to normal ducts, with this moderate signal confined to the periphery of cells (Figure 5D). STK4 then increased dramatically in PanIN-2 and PanIN-3 lesions (Figure 5E), remaining elevated in invasive PDA (Figure 5F). Increased staining was specific to PanIN and invasive epithelial cells,

as the stromal infiltrate lacked STK4 expression, aside from the staining the subset of immune cells mentioned above. Additionally, we note that two animals examined had more sarcomatoid morphology in their primary tumors, and STK4 levels were lower in these than in well-differentiated PDA (supplementary figure 2A).

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 (Figure 5G,H); regions of both primary tumor and metastases possessing more sarcomatoid morphology displayed lower STK4 levels (supplementary figure 2B). The dynamic biphasic expression pattern of STK4 observed by IHC at early stages of disease—notably the increased levels in damaged and transitional acinar structures followed by the drop in PanIN-1 lesions—and subsequent reemergence in higher grade PanIN lesions that persisted to invasive disease stages suggests specificity and warrants further investigation of a role for STK4 in PanIN-to-PDA progression.

STK4 is expressed in human PDA

To determine whether STK4 is also expressed in human tumor epithelia, we performed IHC on sections from a tissue microarray (TMA) containing 25 PDA cases and 8 cores from non-cancerous donors made available through the rapid autopsy program at the University of Nebraska. Individual cores were assessed for the presence or absence of STK4 expression and localization. Of the 25 PDA cores assessed, 23 were evaluable. Of these, 22 showed predominantly nuclear STK4 staining generally accompanied by weak to moderate cytoplasmic staining (Figure 6A-C). STK4 levels in epithelial cells ranged from weak expression (1+) in <1% of cells to strong expression (3+) in 70% of cells, with composite IHC scores ranging

between 0.5 and 208 (mean of 67). While STK4 also appeared in the nuclei of some lymphocytes, it was otherwise absent in the stroma, corroborating our observations in murine PDA.

The composite scoring for normal tissues ranged from 7 to 40 (mean of 20.2). The weak cytoplasmic and weak to moderate nuclear STK4 reactivity observed was only seen in a low percentage of acinar and ductal cells (Figure 6D-F). Importantly, areas that showed these heterogeneous staining patterns were generally confined to the periphery of individual cores. An unpaired 2-tailed Welch's t-test differentiated STK4 levels in PDA from normal tissue with a p-value <0.003. Thus, this preliminary screen strongly implicates STK4 as a novel marker of human PDA.

DISCUSSION

Pancreas cancer remains the deadliest of solid tumors, and its incidence in North America is increasing. While the underlying genetics of PDA are well-characterized this knowledge has not yet translated to improved early detection or prognostic markers, nor to successful targeted therapies. The current FDA-approved plasma marker, CA-19-9, is of limited diagnostic value and is not recommended for routine use in assessing disease recurrence following resection (without accompanying imaging studies or biopsies) and/or to measure response to therapeutic intervention (141). Though extensive studies have focused on the 'omes' of patients to identify early detection markers, many of these have relied on samples collected concurrent 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 true

early detection. In an effort to focus on very early stages of PDA where intervention could be more effective, we chose to use a highly faithful mouse model of PDA in which tissue could be studied from the earliest onset of PanIN formation to advanced disease stages. Using a high dimensional pancreas cancer targeted antibody microarray approach, we identified, validated and tracked putative early detection protein markers and further assessed the spatiotemporal characteristics of one interesting candidate, serine/threonine stress kinase 4 (STK4). The dynamics of STK4 expression during disease progression are intriguing, warranting further study to evaluate a potential functional role in pancreas cancer progression. Thus, this approach also has promise to provide insight into possible roles of individual markers in disease etiology.

Of the 71 putative markers discovered in our array experiments, 42 have been previously implicated in pancreas cancer (see Supplementary Table 1), including up-regulated expression of claudin 18 (CLDN18) (63), matrix metalloproteinase 7 (MMP7) (128), and the secreted kallikrein-related peptidase 6 (KLK6) (142, 143); as well as markers previously reported as down-regulated including E-cadherin (CDH1) and the homeobox transcription factor HOXD13 (144). The panel of 7 proteins whose increased expression we validated and monitored throughout disease progression included some novel—desmocolin 2 (DSC2), replication factor C (activator 1) 4 (RFC4), and STK4—and some previously identified (p19ARF, FN1, BSG and SMAD2) putative markers of PDA. Importantly, 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 (BSG, SMAD2, p19ARF and RFC4) distinguished preinvasive disease in 2-month old *KPC* tissue from normal controls, whereas 6 of the 7 (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 chronic pancreatitis (CP) with p19ARF

able to do so at 2-month disease stage. Of the markers previously implicated in PDA, only FN1 has been previously shown to be elevated at the transcript and protein level (by IHC) in human CP, although expression in PDA tumor tissue was appreciably increased versus expression in CP (145). We therefore suggest this panel of seven proteins may warrant further scrutiny in human PDA, especially since the majority of them have never been comparatively assessed in human PDA and CP.

Tracking our marker panel from preinvasive to invasive and metastatic disease stages revealed that levels of STK4 and BSG only increased between 2-month (preinvasive) and 4-month (invasion with no metastases) *KPC* tissue. Whereas BSG has been previously implicated in pancreas cancer progression—promoting secretion of matrix metalloproteinases from supporting stroma (146)—STK4, or MST1, is a novel biomarker for pancreas cancer. In *KPC* tissue, STK4 staining increased in regions of acinar damage and the emergence of ductal-like structures, commonly termed acinar-to-ductal metaplasia (ADM), and then decreased in true PanIN-1A/B lesions. Increased STK4 was not observed in areas of inflammatory injury in CP. STK4 levels subsequently increased in PanIN-2 and PanIN-3 lesions and remained elevated in invasive and metastatic disease. Throughout this progression in murine PDA, STK4 expression was exclusively cytoplasmic.

In primary human PDA collected at autopsy, we found that STK4 is also expressed at higher levels in tumor epithelia than in normal pancreatic tissue. As a subset of PDA cases were negative or showed negligible staining, further genetic, functional and histological comparisons of STK4-postive and negative tumors could also uncover potential underlying mechanisms that correlate with STK4 status (for example, in glandular PDA compared to more sarcomatoid PDA). In human PDA, however, STK4 staining was found in both cytoplasmic and nuclear

compartments, although nuclear staining predominated. One of the functions of STK4 is to promote apoptosis through its nuclear translocation (147). That STK4 expression in murine PDA was confined to the cytoplasm, whereas it was found predominantly in the nuclear compartment in human PDA, might suggest that STK4 contributes to apoptosis or other signaling processes differently in murine and human PDA.

STK3 and STK4 are the mammalian orthologs of the Drosophila *Hpo* kinase. The role of mammalian hippo kinases in cancer is not yet well delineated: although classically thought to exert pro-apoptotic and antiproliferative effects (148), recent studies suggest that the functions of hippo kinases vary in a tissue-specific and context-dependent manner (149-151). It also remains unknown whether oncogenic *Kras* and mutant *Tp53* also modulate hippo signaling. Whereas both *Kras* and *Trp53* were always mutated in mice, the underlying genetics in the human TMA's are unknown. These genetic differences could also account for differences in STK4 localization in human and murine PDA. Nevertheless, 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, especially the increased levels observed at early stages of disease. Characterization of other hippo pathway members (for example YAP1) 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 genetically engineered mouse models involving STK3/4 knockout (150, 151) mice can also help elucidate the functional implications of STK4 signaling in PDA etiology.

Our experience suggests that markers identified in static studies of resected human tumors may be informed by examination throughout the course of disease progression in faithful genetically engineered mouse models. Such analyses could help focus efforts to the most

relevant early detection markers—those that persist or increase throughout disease progression and/or that respond to treatment—and help place candidate markers into their appropriate pathophysiological context. In turn, these markers could also be potentially applied to monitoring high-risk patients in a clinical setting and help in prioritizing the most suitable therapeutic targets.

FUTURE DIRECTIONS

No clinically relevant markers have emerged since monoclonal antibodies to CA19-9 were isolated in 1979 (26, 44). This is in large part due to a paucity of patient tissue, especially from early disease stages. The results of our array experiments highlight the utility of mouse models for identifying early detection and prognostic markers of PDA as well as markers that may help in disease staging. Using our antibody microarray platform and immunoblotting validation experiments, I respectively identified and validated a panel of 7 tissue markers distinguishing early stages of PDA from normal pancreata, 6 of which also differentiated *KPC* tissue from chronic pancreatitis. Furthermore, as I show with STK4, and as others have shown, markers discovered in GEMM of PDA are relevant to human disease. These results therefore have both clinical and biological implications, which are discussed below, with a focus on future experiments elucidating a role for STK4 in disease etiology.

Clinical implications

As we were also most interested in identifying early (PanIN-2/3 and focally invasive PDA representing stage I disease) tissue markers of disease, I sacrificed mice at preinvasive and early invasive disease stages. Thus, in translating these markers to human PDA, our

experimental design obscured the ability to assign prognostic value to these markers (although, their prognostic significance can still be assessed as discussed below). The panel of tissue markers validated in *KPC* mice is thus perhaps most directly applicable to differentiating early PDA or PanIN lesions from carcinoma that develops from cystic precursor lesions, from benign cystic lesions and from chronic pancreatitis.

It is estimated that approximately 1 in 100 CT exams identify pancreatic cysts, yet only approximately 30% are thought to be malignant (66). Thus, if markers in our panel, including BSG and STK4 can differentiate pancreatic cancer from cystic lesions in fine needle aspirates, this would certainly influence decisions regarding resection or continued monitoring via MRI, PET or CT. Two types of lesions with malignant potential are mucinous cystic neoplasms (MCN) and intraductal papillary mucinous neoplasms (IPMN). While both can progress to invasive carcinoma, PDA that develops from cystic precursors generally portends better prognoses: post-resection survival rates are as high as 40-60% compared to a ~20% 5-year survival rate for patients with resectable PDA that develops from the more predominant PanIN route (152). Similarly to the identification of GNAS mutations as markers for PDA developing from IPMN (51), these studies would collectively help determine the specificity of markers like STK4 for PanIN-to-PDA progression. If BSG, DSC2 and STK4, for example, together differentiate PanIN- from cystic-associated PDA, given the different prognoses described above, a more informed recommendation on whether to include adjuvant therapy following surgery could be made.

Pancreatitis is the most common disease of the exocrine pancreas. Patients with chronic pancreatitis, and in particular those with hereditary chronic pancreatitis, are at increased risk of developing PDA (as high as 40% increased risk for hereditary pancreatitis patients) (153, 154).

Mouse models have also shown the importance of chronic pancreatitis (CP) in accelerating onset and progression of PDA (119, 127). CP can morphologically and pathologically resemble PDA and the two different diseases are thus difficult to distinguish, especially in core needle biopsies and frozen specimens (133). The fact that six of the 7 markers that I validated in the *KPC* model differentiated preinvasive and invasive disease from CP in immunoblotting experiments, and, for STK4 also by IHC, is therefore of importance for translation to a clinical setting.

Additional experiments to assess the clinical applicability of these markers would first include further experiments in mouse models of MCN (123), IPMN (124) and CP, the latter containing the $Prss1^{R122H}$ mutation in cationic trypsinogen identified in most patients with hereditary pancreatitis (155); experiments in this latter model would serve to complement our results from chemically induced CP. In turn, IHC experiments on human PanIN, IPMN and MCN from resected tumor tissue could help determine if our 7-marker panel, or a subset of proteins, translates to identifying the different routes to PDA in human patients. Cystic neoplasms are more available for study than PanIN as they are generally symptomatic and they are macroscopic, making them more resolvable radiologically than microscopic PanIN (152, 156). The utility of these tissue markers could be tested in fine needle aspirates (FNA) removed upon radiologic detection of a suspicious lesion. If subsequent pancreatectomies or pancreaticoduodenectomies reveal PanIN, or our markers differentiate PanIN from associated cystic lesions, then they could be further tested in blinded fashion in later FNA, providing important correlative information to EUS and radiologic findings. Even if IHC experiments using human PDA reveal expression of our panel of markers in cystic lesions, it would be still interesting to determine whether, like GNAS mutations, they garner utility in differentiating

benign lesions from those with malignant potential as MCN and IPMN lesions do not always progress to invasive carcinoma.

While tissue biomarkers of PDA are less rational as early detection markers than their counterparts in plasma, ascites and cyst fluid, they still have practical application in the clinic as discussed above. They also warrant further follow up as prognostic markers following surgical resection. Except for BSG and STK4, the five other markers that I validated (SMAD2, DSC2, FN1, p19ARF and RFC4) showed increased expression with disease progression as measured in 2-month, 4-month and moribund primary tumor tissue. I did not see a dramatic association with survivability and marker expression in the *KPC* aging cohort (see figure 4 from our mouse biomarker studies above) where overall survival ranged from 49 to 318 days, suggesting that the increased protein levels track with disease stage. Levels of these markers were also relatively similar in our 4-month *KPC* tissue lysates, representing disease akin to stage I disease in humans (locally invasive but no apparent lymph node involvement or distant metastasis).

Because all *KPC* mice succumb within 12 months, assessing the prognostic value of markers becomes more difficult. This is better achieved by comparing markers across different GEMM that display more dramatic differences in disease phenotype and latency. In human patients, however, the underlying mutational spectrum is far less uniform than in the *KPC* model and survival rates more variable. Thus, future studies assessing marker expression in resected human tumor tissue and overall survival (see (16)) would be more accurate in the assigning any prognostic value to the five markers in our panel.

Biological implications

Mouse models of PDA have provided insight into mediators of disease initiation and PanIN formation and progression (12, 120, 126, 128, 131, 157, 158). The spatiotemporal approach I employed also enabled me to pinpoint interesting markers of early disease, of which the mammalian hippo kinase STK4 I pursued further. The immunoblotting results showing an increase in STK4 protein levels between preinvasive and invasive *KPC* tissue but not between early invasive and end-stage disease, led to IHC experiments that revealed a very interesting expression pattern implicating this protein in early stages of disease. The increased STK4 staining in metaplastic structures followed by a decrease in early PanIN-1 lesions and subsequent rebound in PanIN-2/3 suggests a potential role for STK4 in the differentiation of $Kras^{G12D/+;}Trp53^{R172H/+}$ acinar cells to tubular complexes and PanIN lesions. Some recent data assessing the role of hippo kinases in pancreatic development also lends support to this theory.

First discovered in screens of suppressors of growth in Drosophila, the mammalian hippo kinases STK3 and STK4 are canonically thought to promote apoptosis and prevent proliferation. The pro-apoptotic functions of STK4 involve caspase-mediated cleavage and translocation to the nucleus, which is also reported to be p53-dependent (147). Hippo kinases exert their antiproliferative functions by acting in a signaling cascade leading to the cytoplasmic sequestration, and thereby functional inhibition, of the downstream oncoproteins TAZ and YAP1, transcriptional regulators that promote cell proliferation and whose over expression result in transformation (148). STK4, however also has anti-apoptotic functions, as evinced by extensive neutropenia in patients lacking functional STK4 (159), and pancreas-specific knockout of STK3/STK4 in mice resulted in *reduced* pancreas size. This is due to onset of an acute pancreatitis-like phenotype resulting from the inability to maintain acinar parenchyma

postnatally (150, 151). Furthermore, while YAP1 has been reported as over expressed in human PDA tissue, its cytoplasmic sequestration is deregulated (149).

Given that: a) STK4 *did not* increase with acinar damage in the setting of chronic pancreatitis; b) STK4 remained exclusively cytoplasmic throughout PanIN-to-PDA progression; and c) the low rates of apoptosis reported in PDA models (130), I hypothesize that STK4 plays a different role than its canonical one as an antiproliferative, pro-apoptotic kinase, in the setting of pancreatic tumorigenesis. Based on the *in vivo* knockout studies, I suggest that increased STK4 levels in regions of acinar damage could represent an attempt to maintain acinar parenchyma following stress in the setting of oncogenic *Kras* and mutant *Trp53*. STK4 might subsequently decrease in PanIN-1 lesions with the establishment of a ductal niche, where after it could then re-emerge in response to increases in and deregulation of YAP1. An important future experiment would be to conduct IHC of YAP1 in serial sections adjacent to our STK4-stained sections to identify whether it is over expressed in PanIN lesions or whether this occurs following the establishment of invasive PDA.

Acinar cells were recently suggested to be more conducive to the formation of precursor PanIN lesions than ductal and centroacinar cells (126). Given its developmental role in the murine pancreas, STK4 could also help maintain a differentiated state conducive to the establishment and persistence of a 'ductal' phenotype upon PanIN establishment. Recent studies also show that cytoplasmic YAP1 can also exert a growth suppressive effect during intestinal stem cell regeneration (160). Thus, increased STK4 observed at later PanIN stages could also help maintain a pool of cytoplasmic YAP1, thereby promoting a differentiated ductal state at the expense of regeneration.

An additional function of elevated STK4 in the setting of mutant *Kras* and *Trp53* could be to prevent apoptosis. As STK4-induced apoptosis has been reported to be p53-dependent, its pro-apoptotic function may be abrogated in the setting of mutant p53. I did not see any induction of apoptosis, as measured by emergence of cleaved caspase-3 upon treatment of *KPC* cells with up to 7 hours of 1 μM staurosporine (STR), and also observed a decrease in STK4 levels with STR treatment in human PDA cells (not shown), which was paradoxically used in the initial studies where hippo kinases were first identified (161).

To address whether STK4 contributes to PanIN and PDA progression and cell maintenance, I have commenced with shRNA STK4 knockdown studies in primary pancreatic ductal cells, using two clones from the murine pGIPZ vector library from Thermoscientific. Pending successful knockdown of STK4, rates of cell proliferation, affects of TGFβ treatment on proliferation and EMT and response to apoptotic stimuli will be compared between empty vector and STK4 shRNA cells. The effects of STK4 knock down on the migratory phenotype and invasive potential by scratch wound assays and matrigel trans-well assays, respectively, will also be compared. Should STK4 levels impact migration and invasion, lung metastasis tail-vein assays could be performed with empty vector and STK4 shRNA cells.

Preliminary experiments have shown that TGFβ treatment leads to decreased STK4 in subconfluent 2-month and 4-month *KPC* cell lines but not in cells collected at end-stage disease (figure 7). There is an accompanying inverse increase in YAP1 in these early cell lines whereas YAP1 was lacking despite treatment in the end-stage cell line (figure 8). Thus, while further experiments in duplicate cell lines are required, these data suggest that STK4 could play an additional function to controlling YAP1 levels in PDA and that STK4 may have different roles at different stages of PDA progression. An alternative explanation is that YAP1 regulation changes

with PDA progression and that YAP1 has different functions at different disease stages. To determine whether the increased YAP1 levels seen with TGFβ treatment is due to decreasing STK4, I will also assess YAP1 levels in our shRNA experiments. I also propose to compare the ability to undergo EMT with TGFβ, rates of cell proliferation and response to apoptotic stimuli, between 2-month, 4-month and end-stage *KPC* cell lines with STK4 knocked down. This would add a temporal component to a potential functional role for STK4 in disease progression, which the results of our IHC experiments also suggest. Complementary to all of these proposed studies discussed above would be re- and over expression studies of STK4 in both shRNA and normal *KPC* cell lines. As no other Hippo pathway members have been directly implicated in PDA, these proposed experiments focusing on STK4 (and STK3 if necessary, see below) and YAP would serve as a starting point to determine the relevance of the pathway as a whole in PDA tumorigenesis.

