Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer-related deaths in the United States and other industrialized countries (1). Despite many efforts, it remains a devastating disease with a 5-year survival rate of less than 5% and a median survival of less than 1 year (1). This grim prognosis is mostly due to the advanced incurable stage in more than 80% of patients (1). The only potentially curative treatment is radical surgical resection (3). Prognosis after resection depends on tumor size (<3 cm), lymph node involvement, and status of the resection margin (3).

Early diagnosis of small or even preinvasive cancers before the onset of metastasis is currently the only means to substantially improve resectability, prognosis after resection, and ultimately survival (4). However, all available diagnostic tools and biomarkers for PDAC fail to detect early or preinvasive cancer and suffer from low specificity and sensitivity (5, 6). The only clinically available serum biomarker for PDAC is CA 19-9, which is of limited use (7). Invasive endoscopic procedures (endoscopic ultrasound and endoscopic retrograde cholangiopancreatography) suffer from potential for injury to the pancreas and are highly operator dependent (5, 6, 8, 9). Cross-sectional abdominal imaging is not reliable enough to allow for screening of high-risk patient populations (10, 11) and fails to detect metastases in up to 30% of patients preoperatively (12, 13). All 3 screening modalities also cannot safely discriminate PDAC from chronic pancreatitis (CP), which is of particular importance because PDAC can arise in the background of CP, and CP often mimics PDAC due to their similar clinical signs and symptoms (14, 15). This similarity makes the identification of biomarkers that distinguish PDAC from CP very challenging (16).

PDAC is believed to progress through precursor lesions termed pancreatic intraepithelial neoplasia (PanIN).
Early PanIN I and II lesions are frequently observed in normal pancreata and CP. PanIN III lesions are considered carcinoma in situ (preinvasive cancer) and possess many of the genetic aberrations of invasive cancer. The detection of PanIN III lesions has a direct impact on clinical treatment decisions (17–19). Thus, the ideal biomarker for PDAC should not only differentiate benign conditions (CP) from malignancy, but also be able to detect small cancers, ideally at the preinvasive PanIN III phase. Novel biomarkers that satisfy these demands have been difficult to identify, and none of the potential candidate biomarkers discovered so far have been able to meet all of the above criteria (20–24). On the basis of findings in vitro and in vivo, and in a genetically engineered mouse model, Plec1 (Plec1) was recently suggested as a biomarker for PDAC (25), but its suitability as a biomarker for primary and metastatic human PDAC and its precursor lesions, and its capability to differentiate PDAC from CP, remain to be assessed.

This study shows that Plec1 is not only a biomarker for human invasive and metastatic PDAC, but may also serve as a marker for preinvasive PanIN III lesions. Plec1 also distinguishes malignant pancreatic disease from CP. In preclinical orthotopic mouse models of PDAC, Plec1 overexpression can be exploited for noninvasive imaging of PDAC and its metastases. These data suggest that Plec1 may indeed be an ideal biomarker for small and preinvasive cancers and that Plec1-targeted imaging of PDAC is feasible. Clinical use of Plec1-based imaging should permit the early diagnosis of small or even preinvasive cancers in addition to metastases, potentially leading to improved resectability rates and survival.

**Clinical Relevance**

Specific biomarkers for the detection of pancreatic ductal adenocarcinoma (PDAC) at an early or preinvasive stage are currently unavailable. Here we report on a pancreatic cancer biomarker, Plec1, that distinguishes PDAC from benign inflammatory diseases such as chronic pancreatitis. Plec1 is identified in 100% of tested PDAC tumors and 60% of preinvasive PanIN III lesions, and is retained in metastatic deposits, characteristics needed for an ideal imaging biomarker. In vivo imaging in orthotopic and liver metastases models of pancreatic cancer using a Plec1-targeted imaging agent for single photon emission/CT resulted in enhanced detection not only of the primary tumor but also of small peritoneal and liver metastases. These data suggest that Plec1 is a specific novel imaging biomarker for PDAC. Although further translational studies in humans will be needed, this study shows that it can be incorporated into present day imaging technologies used in humans and that this target may be used for improved early detection and staging.