Knockdown of STK4 in acinar cells (162) isolated from *KPC* mice could also provide insight into our hypothesis that STK4 is elevated in metaplastic structures as an attempt to maintain an acinar phenotype. Paneling certain stem cell markers in empty vector and STK4 shRNA cells will also be performed in both ductal and acinar STK4 knockdown cells. Similar to described for *KPC* ductal cells, empty vector and shRNA acinar cells would be subjected to stimuli inducing apoptosis, to cerulein treatment to induce differentiation and regeneration, and to TGFβ treatment and the levels of apoptosis, cell proliferation and morphological features would be examined. Naturally, the best method of determining whether STK4 has any causal role in disease initiation and progression would be to cross *KPC* mice with both STK4 and STK3/4 knockout mice, assess any changes in survival, in tumor histology, in tumor latency and disease onset, and in metastatic capability. As such, additional knockdown of STK3 may also be

required in the *in vitro* experiments to eliminate any potential for compensation that may result in no phenotype with STK4 shRNA alone.

The identification of STK4 through proteomic tracking of PDA from inception to end-stages of disease has thus led to additional questions and hypotheses that warrant further follow up. The other 6 markers that I validated by immunoblotting could be similarly further pursued. The progressive increased expression of SMAD2, FN1, p19ARF and RFC4 observed during PDA progression suggests that these might be stromal biomarkers. Indeed, IHC of FN1, a known extracellular marker, confirms this, although I also identified what looks to be high grade PanIN lesions and tumor epithelia expressing FN1 in focally invasive disease (Figure 9), which I can further corroborate by double-staining with the epithelial marker cytokeratin 19 (CK19). Epithelial expression of FN1 at later stages of disease progression warrants follow up to determine whether it might play a role in invasion and metastasis. While SMAD2 did not differentiate PDA from CP tissue, it warrants follow up given the recent report of the importance of stromal-derived TGFβ signaling in metastatic seeding in colorectal cancer (163). Expression of SMAD2 following treatment with stromal targeting agents (129, 130) could also be of interest.

BSG, FN1 and p19ARF all differentiated 2-month *KPC* pancreata from controls, and p19ARF additionally differentiated 2-month *KPC* from CP bulk tissue. Knockout studies have shown that p19ARF actually constrains PDA progression (134), yet it could behave differently in the setting of mutant p53. Recent studies in mutant p53 B-cell lymphoma cells have shown that elevated p14ARF helps stabilize mutant p53 (164). Crossing the p19ARF knockout mouse with *KPC* mice might therefore affect disease differently than in a *Trp53*-/- setting. If IHC staining shows increased epithelial p19ARF, then this functional relationship could be further pursued in the setting of pancreas cancer.

Along with STK4, BSG was the other marker that only increased between preinvasive and early invasive disease stages, and it also differentiated 4-month *KPC* tissue from CP. BSG is implicated in tumor-stroma interactions leading to secretion of MMP2, and is elevated in both tissue and plasma of PDA patients compared to healthy and CP controls (146, 165). Thus, IHC staining for BSG in 2-month and CP tissue should also be performed to help contextualize this biomarker.

Lastly, future antibody microarray experiments could help address questions regarding PDA disease and metastasis, as well identify markers differentiating PanIN lesions from MCN and IPMN. Complementary to the genetic comparisons made between primary and metastatic tumor tissue (166), proteomic comparisons between dissected metastases from various sites and primary tumor tissue can be made to identify proteins relevant to metastatic potential. Elevation of such markers in turn could be used as predictors of recurrence or metastatic spread following resection, and their role in the process of metastasis could be further examined using tail vein metastasis assays, HUVEC cell adhesion assays and their expression levels determined in mouse models lacking overt metastases. PanIN-to-cystic neoplasm comparisons by antibody microarray interrogation could aid in teasing out disease markers that provide insight into mechanisms underlying disease differentiation and that could serve as potential subtype-specific therapeutic targets. Many questions can be asked using our antibody microarray platform, and, as I shown, such experiments can provide valuable information for further, more targeted follow up experiments as well as for making more informed decisions in the clinic.

CHAPTER 3 TABLE AND FIGURES

Table 1. Candidate protein biomarkers of PDA from proteomic comparison of 2-month and 4-month *KPC* tissue to age-matched controls.

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
STMN1	stathmin 1	-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-gamma-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, 70kDa, polypeptide 1	-1.41	0.0306	-2.68	0.0004
TGFBR1	transforming growth factor, beta receptor 1	-1.00	0.0310	1.90	0.0009
DDHD2	DDHD domain containing 2	-1.19	0.0312	0.14	0.7792

AMBP	alpha-1-microglobulin/bikunin precursor	1.64	0.0322	1.20	0.1080
DOK1	docking protein 1, 62kDa (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), beta 1, 88kDa	-1.09	0.0358	-0.55	0.2743
MMP7	matrix metallopeptidase 7 (matrilysin, uterine)	0.99	0.0358	0.52	0.2490
STAT1	signal transducer and activator of transcription 1, 91kDa	-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, 37kDa	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
CDH1	cadherin 1, type 1, E-cadherin (epithelial)	-0.85	0.0424	-0.08	0.8403
BCAR1	breast cancer anti-estrogen resistance 1	-0.74	0.0426	-1.76	0.0009
BAG1	BCL2-associated athanogene	1.10	0.0438	-0.22	0.6725
AMPK	protein kinase, AMP-activated, alpha 2 catalytic subunit	-1.00	0.0439	0.91	0.0635
HSP90AB1	heat shock protein 90kDa alpha (cytosolic), class B member 1	-1.13	0.0446	-1.30	0.0496
GCM2	glial cells missing homolog 2 (Drosophila)	-1.93	0.0446	0.47	0.5872
PKM2	pyruvate kinase, muscle	2.50	0.0447	-1.37	0.3134
JUN	jun proto-oncogene (pS243)	-2.33	0.0455	0.65	0.5433
PRKACA	protein kinase, cAMP-dependent, catalytic, alpha	-0.90	0.0456	-0.14	0.7313
CCND1	cyclin D1	-1.58	0.0456	-0.81	0.2851
KPNA2	karyopherin alpha 2 (RAG cohort 1, importin alpha 1)	0.95	0.0476	0.30	0.5002
ATP6AP1	ATPase, H+ transporting, lysosomal accessory protein 1	-0.82	0.0487	-1.33	0.0034

PTPN11	protein tyrosine phosphatase, non-receptor type 11	-1.26	0.0490	2.95	0.0139

Table 1. Candidate protein tissue biomarkers of PDA. 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 by 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 2- and 4-month time points.

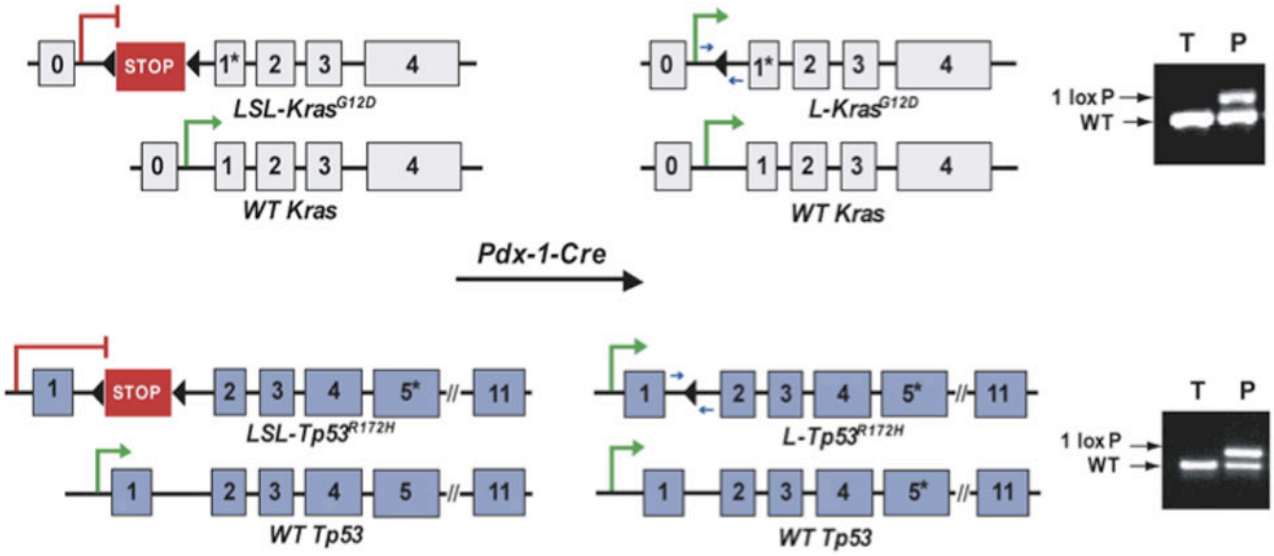

Figure 1. Representative schematic of the LSL-Cre strategy used to target mutant *Kras* and *Trp53* to the murine pancreas to generate *KPC* mice. Lox-stop-lox cassettes and downstream

Kras^{G12D} and *Tp53*^{R172H} mutations are introduced by homologous recombination into one allele of the endogenous *Kras* and *Trp53* loci. Cre recombinase expression, controlled transgenically by the pancreas-specific promoter *Pdx-1* or endogenously by homologous recombination of Cre downstream of another pancreas-specific promoter, *Ptf1a* or *p48* (not shown), drives conditional heterozygous expression of each mutation in all compartments (islet, acinar, ductal) of the pancreas. A specific PCR identifying the presence of the LSL cassette and downstream *Kras* or *Trp53* sequences can be used to identify inheritance of each mutant allele (not shown) in ear clip DNA, while the presence of "1LoxP" site in the genomic DNA from pancreata but not from ear clips, identifies pancreas-specific recombination (shown on the right). Image taken from (131).

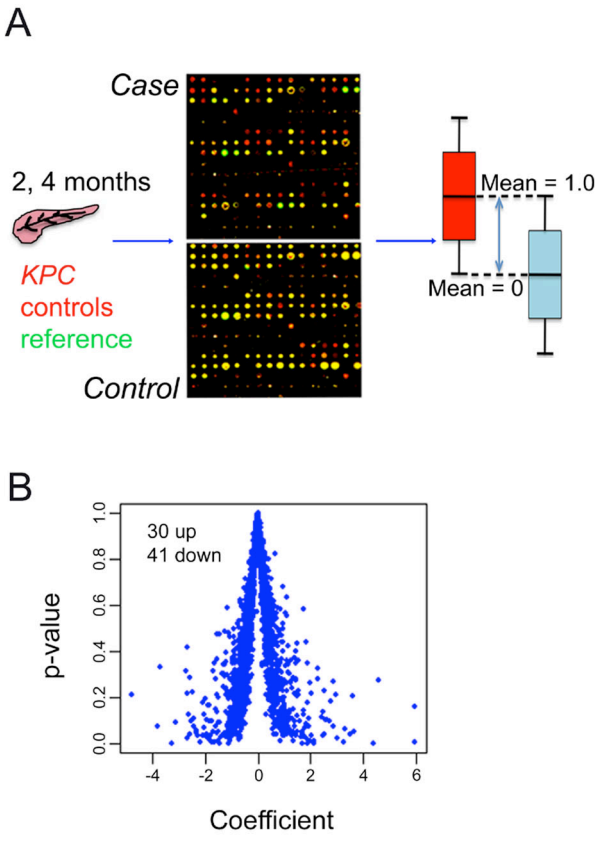

Figure 2. (A) Schematic of antibody microarray protocol. Bulk tissue lysates from the head of the pancreas from 2- and 4-month old *KPC* and littermate controls were labeled with red fluorescent Cy5 dye and a reference pool of wild type tissue with the green fluorescent Cy3. Individual Cy5-labeled samples were pooled with Cy3-reference sample and incubated on each array. Logistic regression analysis ranked candidate markers on their ability to distinguish *KPC* from control tissue with statistical significance. Each unique antibody feature is assigned a semi-quantitative coefficient based on the red/green intensity ratio, representing the number of standard deviations above (+) or below (-) control levels. All controls are normalized to have a mean of 0 and standard deviation of 1.

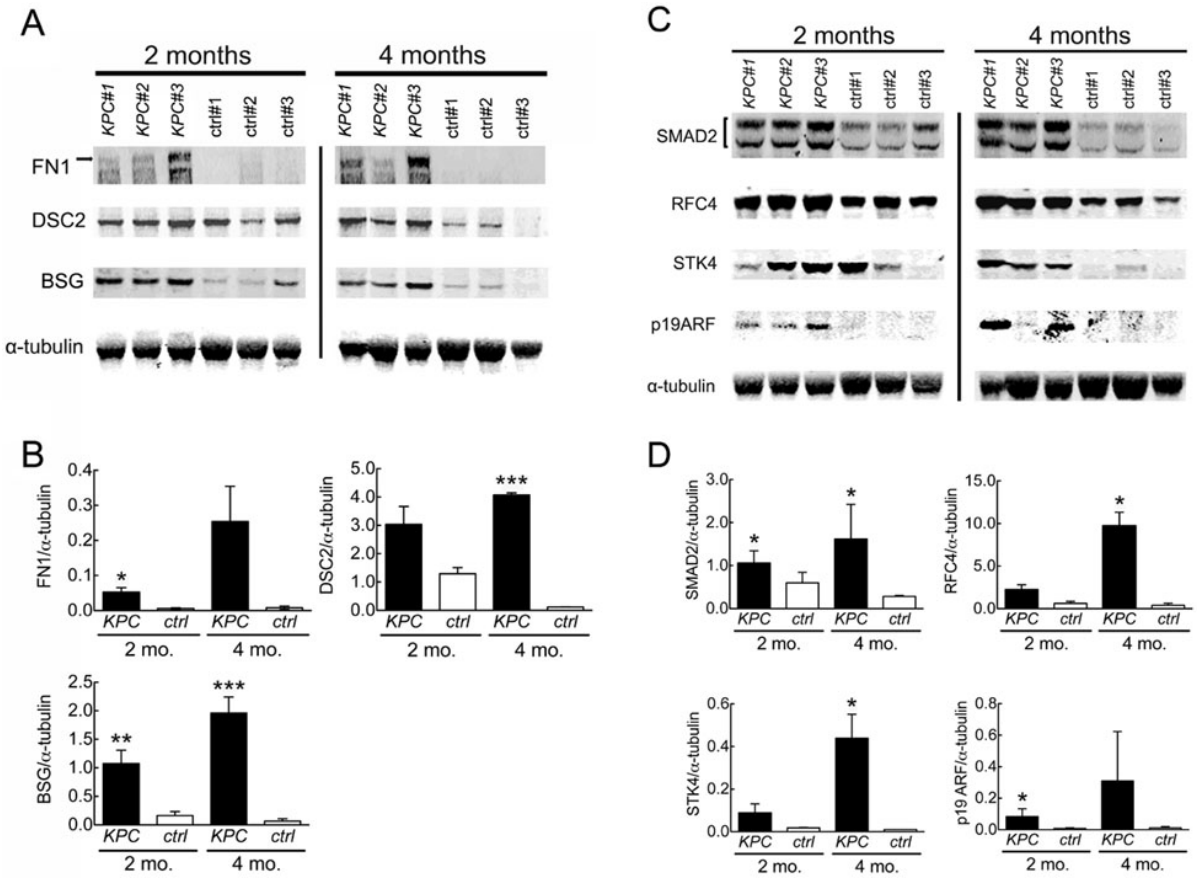

Figure 3. (A, B) The extracellular and membrane proteins FN1, DSC2 and BSG and the intracellular proteins (C, D) SMAD2, RFC4, STK4 and p19ARF distinguish *KPC* from control tissue with statistical significance. Protein levels from immunoblotting experiments (A, C) were quantified using densitometry and normalized to α-tubulin levels from 2 independent experiments of 5 *KPC* and 4 control lysates for each time point (for p19ARF a single experiment was performed). The arrow marks the doublet bands used to quantify FN1; doublet bands outlined were quantified for SMAD2. Statistically significant differences in protein levels between *KPC* and control tissue at individual time points was computed using an unpaired 2-tailed t-test (* = p-value < 0.05, ** = p-value < 0.01; *** = p-value < 0.001) in GraphPad Prism 5.0 (B, D). Mean values and standard deviations from replicate experiments are plotted. Increases in expression of RFC4, BSG and STK4 between 2- and 4-month *KPC* time points were also statistically significant (RFC4 p-value = 0.02, BSG p-value = 0.01, STK4 p-value = 0.05).

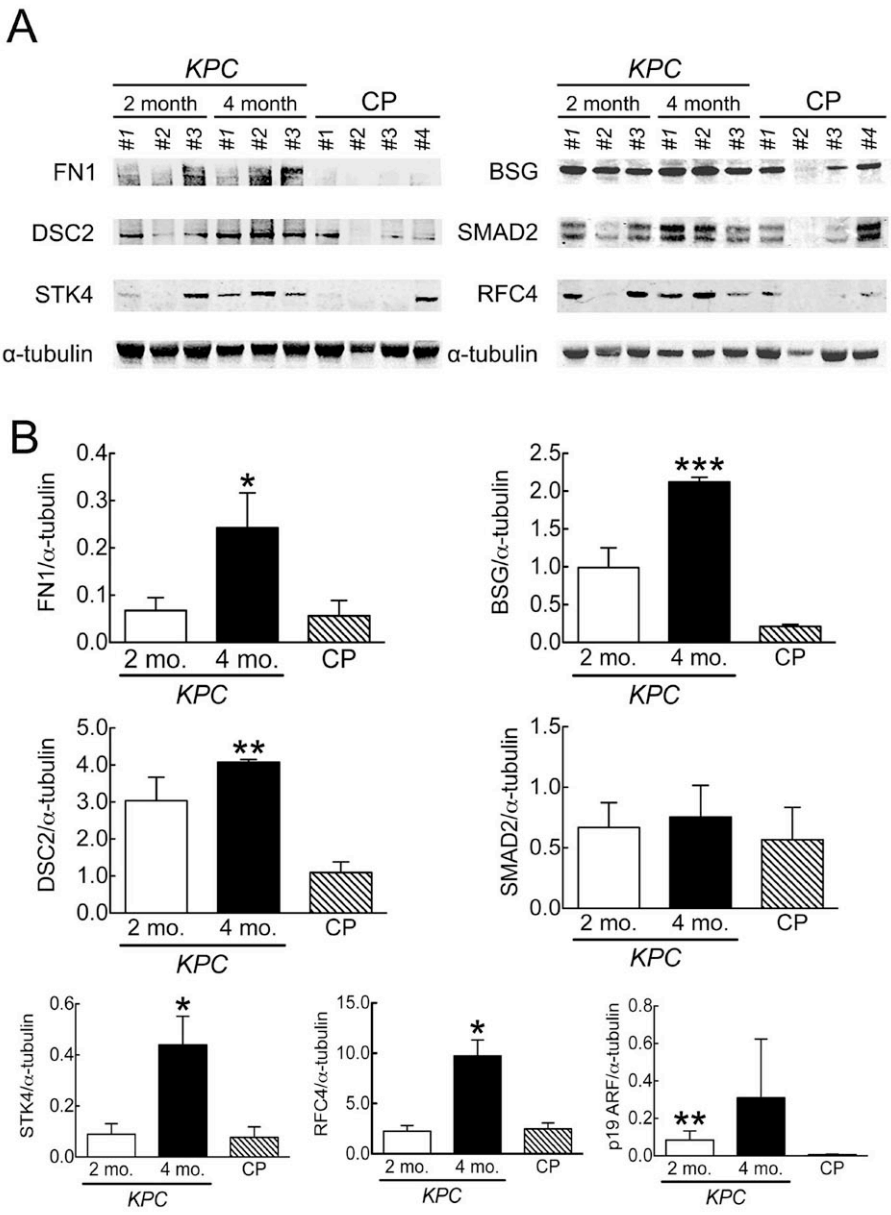

Figure 4. FN1, DSC2, BSG, STK4 and RFC4 distinguish 4-month *KPC* tissue from chronic pancreatitis (CP) tissue and p19ARF distinguishes 2-month *KPC* tissue from CP with statistical significance. Bands from immunoblotting experiments (A) for 2- and 4-month *KPC* tissue and tissue from wild-type animals injected with cerulein for 23 consecutive days, were quantified by

densitometry (B) and normalized to α-tubulin. Mean values and standard deviations from 2 independent experiments of 5 *KPC* and 4 control lysates for each time point (for p19ARF a single experiment was performed and only quantification of densitometry is shown) are plotted. Significance was determined using an unpaired 2-tailed t-test (* = p-value < 0.05, ** = p-value < 0.01; *** = p-value < 0.001).

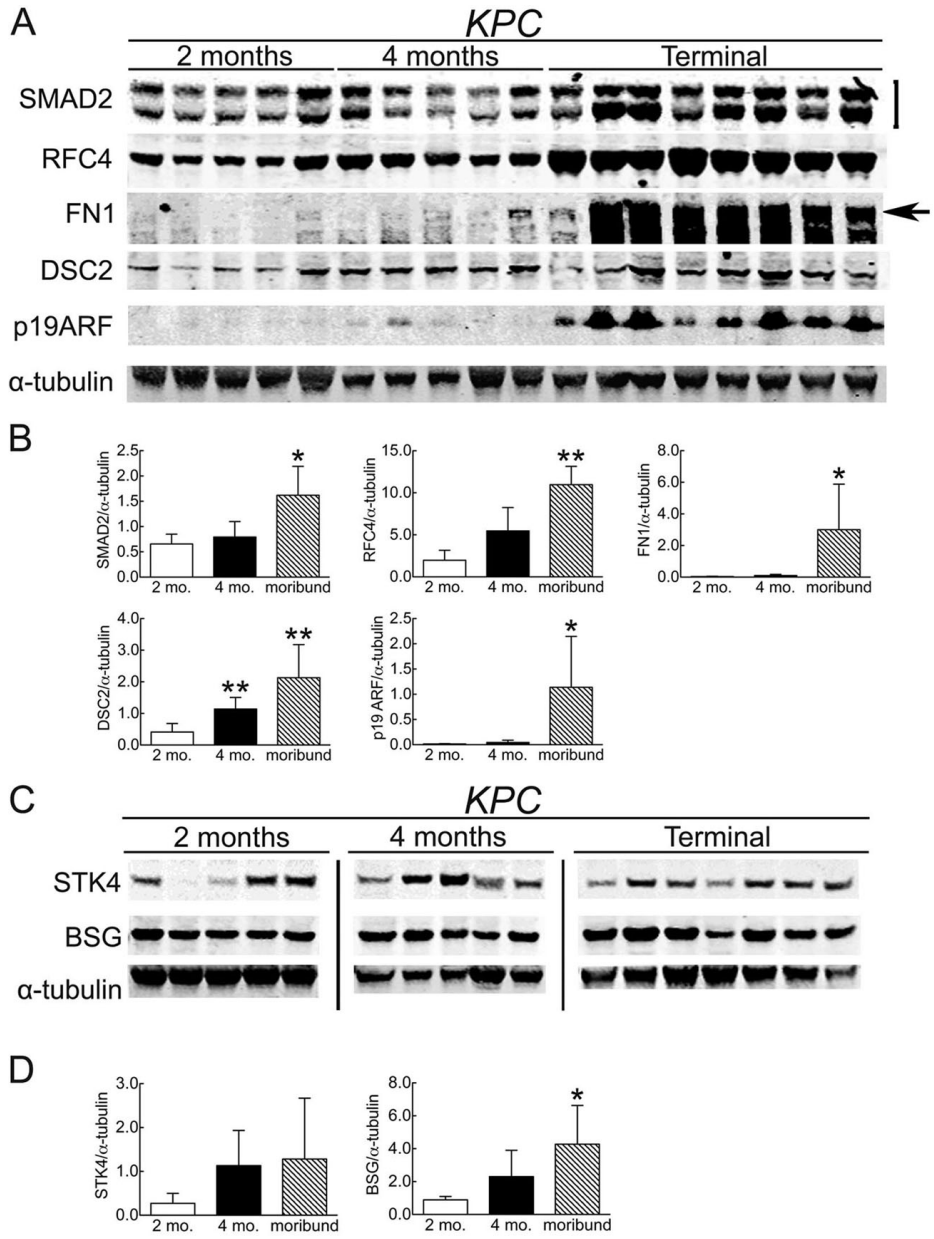

Figure 5. BSG and STK4 protein levels do not increase between 4-month and moribund stages of disease. Lysates from all 5 *KPC* animals from 2- and 4-month time points were run on the same gels as lysates from a separate cohort of 7 or 8 moribund *KPC* animals (A, C) and densitometry used to quantify (B, D) protein levels from 2 independent experiments (a single experiment was performed for p19ARF). Statistical significance was assessed using an unpaired 2-tailed t-test. Significant increases in protein levels between moribund and 4-month *KPC* were observed for

SMAD2, RFC4, FN1 and p19ARF and an increase in DSC2 was seen with a p-value = 0.07 (C). BSG and STK4 levels did not show appreciable increases (p-values = 0.14 and 0.9, respectively).

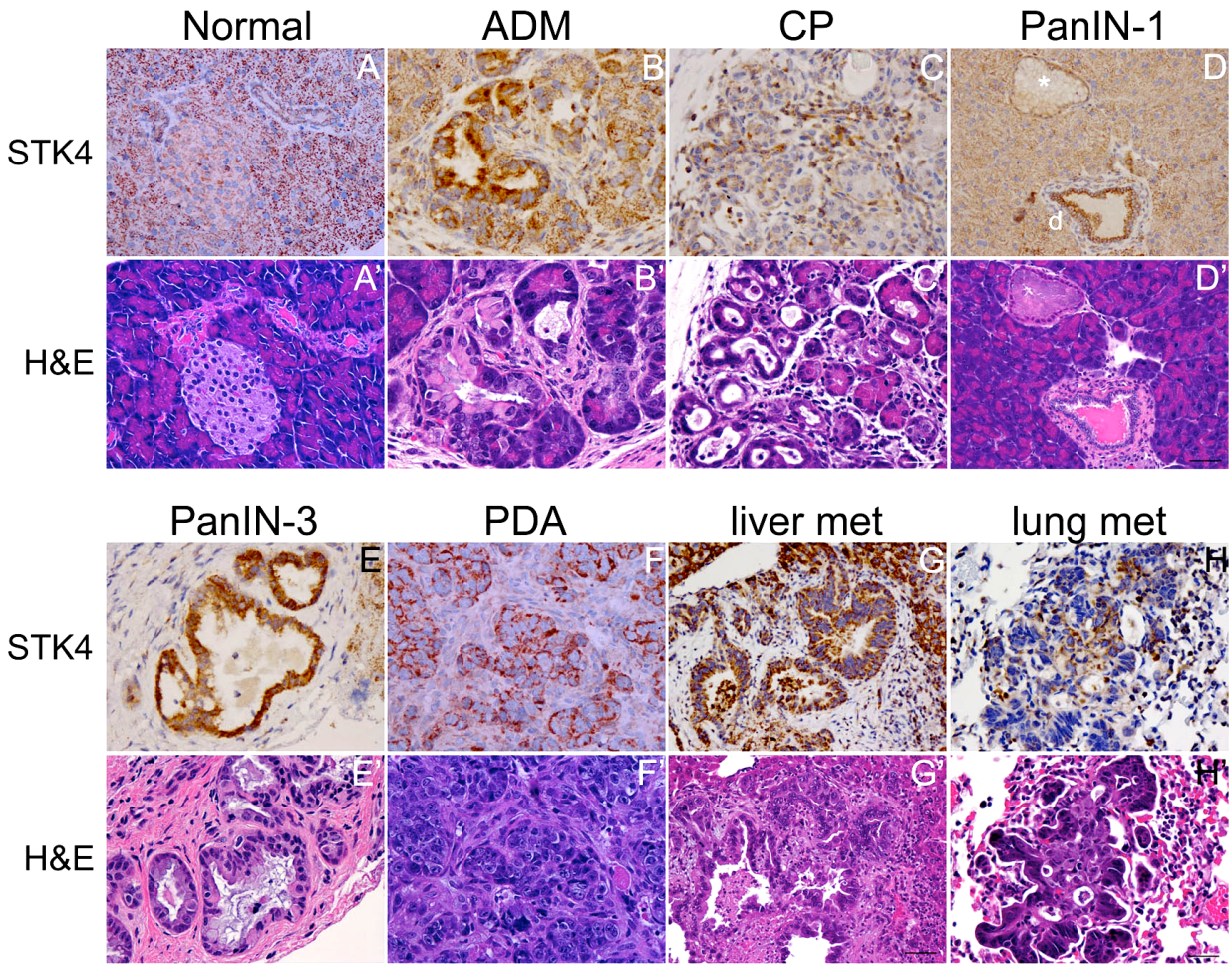

Figure 6. Immunohistochemistry (IHC) of STK4 reveals a bi-phasic cytoplasmic punctate expression pattern during PanIN-to-PDA progression in tumor epithelial cells that is not observed in chronic pancreatitis. Matched serial H&E sections are for each IHC image are included and designated with '. (A) Normal ductules and acini display a cytoplasmic punctate STK4 staining pattern. (B) STK4 expression increases in regions of acinar damage (ADM) in *KPC* tissue but does not increase in regions of acinar damage in chronic pancreatitis (CP) (C).

STK4 expression in PanIN-1 lesions (*) drops below that seen in normal ducts (d) and acini (D). Increased levels were observed again in PanIN-2 (E), PanIN-3 and invasive adenocarcinoma (F). In liver and lung metastases (G, H), STK4 levels remain comparable to what is observed in the primary tumor. Scale bars in C' and H' = 10 μm and are also representative for A, B, E, and F. Scale bars for D', G' = 20 μm.

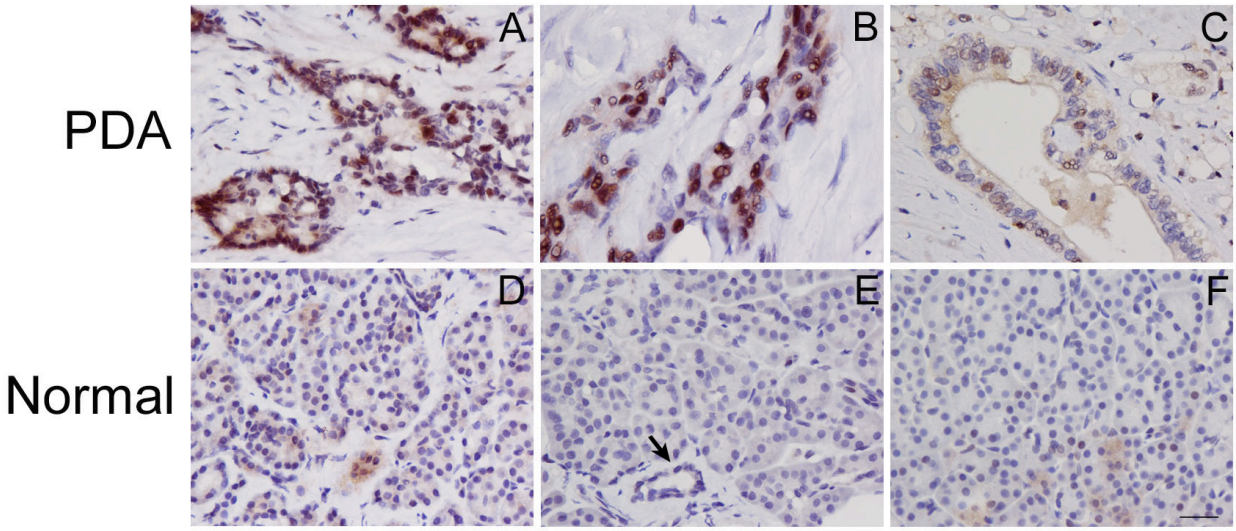

Figure 7. STK4 is expressed in human PDA. IHC of STK4 was performed on tissue microarrays (TMA) made from pancreas tissue collected through the Rapid Autopsy Program at the University of Nebraska. 23 PDA and 8 normal cores were evaluated. Representative images taken from PDA (A-C) and normal (D-F) TMA cores are shown. Scale bar in F = 10 μm is representative for all panels. Arrow in E indicates a normal duct showing a lack of staining, representative of the majority of normal ductal epithelium.

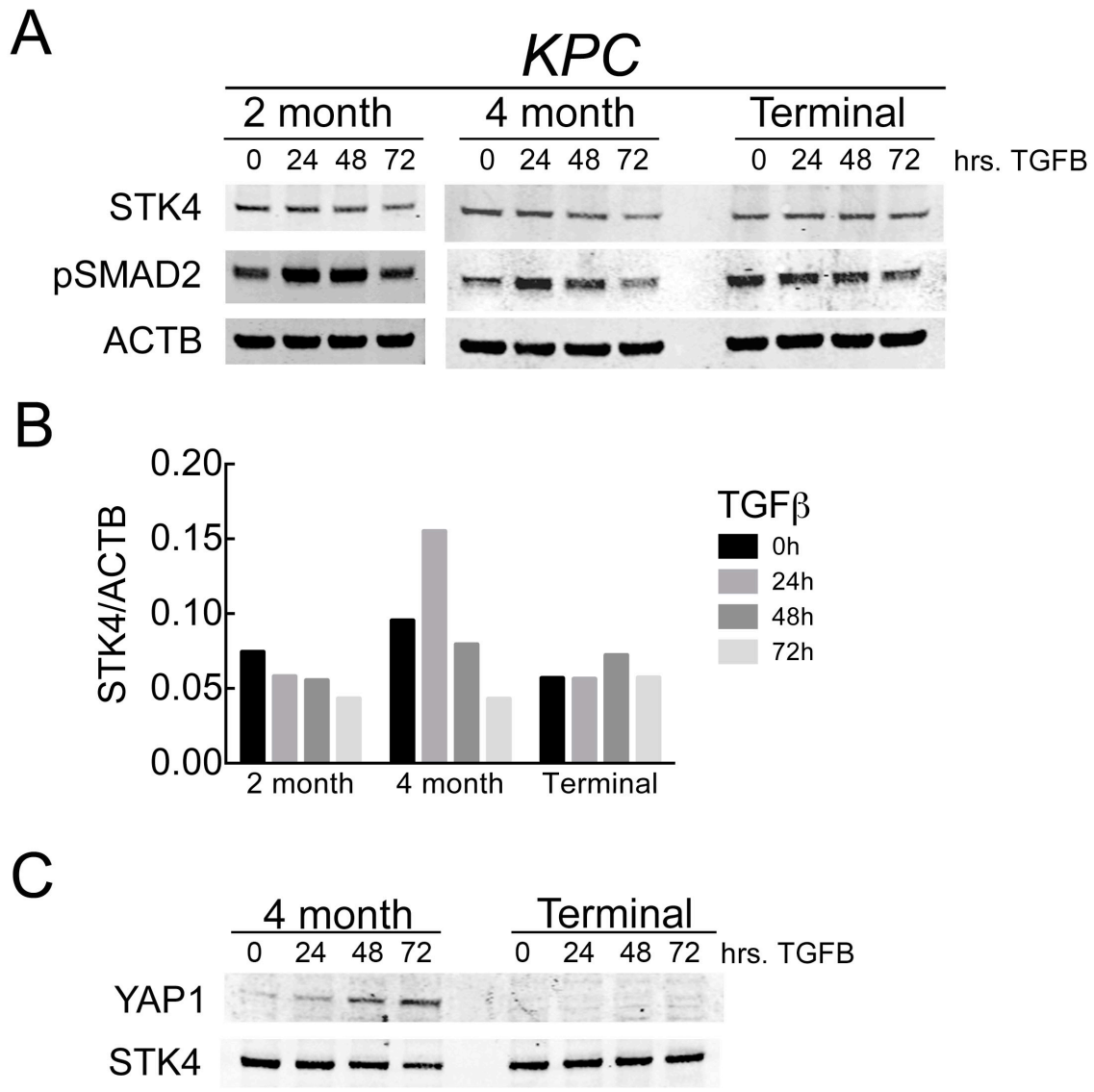

Figure 8.

TGFβ treatment has differential effects on STK4 levels in primary *KPC* cells isolated at different stages of PDA progression. (A) TGFβ treatment (5ng/ml) causes a decrease in STK4 in 2-month and 4-month *KPC* primary cell lines but not in a cell line isolated at terminal disease stages. This could be due to decreased activation of the TGFβ pathway in the end-stage cells, as shown by a less prominent increase in pSMAD2 levels than in 2- and 4-month cell lines. (B)

Densitometric quantification of bands in (A) normalized to β-actin levels. (C) Decreased STK4 in 4-month *KPC* cells with TGFβ treatment is accompanied by a concomitant increase in YAP1 levels. In a terminal *KPC* cell where decreased STK4 does not occur with treatment, YAP1 is lacking. All cells were subconfluent at the commencement of TGFβ treatment.