**Materials and Methods**

**Tissue samples**

All tissues and biological samples were collected with the approval and in accordance with the requirements of the Institutional Review Board of the Massachusetts General Hospital, Boston, Massachusetts.

Paraffin-embedded tissue samples were obtained from the files of the Department of Pathology of the Massachusetts General Hospital, Boston, Massachusetts. All specimens had an established diagnosis at the time of assessment. A total of 4 normal pancreata, 15 CP, 14 PanIN I, 26 PanIN II, 15 PanIN III, 41 PDAC, 8 liver metastasis, 11 lymph node metastasis, 10 with matching primary tumors, and 9 peritoneal metastasis were obtained. For the assessment of Plec1 expression in extrapancreatic human cancer, a commercial tumor tissue microarray (MTU951, US Biomax) was used.

**Mice and cell lines**

All animal procedures were approved by the University of Virginia Animal Care and Use Committee and the Massachusetts General Hospital Subcommittee on Research Animal Care. Nude mice (nu/nu) were purchased from the National Cancer Institute. FVB/NJ mice were purchased from the Jackson Laboratory. Mice were maintained in a germ-free environment and had access to food and water available ad libitum.

The L3.6pl pancreatic cancer cell line was originally derived from a repeated cycle of injecting COLO-357 cells into the pancreas of nude mice, selecting for liver metastases, and reinjecting into the pancreas. AK134 cells were derived from spontaneous PDAC arising in Pft1-Cre; LSL-K-RasG12D; p53 +/- mice in the background of an inbred FVB/NJ strain. The Panc1 pancreatic cancer cell line was obtained from ATCC. All cell lines were routinely verified by morphology and growth curve analysis, and tested for Mycoplasma.

**Animal models**

Three orthotopic mouse models and 1 mouse model of liver metastasis were employed to assess the suitability of Plec1 as an in vivo imaging biomarker. L3.6pl (1 × 10⁶, n = 10) or Panc1 cells (11 × 10⁶, n = 5) in 50 μL of Hank’s Buffered Sterile Saline (HBSS) were injected into the head of the pancreas of nu/nu mice. AK134 (2.5 × 10⁷) were injected into the pancreas of FVB/NJ mice (n = 8). To obtain liver metastasis, 2.5×10⁵ AK134 cells (n = 5) in 50 μL of HBSS were injected into the capsule of the spleen. Seven days (AK134), 10 days (L3.6pl), 4 weeks (Panc1), or 15 days (AK134 liver metastasis) after injection, animals were imaged, sacrificed, and tetrameric synthetic peptide (TPTP) biodistribution assessed. All animals underwent gross inspection of the abdominal cavity and liver for metastasis. Histology was used to confirm macroscopic findings. As control animals, FVB/NJ (n = 2) or nu/nu (n = 5) mice were injected with 50 μL of HBSS into the pancreas and spleen and then imaged 1 to 4 weeks after injection.
Western blot analysis

Pancreatic tissue (50 mg) obtained as snap-frozen surgical specimens was homogenized in RIPA buffer [50 mmol/L Trizma Base (pH 7.4), 1% Triton X-100, 0.25% sodium desoxycholate, 100 mmol/L EDTA, 150 mmol/L NaCl] in combination with a protease inhibitor cocktail (0.001 mg/mL aprotinin, bestatin, pepstatin, leupeptin, and 0.005 mg/mL 20 mmol/L PMSF; Sigma-Aldrich). The lysate was cleared by centrifugation. To ensure equal loading, a highly sensitive and precise copper-based assay was used to determine the protein concentration of each sample (2-D Quant Kit; Abersham Biosciences). A portion of 20 μg of protein per lane were separated via SDS-PAGE and transferred onto a nitrocellulose membrane. Equal transfer was verified by Ponceau staining. Antigen detection was done using a rabbit monoclonal antibody against human Plec1 (Abcam). The secondary antibody was a HRP-coupled goat anti-rabbit polyclonal antibody (Sigma-Aldrich). Bands were visualized with enhanced chemiluminescence (control: rat brain lysate; Santa Cruz Biotechnology).