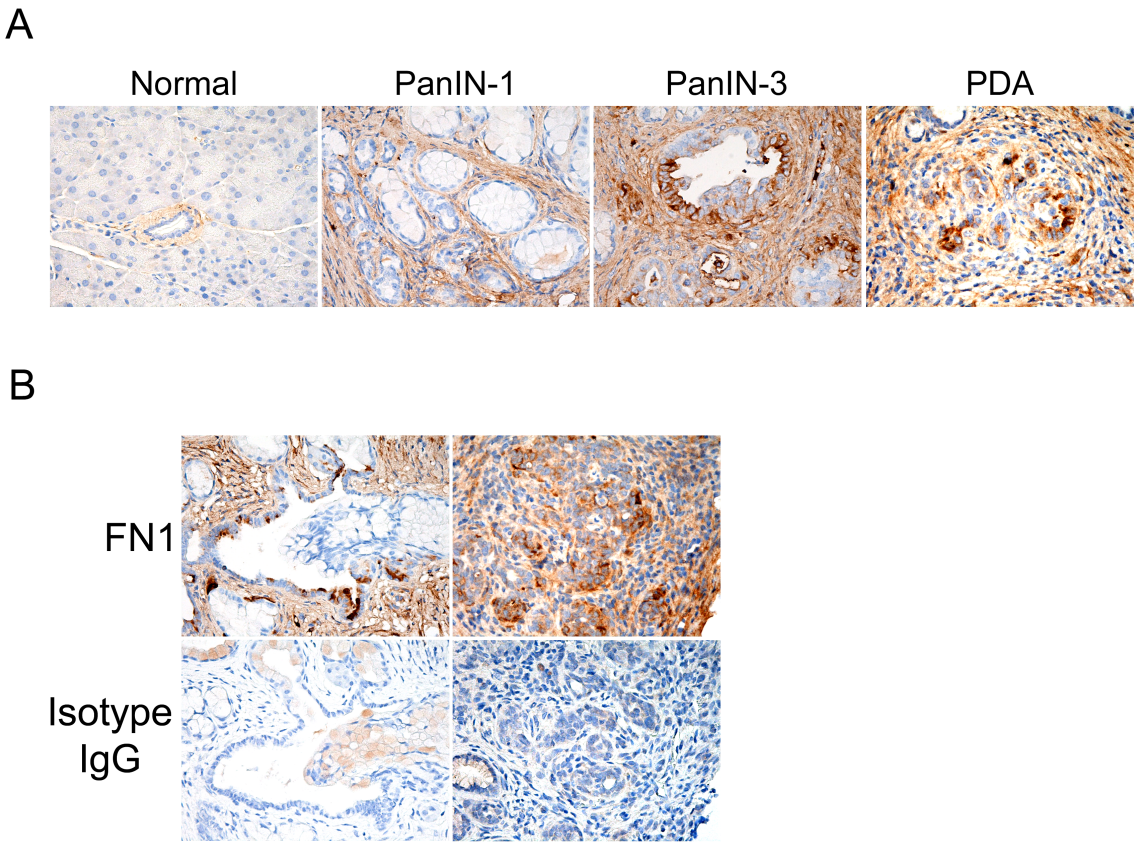

Figure 9. FN1 expression increases in the stroma with progression from PanIN to PDA and is found in tumor epithelia in higher-grade PanIN lesions and focally invasive PDA. (A) FN1 is absent from normal pancreatic parenchyma. Stromal FN1 is dramatically increased as early as PanIN-1 lesions and is evident surrounding higher grade PanIN lesions and focally invasive PDA. Epithelial FN1 also becomes apparent in a subset of PanIN-3 cells and in focally invasive PDA. (B) FN1 staining of a subset of cells in PanIN lesions and tumor epithelia is specific, as it is not observed in adjacent serial sections stained with an isotype control.

CHAPTER 3 SUPPLEMENTARY TABLE AND FIGURES

Supplementary Table 1. Markers identified by antibody microarray interrogation of KPC tissue previously reported by others as putative pancreas cancer markers.

Gene name	Protein name	coefficient	p-value	Previously reported	Method	Neoplasm	Fold Change
KLK6	kallikrein-related peptidase 6	4.40	0.0010	1 ,2, 3	DNA microarray, IHC	PanIN-1A/B, PDA	2.1
TP53	tumor protein 53	-1.11	0.0029	1, 4, 5	DNA microarray, IHC, ELISA	PanIN-2/3, PDA	2.29; GOF mutation/LOH
PKM2	pyruvate kinase, muscle	2.50	0.0036	6, 7, 8	DNA microarray, ICAT	IPMN, CP, PDA	94 (IPMN); 2.33 (CP); 18 (PDA, DNA microarray); 2.3 (PDA, ICAT/MS)
CDH (pan)	cadherin (pan)	-3.27	0.0042	9	IHC	PanIN, PDA	CDH1: increased (PanIN), decreased (PDA)
				10, 11	DNA microarray, IHC, RT-PCR	PDA	CDH11: 5.72 (PDA), increased (CP & PDA)
				12	DNA microarray	PDA	CDH17: 17.5 (PDA)
				13	IHC	PDA	CDH2: increased
				6, 14, 15	IHC, RT-PCR, DNA microarray	PDA	CDH3: increased (PDA); 8.95 (IPMN); >5 (PDA)

				16	DNA microarray	PaNET	CDH5: increased 1.71 in malignant/benign PaNET
				17	DNA microarray	PanIN-1B/2	CDH8: 1.85
LMTK2	lemur tyrosine kinase 2	1.88	0.0044				
FN1	fibronectin 1	2.15	0.0045	11, 18, 19	cDNA microarray, IHC	PDA, CP (stroma)	>2
IRS2	insulin receptor substrate 2	-2.30	0.0048	6, 20	IHC, northern blot, DNA microarray	PDA, IPMN	6.51 (IPMN)
BRAF	v-raf murine sarcoma viral oncogene homolog B1	-1.63	0.0067	21, 22	sequencing	PanIN-1/PDA	GOF mutation
BRCA2	breast cancer 2, early onset	3.24	0.0080	23, 24	sequencing	PanIN-3/PDA	LOF mutations
GCM2	glial cells missing homolog 2 (Drosophila)	-1.93	0.0083				
STAT3 (pY705)	signal transducer and activator of transcription 3 (pY705)	-2.18	0.0086	6, 25, 26, 27	DNA microarray, IHC, antibody microarray	IPMN, PDA	9.95 (IPMN)
STK4	serine/threonine kinase 4	5.98	0.0101				
FABP3	fatty acid binding protein 3	1.98	0.0115				
TNC	tenascin C	1.67	0.0120	28, 29, 30, 31	PanIN-1/2/3, PDA, CP	RT-PCR, DNA microarray, ELISA, IHC	4.32 (PDA); 8.46 (metastases); 1.3 (PDA vs CP); 6.9 (ICAT PDA vs. normal)
HOXD13	homeobox D13	-1.06	0.0122	32	RT-PCR, IHC	PDA	decreased
CTNNB1	catenin (cadherin-associated protein), beta	-1.09	0.0136	6, 9, 33, 34, 35, 36	IHC, RT-PCR	PanIN, PDA, cell lines, ACC,	100-550 (PDA cell lines), 3.5

	1, 88kDa					IPMN, pancreatobl astoma	(IPMN)
CDKNA2	p19 ARF (mouse-specific)	0.88	0.0151				
RBM15	RNA binding motif protein 15	-1.74	0.0152				
USP3	ubiquitin specific peptidase 3	2.12	0.0152				
PRKACA	protein kinase, cAMP-dependent, catalytic, alpha	-0.90	0.0178	37	WB	PDA	Increased
IL18	interleukin 18 (interferon-gamma-inducing factor)	-1.06	0.0178	38, 39, 40	RT-PCR, IHC, ELISA, SAGE	PDA, cell lines, serum	
CCND1	cyclin D1	-1.58	0.0188	9, 18, 41, 42	IHC, northern blot, DNA microarray	PDA, CP, PanIN-2/3, PaNET, IPN,	>3 fold (PDA)
BAD	BCL2-associated agonist of cell death	-0.74	0.0206				
MGMT	O-6-methylguanine-DNA methyltransferase	-1.83	0.0214	43	activity assay, IHC	PDA	
MEK1	mitogen-activated protein kinase kinase 1 (pT291)	-1.06	0.0234				
DSC2	desmocollin 2	3.04	0.0236	44	IHC	PDA	increased
GRB2	growth factor receptor-bound protein 2	-2.68	0.0243				
PTPRB	protein tyrosine phosphatase, receptor type, B	-0.66	0.0273				
NECAB3	N-terminal EF-hand calcium binding protein 3	1.67	0.0294				
SMAD2	SMAD family member 2	1.87	0.0296	19, 45	DNA microarray, northern blot	PDA	3.2 (northern blot); 1.98 (DNA microarray); 1.3 (PDA vs

							CP)
IGF2	insulin-like growth factor 2 (somatomedin A)	-1.10	0.0302	46	ISH	Pancreatoblastoma	increased
RPS6KB1	ribosomal protein S6 kinase, 70kDa, polypeptide 1	-1.41	0.0306	47	DNA microarray	PaNET	downregulated
TGFBR1	transforming growth factor, beta receptor 1	-1.00	0.0310	48	mRNA	cell lines	decreased
BAG1	BCL2-associated athanogene	1.10	0.0312				
DDHD2	DDHD domain containing 2	-1.19	0.0312				
AMBP	alpha-1-microglobulin/bikunin precursor	1.64	0.0322	17, 49, 50	ICAT, DNA microarray	PDA, PanIN	2.72 (ICAT, PDA); -0.2 (PanIN), -3 (DIGE in PDA sera)
DOK1	docking protein 1, 62kDa (downstream of tyrosine kinase 1)	-1.95	0.0331				
MEF2A	myocyte enhancer factor 2A	-1.09	0.0347	2	DNA microarray	PanIN-2	2
CSF3R	colony stimulating factor 3 receptor (granulocyte)	-1.28	0.0347				
MES2	Mes2	2.50	0.0351				
STAT1	signal transducer and activator of transcription 1, 91kDa	-0.68	0.0359	7	DNA microarray	PDA, CP	3 (PDA vs. CP)
FLT1	fms-related tyrosine kinase 1 (VEGFR)	-0.77	0.0360	51	northern blot, IHC	PDA	3.2
STMN1	stathmin 1	-1.52	0.0363	15, 52, 53	DNA microarray, 2-DE	PDA, cell lines	>5 fold (PDA, cell lines)
MITF	microphthalmia-associated transcription factor	-1.05	0.0364				
JUN (pS243)	jun proto-oncogene (pS243)	-2.33	0.0369	54, 55	PDA	IHC	total JUN: increased

FES	feline sarcoma oncogene	1.30	0.0376	56	CGH	tumor xenografts	gene amplification
MAPKAPK2 (pT334)	mitogen-activated protein kinase-activated protein kinase 2	-2.08	0.0376				
RFC4	replication factor C (activator 1) 4, 37kDa	1.68	0.0377				
BSG	basigin (Ok blood group)	0.95	0.0392	6, 48, 57, 58, 59, 60	DNA microarray, RT-PCR, IHC, ELISA, ICAT	IPMN, PDA, serum	18.6 (IPMN), 2.2 (PDA)
KLK14	kallikrein-related peptidase 14	1.70	0.0400				
KRT18	keratin 18	-2.59	0.0400	1	DNA microarray	PDA, PDA cell lines	normalized counts
TP53	tumor protein 53	1.33	0.0400	2, 4, 5	DNA microarray, IHC, ELISA	PanIN-2/3, PDA	2.29; GOF mutation/LOH
MDM2	Mdm2, p53 E3 ubiquitin protein ligase homolog (mouse)	-1.29	0.0400	61	IHC, northern blot	PDA, cell lines	6.4 fold (PDA, mRNA)
CLDN18	claudin 18	3.52	0.0412	6, 62, 63, 64	DNA microarray, IHC	PanIN, PDA, IPMN, MCN	37.85 (IPMN); 12.91 (PDA)
PRDX2	peroxiredoxin 2	0.61	0.0413	2	DNA microarray	PDA	2.02
HRAS	v-Ha-ras Harvey rat sarcoma viral oncogene homolog	0.70	0.0415	7	DNA microarray	PDA, cell lines	6 (PDA), 10 (cell lines)
TACSTD1	epithelial cell adhesion molecule	-1.78	0.0422				
MSI2	musashi homolog 2 (Drosophila)	1.33	0.0422				
MAMDC4	MAM domain containing 4	2.01	0.0424				
CDH1	cadherin 1, type 1, E-cadherin (epithelial)	-0.85	0.0424	9	IHC	PanIN, PDA	CDH1: increased (PanIN),

							decreased (PDA)
BCAR1	breast cancer anti-estrogen resistance 1	-0.74	0.0426				
KPNA2	karyopherin alpha 2 (RAG cohort 1, importin alpha 1)	0.95	0.0438	18	PDA	DNA microarray	4.8 (PDA); 6.9 (cell lines)
AMPK	protein kinase, AMP-activated, alpha 2 catalytic subunit	-1.00	0.0439				
HSP90AB1	heat shock protein 90kDa alpha (cytosolic), class B member 1	-1.13	0.0446	6	DNA microarray	IPMN	11.2
HOXA5	homeobox A5	-1.47	0.0446	17	PanIN-1/2, PDA	RT-PCR, DNA microarray	10 (pooled PanIN samples); 3 fold (PDA)
MMP7	matrix metallopeptidase 7 (matrilysin, uterine)	0.99	0.0447	1, 2, 51, 65, 66	IHC, DNA microarray, SAGE	PanIN, PDA, cell lines, IPMN	2.86 (PanIN-3); 8.79 (PDA)
NLRP7	NLR family, pyrin domain containing 7	-2.61	0.0455				
CAV1 (pY14)	caveolin 1 (pY14)	1.77	0.0456	18, 67, 68	PDA, PDA cell lines	DNA microarray, northern blot, IHC	total CAV1: 2.4, 10 (PDA vs normal); 21.8 (PDA cell lines)
BIRC5	baculoviral IAP repeat containing 5 (survivin)	1.45	0.0476	15, 69, 70 71	RT-PCR, IHC, Western blot	PanIN-2/3, PDA, IPMN	22 (RT-PCR), 5 (DNA microarray); 4.8 (PanIN-1), 10 (PanIN-2), 14 (PanIN-3)
ATP6AP1	ATPase, H+ transporting, lysosomal accessory protein 1	-0.82	0.0487				
PTPN11	protein tyrosine phosphatase, non-receptor type 11	-1.26	0.0490				

Supplementary Table 1. Markers identified by antibody microarray interrogation of *KPC* tissue that have been previously reported by others as putative pancreas cancer markers. A literature search was conducted to determine whether the markers identified by our antibody microarray analysis have been previously implicated as candidate biomarkers of pancreas cancer. Included in the table are the references from our literature search, the methods employed by other groups, the neoplasm in which individual markers were identified, and, if relevant and available, the fold change observed for individual markers in each respective neoplasm.

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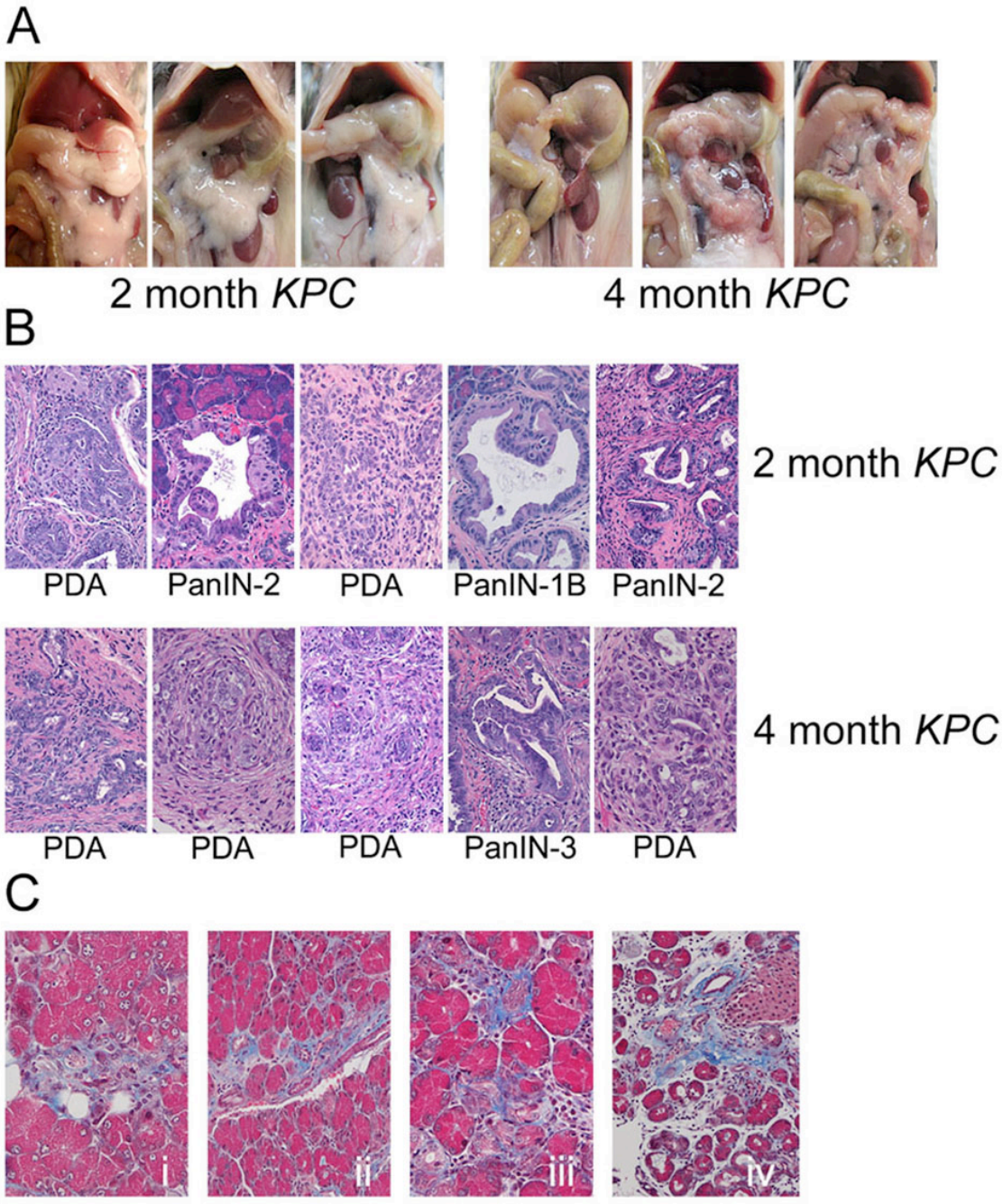
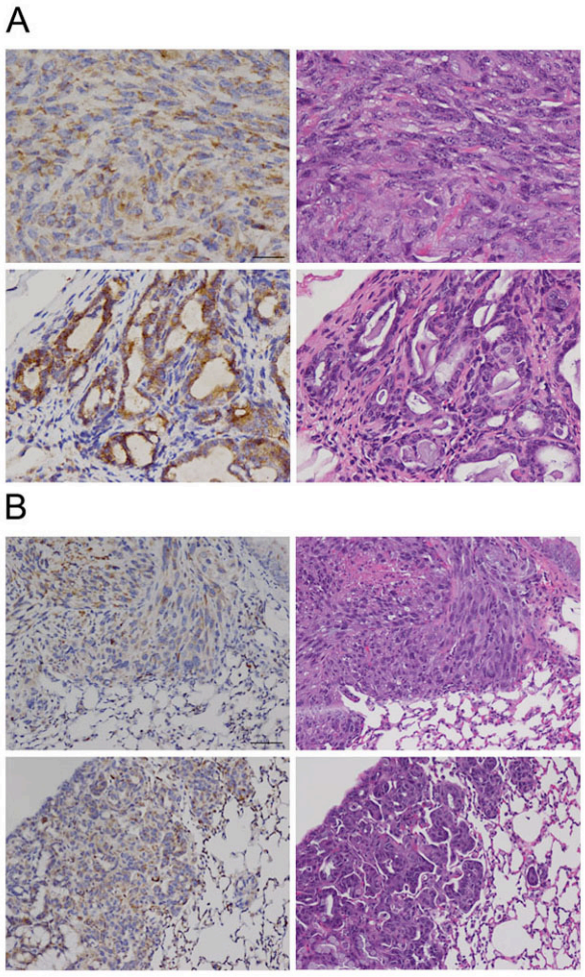


Supplementary figure 1. Assessment of disease progression and evidence of chronic pancreatitis in *KPC* and cerulein-treated animals, respectively. Gross pathology (A) and histological grading of 2- and 4-month *KPC* animals (B) shows that 4-month *KPC* animals exhibit more advanced disease than 2-month *KPC* animals at necropsy. H&E sections 100 μm apart and adjacent to

tissue sections used in the antibody microarray experiments were used for histological assessment. Images are representative of disease stage at the time of necropsy for individual *KPC* animals. In (C) Masson's Trichrome staining of tissue from animals treated with cerulein shows evidence of interlobular fibrosis, a feature of chronic pancreatitis. Collagen is shown in blue and images are from 2 animals (i,ii and iii, iv) in the chronic pancreatitis cohort.



Supplementary figure 2. PDA with sarcomatoid morphology in the primary tumor (A, top) and a matched lung metastasis (B, top) exhibits weaker STK4 staining than a different region of the

primary tumor (A, bottom) and a separate lung metastasis (B, bottom), both with more glandular morphology. Scale bar in A = 10 μm; in B = 20 μm. H&E images were taken from the primary tumor and metastases from a moribund *KPC* animal.

CHAPTER 4

Cross-species antibody microarray interrogation identifies a 3-protein panel of plasma biomarkers for the early detection of pancreas cancer

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Running title: Early detection plasma markers for pancreas cancer

ABSTRACT

Purpose: Pancreatic ductal adenocarcinoma (PDA) is now the third leading cause of cancer death in the United States, and its incidence is on the rise (American Cancer Society, Facts and Figures 2013). Even patients presenting with resectable disease often succumb to recurrent, metastatic disease, making earlier detection critical. To discover early detection biomarkers of PDA, we used a customized, high dimensional antibody microarray platform to interrogate both mouse and pre-diagnostic human plasma.

Experimental Design: Plasma collected at early disease stages from a highly faithful mouse model was examined with our array platform. In parallel, we interrogated a large cohort of pre-diagnostic human plasma provided by the Women's Health Initiative (WHI), in which plasma was drawn up to four years prior to patients succumbing to PDA.

Results: We identified 33 up-regulated markers in murine plasma and 23 up-regulated candidates in our pre-diagnostic human plasma. ERBB2 and TNC, both previously reported in diagnostic samples, were identified across species. In addition, multiple antibodies against ESR1 were up-regulated in WHI case plasma. The AUC for this 3-protein pre-diagnostic marker panel was 0.677, similar to some biomarkers reported in specimens drawn upon surgical resection of PDA.

Conclusions: Our antibody arrays identified putative early detection markers in plasma drawn up to 4 years prior patients succumbing to PDA, providing a proof-of-principle that our platform can identify markers at earlier stages than current screening modalities or clinical symptoms. We suggest these markers, which have been extensively validated here and by others, be assessed in high-risk patients cohorts undergoing more rigorous disease monitoring.

INTRODUCTION

Whereas survival rates for other cancers including breast, colon and prostate have improved in the past two decades, the prognosis for pancreatic ductal adenocarcinoma (PDA), or pancreas cancer, has remained dismal. Five-year survival rates remained unchanged at ~6% from 2002-2008 (American Cancer Facts and Figures, American Cancer Society, 2013), which is of additional concern given the 1.2% increase in incidence from 1999-2010 (SEER Incidence, seer.cancer.gov/faststats/selections). Surgical resection is the only curative option, yet the majority of patients (>80%) still present with unresectable disease at diagnosis, highlighting the need for improved early detection strategies (11). Patients diagnosed with localized, resectable disease have 5-year survival rates that improve from around 5% to a modest 20% (1), with a median post-resection survival of ~17 months (6). These results reflect not only the micrometastatic capability of PDA early in disease progression, but also that staging of disease at resection may not always be precise. Thus, tests that lead to earlier diagnosis are greatly needed to improve upon current survival rates.

The retroperitoneal location of the pancreas further impedes safe and efficient biopsy, making a diagnostic test using readily accessible biological fluids an attractive alternative in the clinic. The only FDA-approved blood-based marker for pancreatic cancer is CA19-9, but with sensitivities and specificities ranging from 60-70% and 70-85%, respectively (22), it is not recommended for use as a screening tool, a diagnostic, nor to determine operability. Rather, CA19-9 is only used to assess response to treatment and/or disease recurrence (44, 141). Thus, numerous studies have focused on identifying serum, tissue, ascites and cyst fluid markers for early detection, yet many of these samples are consequently derived from patients upon diagnosis, at which point almost all will succumb to disease. Biological fluids collected in large,

prospective longitudinal cohort studies provide a source of specimens drawn prior to disease diagnosis. These are especially invaluable for early detection of PDA, where incidences are low and the disease is largely asymptomatic.

I took a parallel proteomic approach to discover and validate early detection plasma biomarkers. First, we used our antibody microarray platform (78, 98, 100), this time customized for interrogation of pancreas cancer samples, in conjunction with plasma drawn from a highly faithful mouse model of pancreas cancer (131) to discover putative early disease markers of PDA. We subsequently looked for cross-species overlap using pre-diagnostic plasma from a large cohort of women who later succumbed to PDA provided by the National Cancer Institute's Women's Health Initiative. Focusing on plasma membrane and secreted proteins identified as up-regulated by our array experiments, our approach identified two markers overlapping between mouse and human datasets, both of which have been previously implicated with PDA. Another marker, ESR1, was of additional interest as a pre-diagnostic candidate as two distinct antibodies detected elevated ESR1 in the pre-diagnostic plasma from the WHI cohort. In an additional set of array experiments on plasma samples collected from patients upon diagnosis, we were able to corroborate elevated expression of the set of three markers. The implications of our findings and their applicability to early detection of pancreas cancer are further discussed for this 3-protein panel of biomarkers.

MATERIALS AND METHODS

Patient samples

Pre-diagnostic samples. Pre-diagnostic pancreas cancer and matched control EDTA-collected plasma samples were provided by the National Cancer Institute's Women's Health Initiative's (WHI) observational hormone replacement therapy study. Control samples were matched based on the following criteria: age at screening, year of WHI enrollment, alcohol consumption at baseline, race/ethnicity, smoking status (never, past, current), diabetes history (yes or no), hormone replacement therapy (none, estrogen only, estrogen and progesterone), blood draw visit (baseline only, baseline and year 3, year 3 only) and follow-up duration (i.e. controls must have been followed and lived for at least the same interval between enrollment and pancreas cancer diagnosis as their matched cases). A reference pool of EDTA-collected plasma was created by pooling plasma drawn from a group of seven female volunteers from the Fred Hutchinson Cancer Research Center aged 27-45. All samples were de-identified and the study was approved by the FHCRC Institutional Review Board.

Diagnostic samples. Diagnostic EDTA-collected plasma samples were provided by the Center for Accelerated Translation in Pancreas Cancer (CATPAC) at the Seattle Cancer Care Alliance, Seattle, Washington. Unmatched control plasma samples were collected and processed using the same methods and laboratory. All patient information provided was done so in accordance with the Institutional Review Board at the Fred Hutchinson Cancer Research Center.

Mouse plasma collection. EDTA-collected mouse plasma samples were acquired from live *Kras*$^{LSL\text{-}G12D/+}$*;Trp53*$^{R172H/+}$*;Cre (KPC)* and age-matched *KP* and *Cre* control mice on a C57BL6/SV129 mixed background following brief anesthesia with isoflurane. Plasma (maximum 200 μl) was collected every two weeks into tubes containing 10 μl 0.5M EDTA. Samples were centrifuged at 6500 rpm for 10 minutes, the supernatant was collected and re-

centrifuged at 13,000 rpm for and additional 10 minutes, and then stored at -80ºC until use in the array experiments.

Mouse tissue collection and induction of chronic pancreatitis. Tissue from the head of the pancreas of 2-month old, 4-month old and end-stage disease *KPC* and age-matched *KP* and *Cre* controls animals was collected at necropsy and flash frozen until use in antibody microarray experiments. Tissues were similarly harvested from a cohort of six 2-month old WT mice injected intraperitoneally with 100 μl cerulein (50 μg/ml) for 23 consecutive days to induce chronic pancreatitis and necropsies were conducted within 6 hours of the final injection. All mouse husbandry was conducted in accordance with a protocol approved by the Institutional Animal Care and Use Committee at the Fred Hutchinson Cancer Research Center.

Sample preparation for antibody microarray analysis

All plasma samples were depleted of IgG and serum albumin using the Proteoprep Immunoaffinity Albumin and IgG Depletion Kit per the manufacturer's instructions (Sigma Aldrich, St. Louis, MO). Depleted case and control plasma samples and mouse tissue lysates (250 μg of total protein for pre-diagnostic, diagnostic and mouse plasma samples and 200 μg for mouse tissue samples) were labeled with N-hyroxysuccinimide (NHS)-Cy5 (GE Health Biosciences, Pittsburgh, PA) and reference plasma and a pool of tissue lysates collected from wild type mice with NHS-Cy3.

KPC and age-matched matched control pancreatic tissue collected at 2 months, 4 months and end-stage disease were weighed and lysed in a 10:1 ratio of lysis buffer to tissue weight using 1% NP-40, 0.25% deoxycholate, 0.25% octyl-β-d-glucopyranoside and 0.25%

amidosulfobetaine-14 supplemented with phosphatase inhibitors, Roche protease inhibitor cocktail (Roche USA, Indianapolis, IN) and 1mM PMSF.

Antibody microarray experiments (printing and hybridization)

Antibody microarray slides were printed and labeled plasma and tissue samples incubated on Nexterion slide H (Schott, Germany) arrays similarly to our previously published method (100). Briefly, antibodies were printed in triplicate in a 16 x 16 block format with 48 blocks per array for a total of 3 x 4096 unique features. Antibodies were printed at a final concentration of 175-350 μg/ml unless their initial concentrations were lower. Following a "case/control versus reference" procedure, individual cases and controls were labeled with NHS-Cy5 and pooled with an equal amount of NHS-Cy3-labeled reference sample to remove dye bias from the analysis. Labeled lysates were incubated on arrays for 1.5 hours, and following a series of washes to remove excess dye, arrays were scanned and analyzed using an Axon Genepix 4200A scanner (Molecular Devices, LLC, Sunnyvale, CA).

Array analysis and statistics

Saturated array spots were flagged and triplicate antibodies with coefficients of variation >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. An average of the median intensity of triplicate spots for each antibody feature was calculated and control signal was subsequently standardized to have a mean signal of zero with a standard deviation of 1. Logistic regression analysis was used for all array experiments. For the WHI pre-diagnostic plasma dataset, the pairwise t-test 'case vs. control,' model was computed after accounting for effects of BMI,

hybridization day and print day; for the CATPAC dataset, the logistic regression model: 'assay~case + hybridization day'; for the mouse plasma and tissue data, logistic regression model: 'case~control + stage,' where 'stage' represents the time point at which plasma/tissue was collected, with day effect accounted for. Candidate protein markers were then ranked based on their effect size (WHI) or coefficient (mouse plasma and tissue); a positive coefficient means the candidate protein is greater in cancer than controls, a negative value means that it is lower and p-values. All normalization procedures and analyses were conducted using R statistical computing software program.

Immunohistochemistry

Four-micron sections of formalin-fixed paraffin-embedded tissues were cut and incubated for 1 hr at 60ºC. Sections were deparaffinized with xylene and rehydrated sequentially. Antigen retrieval in Trilogy pH 8.0 Buffer (Cell Marque, Rocklin, CA) was then performed for 7 minutes in a pressure cooker. All subsequent incubations were performed at room temperature on a Dako Autostainer Plus (Agilent Technologies, Santa Clara, CA). Slides were treated with 3 % hydrogen peroxide for 8 minutes followed by protein blocking using TCT Buffer (0.05M Tris, 0.15M NaCl, 0.25% Casein, 0.1% Tween 20, pH 7.6) for 10 minutes. Tissues were then stained with anti-TNC antibody (1:75) (Novus Biologicals, Littleton, CO) or a matched concentration of rabbit IgG for 60 minutes. Poly-HRP anti-Rabbit IgG Polymer (Leica Microsystems, Buffalo Grove, IL) was then applied for 30 minutes followed by DAB+ substrate-chromagen incubation for 2 applications of 4 minutes each (Agilent. Technologies, Santa Clara, CA). Slides were counterstained with hematoxylin (Agilent) for 2 minutes and then cover slips were added. Serial sections were stained with hematoxylin and eosin for histological analysis. All images were

taken with a Nikon DS-Vi1 brightfield camera using NIS Elements 3.2 Basic Research Image software (Nikon Instruments Inc., Melville, NY).

RESULTS

Antibody microarray interrogation of murine preinvasive and invasive plasma

To discover putative early detection biomarkers of pancreas cancer, we elected to use plasma collected from a highly faithful mouse model of PDA, incorporating endogenous heterozygous pancreas-specific expression of oncogenic $Kras^{G12D}$ and mutant $Trp53^{R172H}$ (131), hereafter referred to as *KPC* mice. Both *Pdx1-Cre* and $p48^{Cre}$ promoters were used to target pancreas-specific expression (12), These mice recapitulate the clinical, histological and molecular characteristics of the human disease (131) and have been extensively used in preclinical therapeutic studies (129, 130, 132). EDTA-collected plasma was acquired every two weeks from *KPC* mice and *KP* and *Cre* age-matched controls from approximately four weeks of age until moribund with disease.

Plasma collected at 6-8 weeks, representing preinvasive disease stages (time point #1 or TP1), and mid-way through the lifepsan of each individual *KPC* mouse (time point #2 or TP2), representive of invasive adenocarcinoma, was compared to controls on our antibody array platform containing 4096 unique features. This platform was tailored for interrogation of pancreas cancer tissue and plasma samples, with a number of antibodies selected based on being identified as markers of PDA by other groups (24); our selection criteria also included enrichment for putative early detection markers of PDA, i.e., markers identified in precursor PanIN-3 lesions. Unpaired logistic regression analysis identified fifty-four proteins for TP1 (21 proteins that were up regulated in *KPC* plasma versus controls and 33 down regulated) and 25

proteins for TP2 (12 up, 13 down) (Figure 1A,B and Table 1). Among the up regulated markers were the plasma membrane proteins IL12RB2, AQP2, PCDH15, ICAM5, OPN3, CD27 (TP1) and RAB7L1, EZR, ERBB2, CCR2 and CSF3R (TP2); and the extracellular or secreted markers TNC, HBA1 (plasma protein), PTHLH (TP1) and B2M and SERPING1 (TP2).

Antibody microarray interrogation of pre-diagnostic human plasma samples

To identify putative protein markers that could be used to detect PDA in asymptomatic individuals, we interrogated pre-diagnostic and matched control plasma samples provided by the Women's Health Initiative. This included plasma from 87 subjects who died from PDA within 4 years of the blood draw and matched controls (see Materials and Methods for matching criteria), thus limiting the impact of any lead-time bias. A breakdown of the sample characteristics is provided in Table 2.

The range of time from blood draw to diagnosis for this sample set was 33 days to just under 4 years, and the time from diagnosis to death ranged from 0 to just under 700 days. The effects of stage at diagnosis on time to death was only significant in patients diagnosed with stage IV (i.e. metastatic) disease (based on using M = 1 in the T, N, M staging system), which comprised 30% of our sample patient population (Figure 1C). Our samples were derived from patients diagnosed with different stages of disease and presenting with apparent disease heterogeneity based on the ranges in days to death following diagnosis. A pair-wise t-test identified a total of 88 candidate markers differentiating pre-diagnostic plasma samples from controls with statistical significance (p-value < 0.05). Twenty-three of these markers were up-regulated in pre-diagnostic plasma and 65 were down-regulated (Figure 1D). The complete list of candidate early detection markers is provided in Table 3.

As the goal was to identify early detection plasma markers that could be used individually or in a panel in a non-invasive blood test, we concentrated on the twenty-three up regulated proteins. We cross-referenced our list to that of the pancreaticcancerdatabase.org and the compendium developed by Harsha, et al. (24), and found that fifteen of these 23 putative markers have been previously associated with pancreatic neoplasms. Eight of these 23 have also been previously reported in the plasma: ERBB2, KRT16, ESR1 (2 antibodies were in the top list), STAT3 (pY705), CLU, SERPINH1, TNC, and PKM.

Cross-species biomarker identification

Comparison of preinvasive and invasive mouse datasets with the pre-diagnostic human dataset shows that antibodies against the extracellular matrix marker TNC and the plasma membrane receptor tyrosine kinase ERBB2 are both up-regulated in case plasma samples relative to controls. Plotting the M-values (the normalized semi-quantitative red/green ratio) of case and control plasma for both pre-diagnostic human and *KPC* preinvasive and invasive plasma shows elevated levels of TNC and ERBB2 (Figure 2A,B).

Elevated ERBB2 has been previously shown in *KPC* PanIN lesions (131), correlating with findings reported here in plasma. To determine whether tissue array analyses corroborated elevated plasma TNC levels, we examined tissue collected from cohorts of *KPC* mice at 2- and 4-months of age, representing pre-invasive and invasive disease stages, respectively. Array analyses also showed increased TNC as represented by the M-value plots of cases and controls (Figure 2A,B). Immunohistochemistry (IHC) revealed increasing levels of TNC with progression from PanIN to invasive PDA (Figure 2D). Of note, stromal deposition of TNC was seen in regions surrounding the earliest PanIN-1 lesions (Figure 2D, i). While others have also

reported modestly elevated levels of TNC in chronic pancreatitis (167)—albeit to a lesser extent than in PDA—we did not see appreciable increases in TNC deposition in an experimental mouse model of chronic pancreatitis (Figure 2D, iv). Thus, elevated plasma TNC and ERBB2 levels identified across species is supported by corresponding increases in primary pancreatic tumor tissue in our study (TNC) and those of others (ERBB2).

ESR1, ERBB2 and TNC as a 3-marker panel for early diagnosis of pancreas cancer

In addition to elevated plasma TNC and ERBB2 identified in both preinvasive mouse and pre-diagnostic human plasma, 2 distinct antibodies against ESR1 were also in the list of significantly up regulated candidates for human pre-diagnostic pancreas cancer plasma (see Table 3) and a third ESR1 antibody had a p-value < 0.06. As these data came from interrogation of plasma exclusively from female patients, we found this an intriguing candidate for further follow up. A receiver operator characteristic (ROC) curve was calculated for the 3 marker panel of ERBB2, ESR1 (the two significant antibodies were included) and TNC for the pre-diagnostic plasma sample set. Together these 3 markers displayed an AUC = 0.677 (Figure 3), with ~30% sensitivity at 90% specificity. By comparison, the AUC for CA19-9 is ~0.78 upon diagnosis (168). An antibody against CA19-9 included on our array had an effect size of -0.04 and a p-value = 0.8, highlighting the lack of diagnostic utility it affords and obviating its use in our pre-diagnostic marker panel.