Immunohistochemistry for Plectin-1

Paraffin-embedded sections were deparaffinized, hydrated with Tris-buffered saline, and blocked with H2O2. Antigen retrieval was achieved by boiling tissue in Retrievit (BioGenex). After blocking with avidin/biotin (http://www.vectorlabs.com/contactus.asp#contact Vector Laboratories) and 5% goat serum in Tris-buffered saline, slides were incubated overnight at 4°C with 1:250 Plec1 antibody (Abcam). Sections were washed 3 times in Tris-buffered saline with Tween, followed by incubation with biotinylated anti-rabbit goat secondary antibody (http://www.vectorlabs.com/contactus.asp#contact Vector Laboratories), than developed using DAB (Invitrogen) and counterstained with hematoxylin. Slides were evaluated using a Y-FL microscope (Nikon).

Expression of Plec1 in nerves within each slide was used as a staining control and reference for staining intensity. Nerves were noted to have a moderate staining intensity. Staining intensity was recorded by 2 independent observers, and in case of discrepant results, evaluated by a third observer. Plec1 staining was classified as negative if the staining intensity was weaker than nerves. It was classified as positive if the staining was as least as strong as nerves.

In vitro competition assay

Tetrameric Plec1-targeted peptide [tPTP-4(BAKTLLPPT-PGSG[PEG5000])KKKDOTAßA-NH2] was synthesized in a GMP grade facility (CS Bio Company). As a control, non-binding tetramer [ncPTP-4(BAKIVMSKQGGS[PEG5000])KKKDOTAßA-NH2] was also synthesized. For Indium labeling, peptide (100 μg) was dissolved in 20 μL PBS, then diluted in 100 μL ammonium acetate buffer (0.1 mol/L, pH 4.5). Indium chloride (5 μCi in water; Cardinal Health) was mixed with the peptide and allowed to equilibrate with mixing at 40°C for 15 minutes. The reaction mixture was purified by size exclusion using a PD10 desalting column pre-equilibrated with Dulbecco’s phosphate-buffered saline. For in vitro peptide validation experiments, cells were incubated at room temperature for 1 hour with tPTP or ncPTP with concentrations ranging from 10−3 to 10−9 and 5 μCi tPTP-In111 in triplicate. After 1 hour, the cells were washed and lysed with 100 μL 1 mol/L NaOH for 5 minutes. The mixture was then transferred to tubes and activity analyzed on a gamma counter.

Imaging

Mice were injected with 1 mCi of 111In labeled tPTP, then imaged 4 hours injection with a microSPECT/CT scanner designed and built at UVA. Computed tomography (CT) acquisition used 200 evenly spaced projections spanning 200 degrees over approximately 5 minutes. Pinhole single photon emission computed tomography (SPECT) scanning was then performed using 2 opposing gamma cameras simultaneously. The 2 cameras were fitted with 0.5 mm diameter tungsten pinholes. Sixty evenly spaced projection views per camera were obtained over 180 degrees, for a total of 120 views at 3-degree increments over 360 degrees. The SPECT acquisition time was approximately 45 minutes. The reconstructed CT voxel size was 0.082 × 0.082 × 0.082 mm on a 640 × 640 × 768 image matrix. The reconstructed SPECT voxel size was 0.65 × 0.65 × 0.65 mm on an 80 × 80 × 80 image matrix. All SPECT images were corrected for radioactivity decay but not for gamma ray attenuation.