Preliminary validation of ESR1, ERBB2 and TNC in diagnostic PDA plasma

We also interrogated the plasma proteome of diagnostic plasma samples collected at the Seattle Cancer Care Alliance and provided by the Center for Accelerated Translation of Pancreas Cancer

(CATPAC). Twenty-four diagnostic plasma samples were compared via array to 24 unmatched control plasma samples also collected at the SCCA using the same methods. Unpaired logistic regression yielded 243 statistically significant (p-value<0.05) candidates with 133 candidates increased in case plasma versus control plasma. When comparing up-regulated markers between the CATPAC and WHI datasets, TNC was again up-regulated with statistical significance (coefficient = 0.86; p-value = 0.004) and up-regulated levels of ESR1 (coefficient = 0.62; p-value = 0.055) and ERBB2 (coefficient = 0.77; p-value = 0.11) trended towards significance in the CATPAC samples. Thus, collectively, array analysis validated two of these markers across preinvasive and pre-diagnostic sample sets, and the 3-protein pre-diagnostic plasma biomarker panel was additionally preliminarily validated in an independent set of diagnostic plasma samples.

CONCLUSION

Pancreas cancer (PDA) remains a uniformly fatal disease, with an estimated 38, 460 patients succumbing to disease out of approximately 45,220 new cases in 2013 (Cancer Facts and Figures, 2013). Patients that undergo surgical resection do have improved survival rates, especially those identified with stage I disease (7); conversely, survival rates after resection are adversely affected by tumor size, lymph node metastases and histologic tumor differentiation (5). These data suggest that the development of screening or testing methods for early detection of PDA should help improve survival times and rates.

Our antibody array platform allowed us to interrogate small volumes of plasma (<50 μl) from individual samples, thereby also identifying interindividual heterogeneity of putative early markers. We took a cross-species approach to discover and preliminarily validate plasma

markers of PDA, looking for overlapping markers in mouse and human plasma using a platform consisting of > 4000 unique features, representing the largest array format available to our knowledge. Importantly, many antibodies included were selected from a pancreas cancer compendium based on reported association with precursor PanIN lesions (24). We reasoned that array interrogation of plasma collected from the *KPC* mouse model at stages of disease where resection would still be possible in patients would serve as a good starting point for initial discovery. Serum proteomic signatures have been previously reported in mouse models of PDA (12, 71). Furthermore, the faithful recapitulation of human disease in mouse models of PDA make them attractive for early detection studies where both tissue and plasma are readily available. These 'discovery' arrays yielded a total of thirty-three proteins that were increased in the plasma of *KPC* mice relative to control plasma, 17 of which are plasma membrane or secreted proteins.

PanIN have been identified in pancreata up to 10 years prior to diagnosis of PDA (31) and elevated blood glucose levels, indicative of late-onset diabetes preceding PDA onset, have been observed up to 5 years before clinical diagnosis (169). Based on these results, we reasoned that clinically relevant plasma markers indicative of incipient PDA could be identified. Array interrogation of pre-diagnostic plasma from the WHI observational study gave us access to samples perhaps most representative of general screening of asymptomatic individuals at increased risk of developing pancreas cancer. From this set of 87 pre-diagnostic plasma samples, also representing the largest pre-diagnostic cohort reported, we identified twenty-three proteins up regulated in case versus control plasma, of which 15 have been previously implicated with PDA.

Looking at the overlap between the seventeen up regulated mouse and 23 up regulated human plasma proteins, only TNC and ERBB2 were present in both datasets. While this overlap is small and could be due to chance, we suggest they warrant further follow up given that both have been previously implicated with pancreas cancer. Elevated ERBB2 levels have been reported in PanIN and PDA in the *KPC* mouse model (131), and increasing levels also correlated with progression from PanIN to invasive PDA in humans (167, 170). Corresponding increases in serum ERBB2 levels have been reported and correlate with metastatic capability and survival (171). Given that 70% of PDA cases overexpress ERBB2 while it is uniformly absent from normal ductal epithelium makes it yet more intriguing as a candidate early detection marker.

We observed increasing levels of TNC in *KPC* mice with disease progression, corroborating similar observations in human PDA (167). A 3 marker panel consisting of TNC and TFPI combined with CA19-9 differentiated diagnostic PDA plasma samples from healthfy controls with an AUC of 0.99, with 100% specificity at 90% sensitivity (172). Interrogating a set of 24 diagnostic plasma samples and unmatched controls using our arrays, we also corroborated elevated levels of TNC in and increased levels of ESR1 and ERBB2 trended towards significance. Elevated ESR1 was of additional interest as estrogen receptor positivity is a defining characteristic of mucinous cystic neoplasms (MCN), which are found predominantly in female patients, and ESR1 staining has also been reported in invasive PDA (173).

Using ERBB2, ESR1 and TNC as a 3-marker diagnostic panel yielded a combined AUC for pre-diagnostic plasma samples of 0.677, establishing a proof of principle that a blood-based assay can identify PDA earlier than current clinical modalities and/or symptoms. Furthermore, we were able to do so in plasma drawn up to 4 years prior to patient diagnosis. However, it is unlikely that this panel could be used for screening in the general population. Somewhat

surprisingly, we did not see any trends in increasing M-values for any of the 3 markers as the time from blood draw to death decreased (Supplementary figure 1), implying a level of heterogeneity in disease latency. This was consistent with the lack of correlation of stage at diagnosis, except for stage IV disease, on time to death as reported here (Figure 2A) and by others (16). Genetic (166) and computational modeling using patient data (174) point to differing disease latencies for pancreas cancer. While studies have shown an association between tumor stage at resection and median survival (10), others have also shown variability in survival rates in patients with both unresectable and resectable disease (16). Our study underscores the difficulty of screening for pancreas cancer in asymptomatic individuals. Nevertheless, given that our analyses were performed on pre-diagnostic plasma samples, a sensitivity of 30% with 90% specificity suggests that these markers may have underlying biological implications and may be of some utility in a panel for screening high-risk individuals. In plasma drawn from patients with clinically diagnosed PDA, plasma TNC had 25% sensitivity with 90% specificity,similar to our pre-diagnostic panel (172). A comparison of our marker panel to diagnostic plasma and serum markers identified upon surgical resection of PDA shows that the AUC value for our panel is also comparable to the AUC values reported for the markers NGAL, SAA and MMP7 (Supplementary Table 1).

Further analyses of this 3 marker panel may be warranted in high-risk cohorts. A variety of at-risk individuals are currently being monitored in pancreas cancer registries including patients with hereditary pancreatitis, those with two or more first-degree relatives with PDA, or those with inherited mutations that predispose them to increased risk of PDA such as individuals with Peutz-Jegher's syndrome (175). The panel of ERBB2, ESR1 and TNC could also be retrospectively tested in conjunction with current screening strategies such as MRI, CT and PET

scans, and with endoscopic ultrasound and fine needle aspiration, which has high diagnostic potential but lacks the ability to detect precursor (PanIN) lesions (156). Should imaging modalities identify suspected lesions that are later confirmed following surgery, plasma levels of these markers could be correlated with tumor histology, disease stage, disease latency and survival times post-resection. Subsequent blinded screening could then help confirm lesions suspected by imaging modalities. ESR1 should be specifically compared between males and females and in association with identified cystic lesions, especially MCN, given that estrogen receptor stromal positivity is a defining feature.

The challenges for pancreas cancer biomarker discovery, validation and implementation are significant. We propose that iterative processes such as described here, can foster the discovery, assessment and contextualization of early detection markers, all which are imperative if they are to become markers for general screening.

FUTURE DIRECTIONS AND CHALLENGES

As the results of our array studies using human plasma samples show, challenges in biomarker studies of PDA, especially early detection studies, remain. While our 3-marker panel of TNC, ESR1 and ERBB2 afforded sensitivities and specificities that are lower than others have reported, these were derived from, to our knowledge, one of the largest reported pre-diagnostic sample sets. As this was effectively an exercise in population-based screening of asymptomatic individuals, a larger sample set such as ours would be expected to affect sensitivities and specificities.

Corroboration or successful validation of biomarker results is one of the keys to successful implementation of diagnostics (74). Thus, it is important to reiterate that our array

platform corroborated the results of other diagnostic studies; notably, similar mouse to human search using mass spectrometry-based techniques also identified TNC as an early detection PDA marker (71), and ERBB2 is also reported by others in precursor PanIN lesions in both mice (131) and humans (170).

To further validate the markers I discovered in our cross-species proteomic studies, follow up experiments in our lab would include corroboration of our array data in both independent sample sets, made available through the Early Detection Research Network (EDRN) at the NIH or through our collaborators, as well as use of other proteomic methods on our pre-diagnostic samples. For the former, validation arrays containing an enrichment of ESR1, ERBB2 and ESR1 antibodies could be envisioned. Interestingly, in a separate array study using a variation on our pancreas cancer array platform to compare plasma collected at diagnosis of PDA from 27 patients with plasma collected from 27 CP patients matched based on age, sex and BMI, antibodies against ESR1 and TNC were both up regulated in PDA plasma (effect sizes of 0.56 and 0.68 and p-values < 0.07 and < 0.1, respectively). Additionally, array results on plasma drawn from patients diagnosed with early stage disease only (stage I/II) compared to controls also showed ERBB2 as a significant candidate (coefficient = 0.48 and p-value < 0.04). Thus, these results provide further validation of these proteins as putative early detection markers.

A combination of ELISA validation of our array results using our pre-diagnostic cases and matched controls as well as IHC of human tumor tissue from rapid autopsy patients made available through our collaborators at the University of Nebraska could help strengthen the support for future studies using ESR1, ERBB2 and TNC as markers of PDA. ELISA would be conducted using selected cases and controls that had the greatest differences in signals for each individual marker. For IHC experiments, the gender of each patient would also be considered

with respect to ESR1 levels. If these markers survive this validation pipeline, then they certainly warrant follow up in larger patient populations, as discussed below.

While a high sensitivity for a biomarker(s) for PDA is necessary for general screening, the specificity is perhaps more important given the low incidence of disease. If a biomarker incorrectly diagnoses a healthy person as having PDA, then this could lead to very invasive procedures with associated morbidities. Furthermore, whereas the sensitivity of a test could be improved by using it to study high-risk patient cohorts, the specificity of this test cannot be similarly modified. Hence our suggestion in the Discussion section of our report that TNC, ESR1 and ERBB2 be tested in people at increased risk of developing PDA. As the pretest probability of linking our markers with PDA is greatly increased when studying high-risk cohorts, this improves the chances of assessing the biological relevance of these markers.

Epidemiological studies have shown that family history, environmental factors and certain genetic predispositions are associated with an increased risk of PDA. Individuals with first-degree relatives (FDR) with PDA are at increasing risk of developing disease as the number of FDR with PDA grows, with a 6.4-fold greater risk in individuals with 2 FDR that increases to 32-fold in those with 3 or more affected FDR. These increases are from an estimated 1.3% lifetime risk of developing PDA (175). In a study of literature reporting on the increased susceptibility to a variety of cancers in patients with germline autosomal dominant mutations in the STK11 gene, or patients with Peutz-Jegher's syndrome (PJS), the likelihood of developing PDA was 132-fold over the normal population, representing a patient cohort with greatest increased risk for PDA (176). In addition, people with chronic and hereditary pancreatitis, most commonly due to mutations in the *PRSS1* cationic trypsinogen gene, are at 26.3% and 53% increased risk, respectively, of developing PDA (153, 154). Other factors contributing to disease

prevalence include a 4 – 7-fold increased risk associated with recent onset diabetes (177), approximately 10% of PDA patients exhibit germline mutations in the mismatch repair gene *BRCA2* (with around 5-fold increased risk) (178) and patients with germline familial atypical multiple mole melanoma (FAMMM) or *p16/INK4A* mutations carrying a 13-fold increased risk (179).

Screening such patient cohorts could have benefits by identifying lesions at early stages, where resection could greatly increase survival times. Indeed, studies have reported success at identifying abnormal lesions, some of which led to surgical intervention, in asymptomatic at-risk individuals enrolled in Pancreatic cancer tumor registries. In a study conducted at Memorial Sloan Kettering Cancer Center, at-risk patients were enrolled and screened using non-invasive magnetic resonance cholangiopancreatography (MRCP) followed by endoscopic ultrasound (EUS) with fine needle aspiration if MRCP identified any potential abnormalities. Of 109 patients that had completed one screening cycle, 18 displayed abnormal radiographic findings, 15 of which went on to have EUS, which in turn confirmed the radiographic findings in 9 patients. Thus, 9 of 109 patients (8.3%) with suspicious lesions were identified in this screening program after just one cycle of screening (180). Of the 6 patients who underwent surgery, a T3N0 PDA was discovered in one patient, two patients had main branch IPMN, one patient had a single PanIN-3, one a PanIN-2 and the final patient had serous cystadenoma with a PanIN-1 (180). In another study enrolling 51 asymptomatic individuals, 12% of enrollees that underwent EUS or MRCP had malignant (one patient with stage IV unresectable disease, one with resectable PDA) or pre-malignant (four patients with branch duct IPMN and multifocal PanIN-2) pancreatic lesions, resulting in total or partial pancreatectomies being performed (181). Another study of 216 individuals, including familial breast-ovarian cancer patients with at least 1 affected

first- or second-degree relative with PDA, individuals with PJS, and relatives of patients with familial PDA having at least 1 affected first-degree relative, found pancreatic lesions in 92 enrollees (5 dilated main pancreatic ducts, 84 cysts and 3 solid lesions) (156).

Based on the successes of these studies and others, an International Cancer of the Pancreas Screening (CAPS) consortium was recently formed to "help organize global pancreatic screening" and topics including which patients to screen, at what age screening should commence, when surgery should be conducted and assessing performance outcomes, were discussed (175).

It is in this setting, in which asymptomatic individuals at increased risk for developing PDA are followed using screening modalities such as MRI and (if necessary) EUS and pathological findings, surgeries and survival outcomes are documented, that our pre-diagnostic plasma markers could be studied with potential increased clinical relevance. For example, if elevated plasma TNC and ERBB2 are elevated in patients with suspected lesions at screening, their levels can then be further linked to pathological findings and/or patient outcomes. If they are both elevated in individuals who present with PanIN-2 or PanIN-3 lesions, but not in those with benign lesions, then in turn they might be used in conjunction with MRI, for example, to determine whether EUS is necessary. Such markers could also be used more simply for further assessing increased risk, thereby providing support for enrolling patients with relatives with familial pancreas cancer into such observational studies.

To this end, plasma markers such as ERBB2, ESR1 and TNC could serve as complementary confirmation, and perhaps eventually as surrogate markers, for the presence of early and resectable stages of disease in non-invasive blood tests. As PanIN lesions are still

more difficult to identify preoperatively, reflecting a caveat to solely using imaging in screening (156), markers elevated in both plasma and/or tissue that distinguish PDA from MCN and IPMN in preclinical models could help confirm the presence of PanIN.

Translation of biomarkers to the clinic therefore requires a coordinated collective effort across institutions. By integrating data on markers discovered by various groups, their utility to larger patient cohorts can be better determined. Along with incorporation of early detection markers showing promise into screening programs for high-risk patients, markers for early stage disease can also be applied to patient follow up subsequent to surgical resection and/or intervention with adjuvant therapy. With technological advances in genomics and proteomics improving our abilities to detect low abundance markers of disease, and the establishment of pancreas cancer screening programs, the infrastructure is there to further test putative biomarkers of disease. As these markers are more directly linked with disease characteristics and outcomes, their utility in helping save lives will become clearer.

CHAPTER 4 TABLES AND FIGURES

Table 1. Candidate plasma biomarkers from analysis of *KPC* TP1 and TP2 (preinvasive and invasive) plasma antibody microarray interrogation

Time point 1 (TP1)			
Gene name	Protein name	Coefficient TP1	p-value TP1
TP53	tumor protein p53	1.66	0.0068
ANXA2	annexin A2	-1.55	0.0113
IL12RB2	interleukin 12 receptor, beta 2	2.96	0.0135
AQP2	aquaporin 2 (collecting duct)	1.05	0.0140

TNC	tenascin C	1.76	0.0153
CREB1	cAMP responsive element binding protein 1	-7.85	0.0190
VIL2 (EZR)	ezrin	-1.75	0.0212
NFKB1	Nuclear Factor Of Kappa Light Polypeptide Gene Enhancer In B-Cells 1	-2.37	0.0222
FOS	FBJ murine osteosarcoma viral oncogene homolog	1.57	0.0225
SPINK1	serine peptidase inhibitor, Kazal type 1	-2.17	0.0227
PTEN	phosphatase and tensin homolog	-1.15	0.0237
AKAP12	A kinase (PRKA) anchor protein 12	2.24	0.0264
UBE2S	ubiquitin-conjugating enzyme E2S	0.98	0.0272
PLCG1	phospholipase C, gamma 1	-3.16	0.0278
KI67	monoclonal recognizing Ki-67	1.79	0.0281
APAF1	apoptotic peptidase activating factor 1	1.86	0.0291
AMBRA1	autophagy/beclin-1 regulator 1	1.03	0.0296
MKI67	monoclonal recognizing Ki-67	2.00	0.0301
ADAM28	ADAM metallopeptidase domain 28	-1.82	0.0302
YWHAZ	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	-1.94	0.0303
FANCF	Fanconi anemia, complementation group F	0.96	0.0309
WDR1	WD repeat domain 1	-1.60	0.0313
IRS1	insulin receptor substrate 1	-3.10	0.0317
MAPK3/MAPK1 (pThr202/Tyr204)	mitogen-activated protein kinase 1/mitogen-activated protein kinase 3	-1.16	0.0333
FTH1	ferritin, heavy polypeptide 1	1.77	0.0333
PCDH15	protocadherin-related 15	0.98	0.0335
HBA1	hemoglobin, alpha 1 (CD31)	0.83	0.0336
STAT3	signal transducer and activator of transcription 3 (acute-phase response factor)	-2.43	0.0341
RPS5	ribosomal protein S5	-3.73	0.0360
PIK3CA	phosphoinositide-3-kinase, catalytic, alpha polypeptide	0.88	0.0362
LASP1	LIM and SH3 protein 1	-3.26	0.0368
FKBP4	FK506 binding protein 4, 59kDa	-2.27	0.0393
ICAM5	intercellular adhesion molecule 5, telencephalin	1.70	0.0406
PRKAR2A	protein kinase, cAMP-dependent, regulatory, type II, alpha	-1.36	0.0419
TIMP1	TIMP metallopeptidase inhibitor 1	-1.70	0.0421
GADD45G	growth arrest and DNA-damage-inducible, gamma	1.54	0.0429
DPYSL3	dihydropyrimidinase-like 3	-1.00	0.0433
CLU	clusterin	-0.77	0.0434
THBS2	thrombospondin 2	-1.07	0.0434
LASP1	LIM and SH3 protein 1	-2.46	0.0440
OPN3	opsin 3	1.57	0.0448

ABL1	c-abl oncogene 1, non-receptor tyrosine kinase	-11.12	0.0454
IL1B	interleukin 1, beta	-1.32	0.0464
IL5	interleukin 5 (colony-stimulating factor, eosinophil)	-1.22	0.0464
MT3	metallothionein 3	-0.79	0.0466
EPRS	glutamyl-prolyl-tRNA synthetase	-1.61	0.0469
PTHLH	parathyroid hormone-like hormone	1.24	0.0470
TPI1	triosephosphate isomerase 1	-1.24	0.0475
KLK5	kallikrein-related peptidase 5	-0.88	0.0477
REG4	regenerating islet-derived family, member 4	-1.45	0.0480
CD27	CD27 molecule	1.40	0.0483
NTNG1	netrin G1	-1.48	0.0493
RAD54L	RAD54-like (S. cerevisiae)	-3.95	0.0497
AFP	alpha-fetoprotein	-1.30	0.0500
Time point 2 (TP2)			
Gene name	Protein name	Coefficient TP2	p-value TP2
FANCF	Fanconi anemia, complementation group F	1.01	0.0193
B2M	beta-2-microglobulin	2.44	0.0264
RAB7L1	RAB7, member RAS oncogene family-like 1	1.68	0.0343
EZR	ezrin	1.92	0.0344
NF2	neurofibromin 2 (merlin)	1.03	0.0428
ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)	2.09	0.0443
CCR2	chemokine (C-C motif) receptor 2	1.55	0.0451
LIN13	unknown gene (C. elegans)	2.10	0.0459
SUFU	suppressor of fused homolog (Drosophila)	3.38	0.0472
SERPING1	serpin peptidase inhibitor, clade G (C1 inhibitor), member 1	1.58	0.0475
CSF3R	colony stimulating factor 3 receptor (granulocyte)	1.09	0.0478
MCM6	minichromosome maintenance complex component 6	1.44	0.0485
RELA	v-rel reticuloendotheliosis viral oncogene homolog A (avian)	-2.42	0.0177
ALPPL2	alkaline phosphatase, placental-like 2	-1.34	0.0188
FANCF	Fanconi anemia, complementation group F	1.01	0.0193
B2M	beta-2-microglobulin	2.44	0.0264
BAD	BCL2-associated agonist of cell death	-1.29	0.0289
SPINT2	serine peptidase inhibitor, Kunitz type, 2	-1.30	0.0293
BCR	breakpoint cluster region	-2.70	0.0298
FABP3	fatty acid binding protein 3, muscle and heart (mammary-derived growth inhibitor)	-1.87	0.0331
RAB7L1	RAB7, member RAS oncogene family-like	1.68	0.0343

	1		
EZR	ezrin	1.92	0.0344
SMAD2	SMAD family member 2	-5.31	0.0372
MET	met proto-oncogene (hepatocyte growth factor receptor)	-2.18	0.0387
PTPRF	protein tyrosine phosphatase, receptor type, F	-1.51	0.0389
POLE4	polymerase (DNA-directed), epsilon 4, accessory subunit	-1.63	0.0403
EIF4E	eukaryotic translation initiation factor 4E	-2.17	0.0414
NF2	neurofibromin 2 (merlin)	1.03	0.0428
ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2	2.09	0.0443
CCR2	chemokine (C-C motif) receptor 2	1.55	0.0451
CCNA2	cyclin A2	-1.62	0.0452
HAPLN1	hyaluronan and proteoglycan link protein 1	-1.79	0.0457
LIN13	lin13 (C. elegans)	2.10	0.0459
SUFU	suppressor of fused homolog (Drosophila)	3.38	0.0472
SERPING1	serpin peptidase inhibitor, clade G (C1 inhibitor), member 1	1.58	0.0475
CSF3R	colony stimulating factor 3 receptor (granulocyte)	1.09	0.0478
MCM6	minichromosome maintenance complex component 6	1.44	0.0485

Table 1. Candidate biomarkers for *KPC* preinvasive and invasive plasma drawn between 8-10 weeks and mid-way through disease progression, respectively. Antibody microarray interrogation and logistic regression analysis were used to identify candidate plasma proteins differentiating *KPC* from age-matched control plasma samples with statistical significance. Plotted are markers with p-value < 0.05 and their semi-quantitative normalized coefficients, representing the degree to which each marker is elevated or down regulated in *KPC* versus control plasma. Candidates are listed based on ascending p-values.

Table 2. Patient characteristics for WHI cases and matched controls

Age	Cases	Controls
50-59	14	14
60-69	32	33
70-80	41	40

Ethnicity	Cases	Controls
Asian	4	5
Black	4	3
White	76	76
Other	3	3

Smoking status	Cases	Controls
Never	40	40
Current	8	7
Past	39	40

HRT status	Cases	Controls
Estrogen alone	15	17
Estrogen + progesterone	16	16
None	56	54

BMI	Cases	Controls
Normal	29	38
Overweight	35	27
Obese	22	21
NA	1	1

Stage at diagnosis for WHI cases

Stage	N
IA	3
IB	5
IIA	19
IIB	12
III	17
IV	26
Unknown	5
	87

Table 2. Statistics for plasma samples drawn from women enrolled in the Women's Health Initiative's (WHI) hormone replacement therapy (HRT) observational study and used in our antibody microarray analysis. Cases and controls were matched on an extensive list of criteria (see Materials and Methods) including age, ethnicity, smoking status, HRT status and BMI, whose breakdown are shown here. The stage of PDA at diagnosis was determined using the T, N, and M classification system for pancreas cancer, with information provided by the WHI.

Table 3. Candidate plasma biomarkers from analysis of WHI pre-diagnostic plasma antibody microarray interrogation

Gene name	Protein name	Effect size	p-value
DPP10	dipeptidyl-peptidase 10 (non-functional)	-0.43	0.0003
ALDH1A1	aldehyde dehydrogenase 1 family, member A1	-0.76	0.0006
AAMP	angio-associated, migratory cell protein	-0.36	0.0028
PCTK1 (CDK16)	cyclin-dependent kinase 16	-0.41	0.0052
BCL3	B-cell CLL/lymphoma 3	-0.36	0.0054
EIF2C2 (AGO2)	argonaute RISC catalytic component 2	-0.43	0.0064
ITGB1	integrin, beta 1	-0.36	0.0079
SEPT5	septin 5	0.42	0.0083
IL2RA	interleukin 2 receptor, alpha	0.42	0.0085
MAP2K3	mitogen-activated protein kinase kinase 3	-0.33	0.0104
GADD45G	growth arrest and DNA-damage-inducible, gamma	-0.31	0.0109
ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2	0.59	0.0122
KRT16	keratin 16	0.34	0.0129
ESR1	estrogen receptor 1	0.63	0.0132
FLT3	fms-related tyrosine kinase 3	-0.32	0.0143
NPTX2	neuronal pentraxin II	-0.32	0.0148
THSD1	thrombospondin, type I, domain containing 1	-0.36	0.0155
N-PAC (GLYR1)	glyoxylate reductase 1 homolog (Arabidopsis)	-0.26	0.0162
TYK2	tyrosine kinase 2	-0.33	0.0162
ICAM1	intercellular adhesion molecule 1	-0.28	0.0168
PLEK	plekstrin	-0.35	0.0173
TBC1D3	TBC1 domain family, member 3	-0.25	0.0179
GPR125	G protein-coupled receptor 125	-0.35	0.0180
GATA3	GATA binding protein 3	0.28	0.0181
TLX3	T-cell leukemia homeobox 3	0.36	0.0190

ITGB1	integrin, beta 1	-0.21	0.0195
CDK2AP1	cyclin-dependent kinase 2 associated protein 1	0.38	0.0205
COPB2	coatomer protein complex, subunit beta 2 (beta prime)	-0.24	0.0207
SPINT2	serine peptidase inhibitor, Kunitz type, 2	-0.31	0.0209
IL16	interleukin 16	-0.30	0.0218
PAK2	p21 protein (Cdc42/Rac)-activated kinase 2	-0.27	0.0222
LCP1	lymphocyte cytosolic protein 1 (L-plastin)	-0.34	0.0224
STAT3 (pY705)	signal transducer and activator of transcription 3	0.38	0.0224
CLU	clusterin	0.37	0.0226
HIF1A	hypoxia inducible factor 1, alpha subunit	-0.29	0.0227
SERPINH1	serpin peptidase inhibitor, clade H	0.45	0.0227
HOXD13	homeobox D13	0.33	0.0229
IGFBP2	insulin-like growth factor binding protein 2, 36kDa	-0.33	0.0231
CTSE	cathepsin E	-0.31	0.0238
STAT3	signal transducer and activator of transcription 3	-0.25	0.0241
EGFR	epidermal growth factor receptor	-0.27	0.0249
APOL1	apolipoprotein L, 1	-0.21	0.0255
AR	androgen receptor	-0.35	0.0267
BCL2	B-cell CLL/lymphoma 2	0.40	0.0276
UBE2S	ubiquitin-conjugating enzyme E2S	-0.31	0.0286
PRDX3	peroxiredoxin 3	-0.32	0.0290
PEBP1	phosphatidylethanolamine binding protein 1	-0.29	0.0293
ABL1	c-abl oncogene 1, non-receptor tyrosine kinase	-0.27	0.0306
BIRC5	baculoviral IAP repeat containing 5	-0.21	0.0307
MFI2	antigen p97 (melanoma associated) identified by monoclonal antibodies 133.2 and 96.5	-0.27	0.0311
STAT6	signal transducer and activator of transcription 6, interleukin-4 induced	-0.31	0.0313
XRCC3	X-ray repair complementing defective repair in Chinese hamster cells 3	-0.30	0.0321
CHEK1	checkpoint kinase 1	-0.26	0.0323
IL1A	interleukin 1, alpha	0.30	0.0326
ZNF331	zinc finger protein 331	-0.34	0.0326
HDAC2	histone deacetylase 2	-0.34	0.0330
MLLT10	myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 10	0.33	0.0346
ATP8B1	ATPase, aminophospholipid transporter, class I, type 8B, member 1	-0.34	0.0348
RAB5A	RAB5A, member RAS oncogene family	-0.26	0.0361
BRCA1	breast cancer 1, early onset	-0.27	0.0363
DDB2	damage-specific DNA binding protein 2, 48kDa	0.26	0.0368
CD20 (MS4A1)	membrane-spanning 4-domains, subfamily A, member 1	0.21	0.0371
PTPN11	protein tyrosine phosphatase, non-receptor type 11	-0.14	0.0375
BRAF	v-raf murine sarcoma viral oncogene homolog B1	0.27	0.0379
FN1	fibronectin 1	-0.44	0.0382
FCRL5	Fc receptor-like 5	-0.27	0.0389
TNC	tenascin C	0.35	0.0402
ELP2	elongator acetyltransferase complex subunit 2	-0.24	0.0405
KIF16B	kinesin family member 16B	-0.34	0.0419

PRDX3	peroxiredoxin 3	-0.11	0.0421
STEAP2	STEAP family member 2, metalloreductase	0.29	0.0421
NANOG	Nanog homeobox	-0.27	0.0422
CD44	CD44 molecule (Indian blood group)	-0.24	0.0446
ALDH1A1	aldehyde dehydrogenase 1 family, member A1	-0.24	0.0447
TGFB2	transforming growth factor, beta 2	-0.36	0.0456
ANKRD30A	ankyrin repeat domain 30A	-0.25	0.0463
ORM1	orosomucoid 1	-0.26	0.0466
TMEM173	transmembrane protein 173	-0.44	0.0469
CHEK1	checkpoint kinase 1	-0.32	0.0470
KRT14	keratin 14	-0.32	0.0477
PKM2	pyruvate kinase, muscle	0.39	0.0478
MUTYH	mutY homolog (E. coli)	-0.24	0.0480
CLTC	clathrin, heavy chain (Hc)	-0.26	0.0483
NDRG1	N-myc downstream regulated 1	0.45	0.0484
DCLK3	doublecortin-like kinase 3	-0.27	0.0484
PEBP1	phosphatidylethanolamine binding protein 1	-0.28	0.0487
ESR1	estrogen receptor 1	0.43	0.0498
ARMCX1	armadillo repeat containing, X-linked 1	-0.24	0.0499

Table 3. Candidate pre-diagnostic plasma biomarkers for human pancreas cancer. Antibody microarray interrogation and a pair-wise t-test were used to identify candidate plasma proteins differentiating pre-diagnostic pancreas cancer plasma from age-matched control samples with statistical significance. The semi-quantitative effect size (normalized red/green coefficient across all case and control samples) and accompanying p-value for markers are listed in the order of ascending p-values.

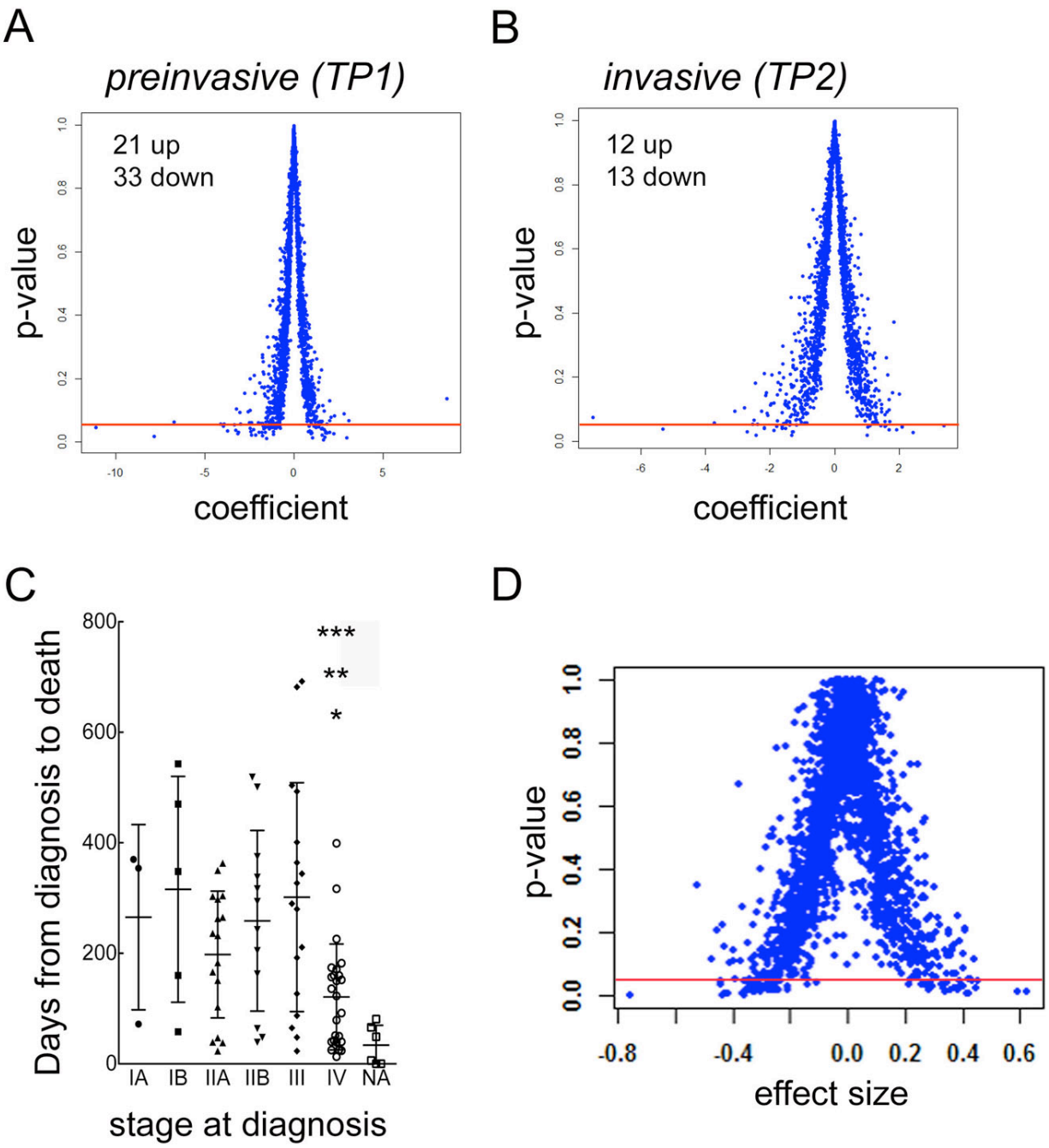


Figure 1. Cross-species antibody microarray array results for murine *KPC* preinvasive (A) and invasive (B) pancreas cancer plasma and for human pre-diagnostic plasma samples from the WHI (D). Plasma samples were drawn up to four years prior to death from PDA, and patients were diagnosed with all stages of disease, as determined using the T, N, M classification system and the patient information provided by WHI. The stage at diagnosis only influenced time from

diagnosis to death in patients with stage IV disease (C). Statistical significance was computed using an unpaired 2-tailed t-test in GraphPad Prism 5.0 * p-value < 0.05 for stage IA and IIA vs. IV; ** p-value < 0.01 for stage IB and IB vs. IV; *** p-value < 0.001 for stage III vs IV. Samples from patients where stage at diagnosis could not be determined were not included in these comparisons. Volcano plots depict the p-values and accompanying semi-quantitative coefficients (murine) and effect sizes (human) for each antibody feature on the array platform as determined using logistic regression analysis for the mouse arrays and a pairwise t-test for the human arrays. Candidate markers with p-values < 0.05 are found below the red line (estimated) and the number of significant candidates—both up regulated and down regulated in disease versus control plasma—are indicated in the top left corner for each dataset.

Figure 2.