Biodistribution and blood half-life

After mice were imaged via SPECT/CT, animals were sacrificed and their organs harvested and placed into pre-weighed Eppendorf tubes. Each tube was then reweighed to determine the weight of the organ and the radiation measured. Tubes containing organs were analyzed on a gamma counter. To determine the plasma lifetime of the probe, a mouse injected with the tPTP-Peg-111In was bled 0, 15, 30, 45, 60, and 120 minutes postinjection, and the sample analyzed on a gamma counter. Tissue samples were then placed in histology cassettes and fixed for paraffin embedding. After the radioactivity in the tissue samples decayed, the blocks were sectioned on a microtome and evaluated by hematoyxlin and eosin (H&E).

Results

Plec1 expression intensity and pattern distinguish malignant from benign pancreatic disease

To determine whether Plec1 can be used as a marker for the detection of PDAC, immunohistochemistry (IHC) of human tissue sections was performed. Plec1 expression was scored as negative in all benign tissues (4 of 4 normal pancreata and 15 of 15 CP). In contrast, 41 of 41 PDAC stained strongly positive for Plec1 (Fig. 1 A and B). Similarly, Western blotting of pancreatic tissue lysates detected no Plec1 in normal pancreas or CP whereas it is present in each lysate from PDAC (Fig. 1C). Thus, Plec1 is identified in all PDACs and clearly distinguishes malignant from benign pancreatic disease (Fig. 1).
Figure 1. Plec1 immunohistochemistry and Western blot. A, representative images of the evaluated normal pancreata, chronic pancreatitis (CP), PanIN, PDAC, xenografted PDAC, and PDAC metastasis sites (liver, lymph node, and peritoneum). Overview (top) and detailed view of the black box (bottom). Chronic pancreatitis and normal pancreas do not express Plec1. PanIN III has a membranous staining pattern. PDAC and PDAC xenograft tissue stain moderately to strongly cytoplasmic and membranous for Plec1. Common PDAC metastasis sites do not show significant Plec1 expression, whereas the tumor cells stain intensely for Plec1. B, distribution of staining intensity and staining pattern in the specimens. All PDAC cases were Plec1-positive, whereas normal pancreas and CP did not express Plec1. All PanIN I and most II lesions were Plec1-negative, whereas the majority of PanIN III lesions were Plec1-positive. The cellular localization of Plec1 also changes during carcinogenesis. The protein is found only in the membrane in 33% of PanIN III lesions, whereas 27% of PanIN III and all PDAC show membranous and cytoplasmic Plec1 expression. C, quantitative Western blot for Plec1 from 50 mg of pancreatic tissue (snap-frozen surgical specimens). No Plec1 was detected in the normal pancreas and CP, whereas it was present in each PDAC.
To determine whether Plec1 expression changes during carcinogenesis, tissue sections of PDAC precursor lesions, PanINs, were evaluated by IHC. Although 0% PanIN I and only 3.85% PanIN II are Plec1-positive, 60% of PanIN III and 100% of invasive PDAC (Fig. 1A and B) were Plec1-positive. Plec1 expression thus increases during pancreatic carcinogenesis and discriminates early-stage PanIN I and II lesions from PanIN III and PDAC. During carcinogenesis, the cellular localization of Plec1 also changes. Plec1 was identified in the cytoplasm and/or on the cell membrane. Although its expression was restricted to the cell membrane in 33% of PanIN III (5/15), a membranous and cytoplasmic expression of Plec1 was observed in 26.67% of PanIN III (4 of 15) and 100% of invasive PDAC (41 of 41). Sensitivity and specificity of Plec1 for differentiating PanIN III and PDAC from normal pancreata, CP, and lower-grade PanIN lesions were 87% and 98%, respectively. Sensitivity for invasive cancers (PDAC) alone was 100%.