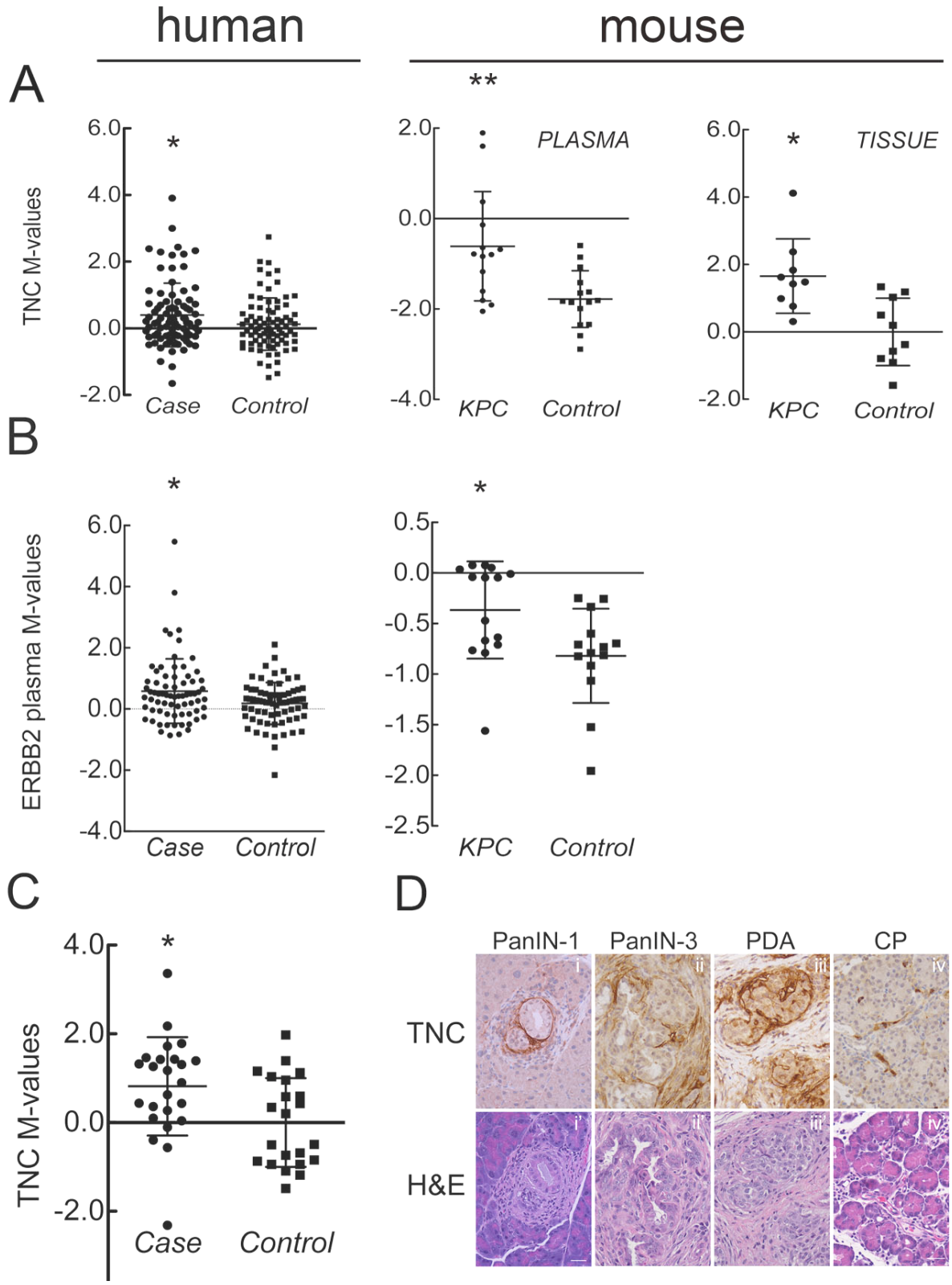

human
mouse
A
TNC M-values
6.0
4.0
2.0
0.0
-2.0
*
Case
Control
**
PLASMA
2.0
0.0
-2.0
-4.0
KPC
Control
TISSUE
6.0
4.0
2.0
0.0
-2.0
*
KPC
Control
B
ERBB2 plasma M-values
6.0
4.0
2.0
0.0
-2.0
-4.0
*
Case
Control
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
*
KPC
Control
C
TNC M-values
4.0
2.0
0.0
-2.0
*
Case
Control
D
PanIN-1
PanIN-3
PDA
CP
TNC
H&E
i
ii
iii
iv

Figure 2. Cross-species identification of elevated TNC and ERBB2 in *KPC* pancreas cancer plasma, in human pre-diagnostic and, for TNC, in diagnostic plasma samples. M-value blots for TNC (A, C) and ERBB2 (B) show elevated plasma levels in human pre-diagnostic and mouse *KPC* plasma, and, for TNC, additionally in diagnostic human plasma (C) and *KPC* tissue (D). Semi-quantitative, normalized M-values (red/green coefficients) for case and control samples are plotted along with the mean and standard deviations for each dataset. Paired 2-tailed t-tests were used to determine statistical significance for human pre-diagnostic plasma and unpaired 2-tailed t-tests for mouse plasma and tissue and human diagnostic plasma datasets. All statistical analyses were conducted in GraphPad Prism 5.0. * p-value < 0.05; ** p-value < 0.01. In (D), Immunohistochemistry (IHC) of TNC (i-iv) and accompanying serial sections stained with hematoxylin and eosin (i' - iv') in *KPC* tissue shows the emergence of TNC expression at early preinvasive stages (PanIN-1) (i, i') that increases with progression to invasive PDA. IHC of TNC associated with a PanIN-3 and invasive adenocarcinoma are shown in ii and iii, respectively. TNC deposition does not increase, however in a model of chronic pancreatitis (iv).

Figure 3.

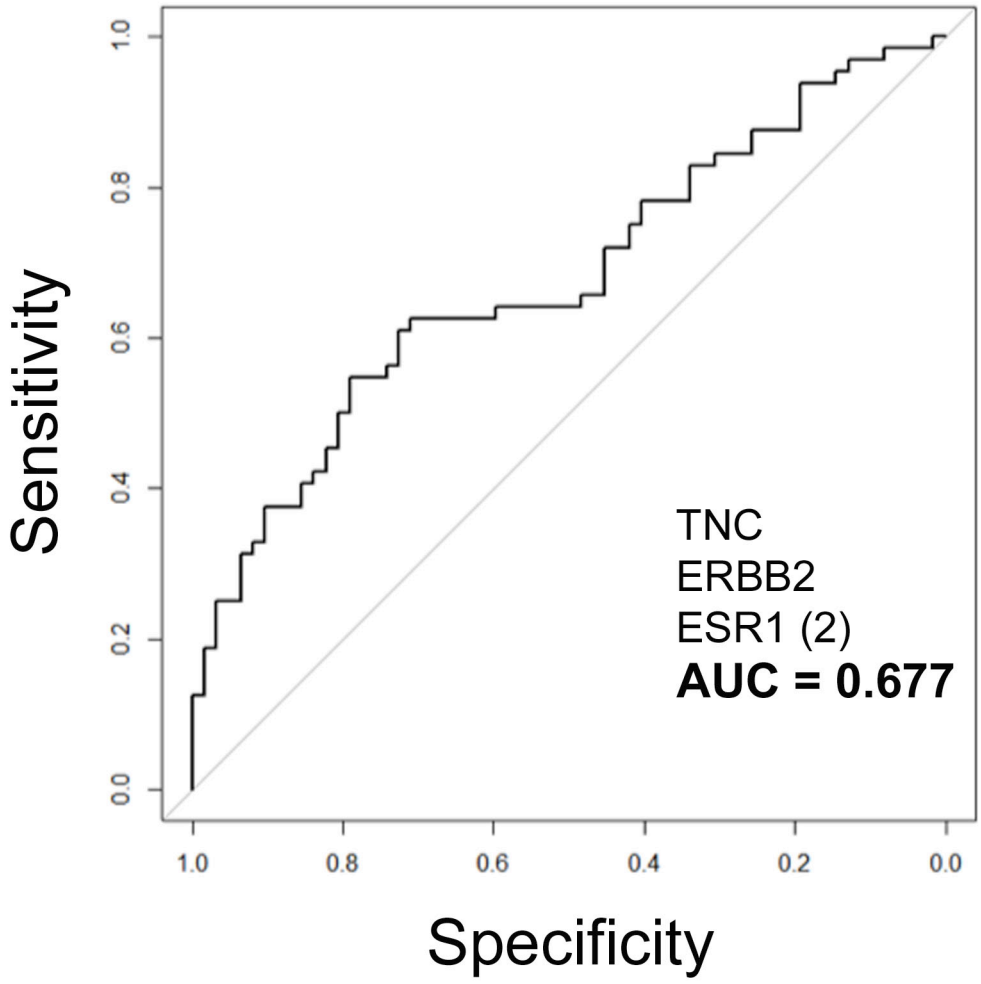


Figure 3. Receiver operator curve (ROC) analysis of a 3-marker for the WHI pre-diagnostic plasma sample set. Specificity and sensitivity for ESR1, ERBB2 and TNC as a panel differentiating pending PDA from controls in pre-diagnostic samples are plotted on x- and y-axes, respectively. The combined area under the curve (AUC) for this panel = 0.677.

CHAPTER 4 SUPPLEMENTARY TABLE AND FIGURE

Supplementary Table 1. Comparison of diagnostic markers from plasma and serum collected at surgery to the 3-protein pre-diagnostic plasma biomarker panel

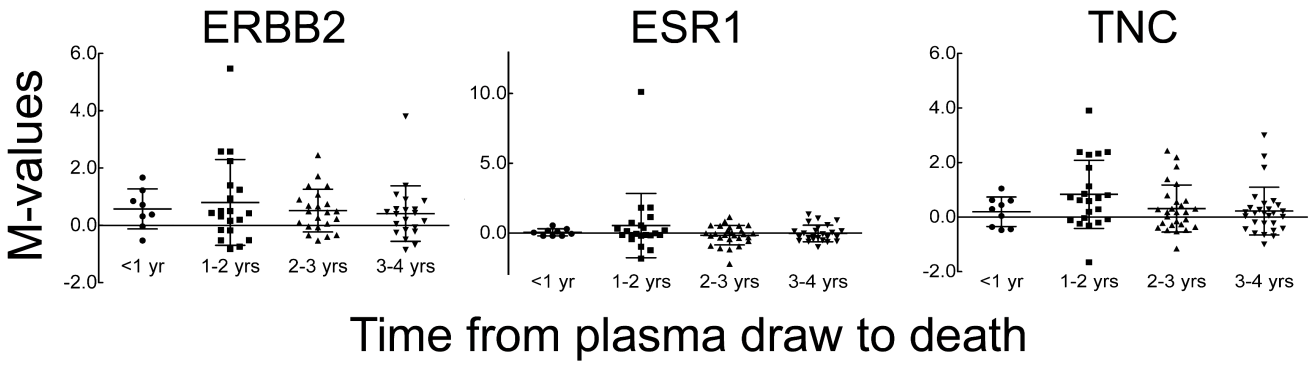
Marker panel	Sample size	Methods	AUC	Reference
CA19-9, ICAM1, OPG	107 PDA 70 Benign 83 Normal	Luminex immunoassay	0.91 (PDA vs. non-PDA)	Brand et al., 2011
CA19-9, CEA, TIMP1	107 PDA 70 Benign 83 Normal	Luminex immunoassay	0.83 (PDA vs. Benign)	Brand et al., 2011
MUC1	53 PDA 43 Normal 87 pancreatitis	ELISA	0.88 (PDA vs. non-PDA) 0.85 (PDA vs. pancreatitis)	Gold, et al., 2006
NGAL	16 PDA 16 pancreatitis 8 Normal	ELISA	0.75 (PDA vs non-PDA)	Moniaux et al., 2008
CA19-9, MIC1	50 PDA 50 chronic pancreatitis 50 Normal	ELISA	0.91 (PDA vs. non-PDA)	Koopmann et al., 2006
CA19-9, CEACAM1	81 PDA 53 chronic pancreatitis 61 controls	ELISA	0.95 (PDA vs Normal) 0.75 (PDA vs CP)	Simeone et al., 2007
HSP27	35 PDA 37 Normal	ELISA	0.985	Melle et al., 2007
MMP7	29 PDA 31 chronic pancreatitis 9 benign disease	ELISA	0.73 (PDA vs. benign)	Kuhlmann et al., 2007
TNC, TFPI	36 PDA 19 Normal	ELISA	0.88	Balasenthil et al., 2011
SAA, HP	75 PDA 224 Non-PDA	ELISA, colorimetric	0.792 (HP) 0.691 (SAA)	Firpo, et al., 2009
ERBB2, ESR1, TNC	87 PDA	Antibody	0.677	Current study

	microarray
87 Normal	

Non-PDA = normal & benign disease

Supplementary Table 1. A comparison of receiver operator characteristic (ROC) area under the curve values for the 3-marker panel of ESR1, ERBB2 and TNC in pre-diagnostic plasma to those for other serum and plasma biomarkers discovered in diagnostic plasma samples. Included are the number and type of samples compared in each study, the method used to identify the AUC values and the source of the study. The AUC of 0.677 for our pre-diagnostic 3-marker panel is only modestly lower than some of the reported diagnostic markers.

Supplementary figure 1.



Supplementary figure 1. Plasma M-values for ERBB2, ESR1 and TNC do not change as the time between blood draw and death decreases. M-values for pre-diagnostic case samples drawn < 1 year, between 1 and 2, 2 and 3 and 3 and 4 years were bulked together and plotted along with the mean M-values and standard deviations. 2-tailed unpaired t-tests were used to determine significance. Differences between M-values as a function of time from draw to death were not significant for all comparisons.

CHAPTER 5

PERSPECTIVES

The biomarkers identified in the array studies in mice and humans provide some promising candidates that warrant further pursuit. From a biological perspective, the identification of a hippo pathway member as being up regulated in metaplastic lesions and, later, in high-grade PanIN lesions potentially implicates a new pathway in pancreatic carcinogenesis. Coupled with the recent findings showing the importance of hippo kinases in maintenance of pancreatic parenchyma (150, 151) and a role for cytoplasmic YAP1 in counterbalancing Wnt-mediated stem cell regeneration (160), it is conceivable that hippo signaling could play a role in maintaining a more differentiated cancer phenotype. Thus, it is interesting that I saw decreased STK4 in more sarcomatoid PDA and metastases and that TGFβ treatment, known to induce EMT and reduce cell proliferation in *KPC* cells (123), decreased STK4 and concomitantly increased YAP1. The STK4 shRNA knockdown results will help determine whether hippo kinases play a functional role.

From a biomarker perspective, STK4 holds promise as a marker helping diagnose PDA, even at early stages, and differentiate it from CP. If, for example, MRI or CT scans suggest a possible pancreatic lesion, use of markers such as the seven markers I validated in our mouse studies, might help corroborate PDA versus CP from EUS-FNA biopsies. Markers should first be tested in resected tumor tissue, as was performed with STK4 in autopsy tissue. Then, preliminary assessment should occur in high-risk individuals (HRI) currently enrolled in pancreatic registries (175), before they are potentially applied as diagnostics in the general population. Similarly, TNC and ERBB2, which have been identified by mass-spectrometry,

IHC, ELISA and array platforms, could be paneled in plasma collected in these HRI as well as plasma collected from patients prior to their tumor resection. Their utility as diagnostic and prognostic markers, respectively, might then bear significance when linked with identification of solid lesions or with survival times, respectively.

As I saw with STK4, not all human PDA tumors will express a marker of interest, so it becomes important to identify which clinicopathological features can be linked with expression of potential biomarkers of disease. This is not surprising given the genetic heterogeneity of primary and metastatic disease. The context of markers can be explored in resected tumor tissue vis-à-vis the stage, grade, lymph node status, mutational spectrum, etc. Approximately 40% of PDA tumors studied show mutations in all of the driver genes (*KRAS*, *TP53*, *DPC4*, *CDKN2A*), and recently, the number of inactivated driver mutations per tumor was linked with poorer patient survival and higher metastatic disease burden (33, 50). Thus, is STK4, for example, only found in patients presenting with *TP53* mutations but retaining functional *CDKN2A*, as is the case in *KPC* mice? For plasma markers such as TNC and ERBB2, how do their levels fare with regards to stage at diagnosis?

This theme of placing biomarkers in their context to improve their diagnostic, prognostic and predictive values is challenging to apply to an aggressive disease like PDA. As discussed in the 'Future Directions' section for cross-species identification of putative early plasma markers of PDA, the low incidence of PDA in the general population makes testing markers for general screening difficult. Hence the studies of markers in HRI, where increased risk and earlier onset of disease and the creation of pancreas cancer registries for monitoring disease, tips the scale in favor of identifying early lesions in asymptomatic individuals. Yet the frequency of HRI in the general population is low, too, such that having large enough numbers to identify trends in

biomarker expression remains difficult. For example, the 132-fold increased risk of developing PDA in patients with Peutz-Jegher's Syndrome was based on 6 of 210 patients presenting with pancreas cancer (176). Of the 1040 HRI screened to date, 70 of these have had a lesion or potential lesion resected (175). These numbers are bound to increase, but are indicative the challenges of applying markers even in this setting.

It also remains unclear what constitutes true early detection from the perspective of survival benefit. Longer survival times have been reported when stage I disease is resected (10, 16, 175) and especially when the tumor is resected with negative (R0) margins (15, 182). Detection and treatment of PanIN-3 lesions, or carcinoma in situ, has been suggested especially in HRI since patients with tumors <2 cm, negative lymph nodes and no evidence of metastases still succumb to disease following resection. Yet, it is unclear how fast PanIN progress to PDA and with what frequency. Canto et al. cite a study suggesting just <1% of PanIN lesions progress to PDA (175). Studies comparing the presence and absence of PanIN lesions in the transection margin of R0 resected tumors showed no differences in survival between patients without PanIN, patients with low-grade PanIN and patients with PanIN-3 at their resection margin (15). Of course, these may represent more indolent PanIN failing to progress to invasive carcinoma, and therefore could be misleading with regards to the utility of detecting and treating early PanIN.

To address whether identification and treatment of PanIN in HRI might show survival benefits, methods to identify PanIN need improving. In a study of 216 asymptomatic HRI comparing the diagnostic utility of MRI, CT and EUS-FNA, of the 4 patients where solid lesions were identified, these were 'not concerning' for malignancy by MRI, CT and EUS-FNA, thus indicating that PanIN cannot be successfully identified preoperatively (156). Most often, PanIN associated with cystic lesions are discovered in the pathology report. This is again where a

marker panel, inclusive of STK4, BSG and p19ARF, which differentiate preinvasive and early invasive PDA from CP in mice, as well as SMAD2, which did not show differences, could be useful. If these markers are validated in resected human PDA tissue as I have done for STK4 in autopsy tissue, then they might be applied to analyses of FNA, where elevated protein levels (separate FNA for cytological and protein preparations could be required) would point towards the presence of PanIN or early invasive PDA. BSG and STK4 would hold especial promise since they are specific to the epithelial component of tumor tissue (146). As discussed above in the 'Future Directions' section for our mouse biomarker studies, if these markers also differentiate PanIN from other precursor lesions such as IPMN, then combining them with the presence of *KRAS* and *GNAS* mutations could help identify more malignant IPMN with associated PanIN, further differentiating suspected benign from malignant lesions.

The success of protein markers, especially plasma markers, is thus predicated on further validation in controlled studies with defined goals. Panels and various combinations of markers will most plausibly be of future benefit serving to complement imaging, tumor histology and clinical outcomes. Only through such retrospective studies can promising markers of PDA be effectively identified and then applied in more general screening of larger populations. It will also be interesting to track putative markers in resected tumors and plasma collected from long term survivors of pancreas cancer. If plasma markers were absent in follow up studies in the small number of PDA survivors, these would serve as excellent prognostic indicators. The lack of markers in resected tissue from survivors may also suggest that these are markers of untreatable disease.

As technologies improve, just as our array platforms have improved over progressive iterations, low abundance plasma markers and PDA-specific disease markers will be more

readily identified. Given that CA19-9, which was discovered close to 30 years ago and was given FDA approval a decade ago, was identified using immune-based technologies, I think antibody microarray platforms are suited to further validate and serve as platforms in a more clinical setting such as studying HRI cohorts. With research in PDA garnering more attention recently (see the case study of PDA in "Cancer Facts and Figures 2013" by the American Cancer Society) the hope is that more individuals with other family members diagnosed with pancreas cancer will enroll in Pancreas Cancer Registries. The extensive and collaborative effort into identifying relevant biomarkers of PDA, into further characterizing drivers of early carcinogenesis and metastasis and understanding the role of the tumor microenvironment and immune component of PDA, has made measurable progress on our understanding of the disease. The next step will be to integrate this acquired knowledge and effectively apply it in a clinical setting so that we achieve the ultimate goal of improving patient survival times and rates.

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