Plec1 is not expressed in most normal human tissue and is retained in PDAC metastasis

IHC of a human tissue microarray revealed that Plec1 is not expressed by most normal tissue, with the exception of the skin and genitourinary tract (Fig. 2). Specifically, it is not expressed in the liver, lymph node, lung, or peritoneum. PDAC has a propensity to metastasize early to these sites. To evaluate the suitability of Plec1 as a biomarker for metastatic disease, IHC of metastatic deposits was performed. All metastatic foci assayed retained their Plec1 expression, clearly identifying and highlighting metastatic deposits in the liver (8 of 8), lymph nodes (11 of 11), and peritoneum (9 of 9). The 10 lymph node metastases had the same pattern and staining intensity as their matched primary tumor (Fig. 1A).

Plec1 is an ideal biomarker for detecting pancreatic cancer, but may also be an ideal biomarker for detecting other cancers, such as esophageal, stomach, and lung cancers, where a differential expression between normal and cancerous tissue was also noted in the tissue microarray. The discovery that Plec1 can be used to highlight tumors and their potential metastatic foci suggests that smart imaging agents targeting this marker could be used to improve diagnosis and staging (Fig. 2).

Plec1-targeting probes can be used for noninvasive imaging of PDAC

To determine whether Plec1 can be used as an imaging biomarker to facilitate the detection of human PDAC in vivo, we employed Plec1-targeted peptides derived from a phage display screen (25) to synthesize a tPTP that functions as a clinically relevant imaging agent for SPECT. In vitro validation of the specificity of the tPTP was performed by competition assay with labeled tPTP and nonrelated control PTP (nrPTP). The \( K_i \) (inhibition dissociation constant) for tPTP was \( 8.3 \times 10^{-7} \) mol/L versus \( 2.86 \times 10^{-6} \) mol/L for nrPTP (Fig. 3A). Animals bearing orthotopically injected human pancreatic cancer cells (L3.6pl or Panc1) were administered tPTP and imaged via SPECT/CT 4 hours after tPTP injection. In both L3.6pl and Panc1 animals, imaging illuminated the tumor in the pancreas (Fig. 3B and data not shown). Panc1...
orthotopically injected animals did not form metastatic disease. Consistent with this finding, no tPTP uptake was identified outside the pancreas. However, 2 of 10 mice injected with L3.6pl at autopsy were found to have peritoneal metastases. tPTP SPECT/CT imaging was able to accurately detect the peritoneal metastases in these 2 animals (Fig. 3B, L3.6pl). Likewise, in a syngeneic mouse model of PDAC, tPTP was able to highlight the primary tumor and associated metastases in the peritoneum (Fig. 3B, AK134). In contrast, only the kidneys were visible in control animals (Fig. 3B, null).

To validate and quantitate imaging results, biodistribution studies were performed to confirm tumor-specific tPTP accumulation. Biodistribution results showed that the pancreatic tumors from all 3 cell lines had a statistically significant 1.9- to 2.9-fold higher uptake when compared with pancreata from control animals (Fig. 3C, P < 0.01). The probe was identified in the kidneys, which are its main route of elimination (Fig. 3C). H&E staining and of sectioned pancreas and peritoneal metastases showed the presence of Plec1-expressing tumors in the pancreas and peritoneum (Fig. 3D). These data show that Plec1-targeted imaging using tPTP functions as a highly specific imaging tool for PDAC, clearly distinguishing PDAC and its metastases from their adjacent normal tissues.

To determine the ability of tPTP to highlight liver metastases, AK134 cells were used in a well-known model of liver metastases that form after intrasplenic injection. Animals injected with tPTP were imaged 4 hours later, followed by biodistribution measurements. Metastases in the liver were readily identified via tPTP-mediated SPECT/CT imaging (Fig. 4A). Biodistribution analysis confirmed the imaging results, with a 1.7-fold increase in tPTP accumulation in livers that had metastases over livers from animals devoid of tumors (P < 0.01; Fig. 4B). H&E confirmed the presence of metastatic disease in livers that were positive via tPTP-mediated SPECT imaging (Fig. 4C). These data show that Plec1-targeted imaging sing tPTP functions as a highly specific imaging tool for PDAC, highlighting PDAC and its metastases.

Discussion

In this study we show that Plec1 expression identifies preinvasive PanIN III lesions as well as primary and meta-
static human PDAC. Plec1 expression intensity also clearly discriminates cancers from benign conditions, in particular CP. In a preclinical orthotopic mouse model of PDAC, Plec1-targeted noninvasive imaging detects primary and metastatic PDAC. Taken together, these data suggest that Plec1 may indeed be an ideal biomarker for PDAC.

The use of Plec1 as a biomarker offers several advantages over current clinical diagnostic tools and markers. Unlike CA 19-9, which lacks sensitivity and specificity (7), Plec1 is a specific biomarker for invasive and preinvasive pancreatic cancer. In contrast to conventional cross-sectional abdominal imaging (10–13), Plec1-based imaging would highlight small, currently unidentifiable metastatic foci preoperatively, thus substantially improving preoperative staging. Due to its ability to potentially highlight preinvasive PDAC, it has the potential to be rapidly developed into present-day screening protocols for high risk patients with the promise of detecting PDAC prior to invasion.

Due to its unique properties, the use of Plec1 as a biomarker for preinvasive and invasive PDAC also holds promise to be superior to other recently described biomarkers. Lactose-binding protein, which is overexpressed in acinar cells that surround PDAC, is ideal to detect small cancers within the pancreas, but cannot detect small metastases (24). Neutrophil gelatinase–associated lipocalin delineates PanIN lesions and some invasive cancers, but does not identify poorly differentiated adenocarcinoma (23). MUC1 and MUC4 are overexpressed in a subset of invasive adenocarcinoma, but their expression does not distinguish early PanIN I/II from preinvasive (PanIN III) and invasive cancers. MUC4 is also aberrantly expressed in CP (20, 21). In contrast, Plec1 detects all PDAC and their small metastatic foci as well as the majority of preinvasive cancers (PanIN III) in addition to discriminating PDAC from CP.

To date, the role of Plec1 overexpression in pancreatic cancer is unknown. Plec1 itself is a cytolinker protein of the plakin family. Plakins connect intermediate filaments to desmosomes and hemidesmosomes, stabilize cells mechanically, regulate cytoskeleton dynamics, and serve as a scaffolding platform for signaling molecules. Plakins were first described as essential for skin and skeletal muscle integrity (26) and mutations of the Plec1 gene were therefore initially identified in skin disease, such as epidermolysis bullosa (27, 28). A recent study, in which Plec1 was found to interact with the breast cancer susceptibility gene 2 (BRCA2; ref. 29), provides more insight into the protein’s potential role in cancer. BRCA2 mutations are associated with an increased risk of pancreatic cancer (30, 31). BRCA2 itself plays an important role in DNA damage repair and is mainly found in the cell nucleus, but has also been identified in the centrosome. The Plec1/BRCA2 interaction is involved in the regulation of centrosome localization and Plec1 misexpression leads to displacement of the centrosome. This may contribute to genomic instability and therefore cancer development (29). We found that Plec1 expression is acquired during the transition from PanIN II to PanIN III. As lesions progress, Plec1 is not retained at the cell membrane, but rather is ubiquitously expressed in the cancer cells. It thus appears that Plec1 overexpression and cytoplasmic localization begin at the stage of PanIN III and further increase as lesions progress to invasive cancer.
In summary, Plectin-1 may be the best novel biomarker for PDAC identified to date. Although further studies are needed to verify Plectin-1 as a target in humans, strategies designed to image Plectin-1 could substantially improve detection and staging, thus contributing to improved resectability, prognosis, and ultimately survival in pancreatic cancer.

Disclosure of Potential Conflicts of Interest

The authors declared no potential conflicts of interest.

References


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Dirk Bausch, Stephanie Thomas, Mari Mino-Kenudson, et al.


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