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Detection of Pancreatic Ductal Adenocarcinoma in Mice by Ultrasound Imaging of Thymocyte Differentiation Antigen 1

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Abstract

BACKGROUND & AIMS—Early detection of pancreatic ductal adenocarcinoma (PDAC) allows for surgical resection and increases patient survival times. Imaging agents that bind and amplify the signal of neovascular proteins in neoplasms can be detected by ultrasound, enabling accurate detection of small lesions. We searched for new markers of neovasculature in PDAC and assessed their potential for tumor detection by ultrasound molecular imaging.

METHODS—Thymocyte Differentiation Antigen 1 (Thy1) was identified as a specific biomarker of PDAC neovasculature by proteomic analysis. Upregulation in PDAC was validated by immunohistochemical analysis of pancreatic tissue samples from 28 healthy individuals, 15 with primary chronic pancreatitis tissues, and 196 with PDAC. Binding of Thy1-targeted contrast microbubbles was assessed in cultured cells, in mice with orthotopic PDAC xenograft tumors expressing human Thy1 on the neovasculature, and on the neovasculature of a genetic mouse model of PDAC.

RESULTS—Based on immunohistochemical analyses, levels of Thy1 were significantly higher in the vascular of human PDAC than chronic pancreatitis ($P=.007$) or normal tissue samples ($P<.0001$). In mice, ultrasound imaging accurately detected human Thy1-positive PDAC xenografts, as well as PDACs that express endogenous Thy1 in genetic mouse models of PDAC.

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The authors declare no conflicts of interest.

Author contributions:

JKW and AML designed research; KF, HW, AML, RC, SM, MAP, TC, TAB, and JKW performed research; KF, HW, RC, LT, TAB, and JKW analyzed data; JKW wrote the manuscript, and all authors edited the manuscript.

CONCLUSION—We have identified and validated Thy1 as a marker of PDAC that can be detected by ultrasound molecular imaging in mice. The development of a specific imaging agent and identification of Thy1 as a new biomarker could aid in the diagnosis of this cancer and management of patients.

Keywords

Molecular imaging; pancreatic cancer; CD90; diagnostic test

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer death in the USA.¹ The American Cancer Society estimated 43,920 new diagnoses of PDAC and 37,390 deaths from this cancer in 2012 in the USA.¹ Survival from pancreas cancer is stage dependent. Because the disease is diagnosed at advanced tumor stage, patients have a median survival of less than one year; only 5% of patients survive five years after diagnosis.^{2, 3} Unfortunately, current chemotherapy and radiotherapy approaches offer only moderate survival benefits and surgery for localized disease is only possible in 15–20% of patients at the time of diagnosis.^{4, 5} Therefore, earlier detection of PDAC that allows potentially curable surgical resection offers our best hope to improve patient survival.⁶

One potential strategy for earlier detection of cancer, including PDAC, involves screening moderate and high-risk patients with a highly accurate and inexpensive blood biomarker test (or combination of biomarkers) followed by a second-level, imaging-based test to confirm a positive biomarker result and anatomically localize the cancer.⁷ Serum CA-19-9, currently the only clinically used blood biomarker, lacks the sensitivity and, more importantly, the specificity needed to detect early-stage PDAC; active research to discover more accurate blood-based or saliva-based biomarkers is underway.⁸

Imaging tests are also limited in accuracy for early detection of pancreatic cancer. Currently used imaging techniques, including abdominal computed tomography (CT), magnetic resonance imaging and cholangiopancreatography (MRI/MRCP), as well as transabdominal and endoscopic ultrasound (EUS), can be unreliable and/or non-specific in detecting early stage PDAC, particularly for lesions smaller than 1 cm.^{9, 10} Furthermore, chronic pancreatitis, a risk factor for PDAC¹¹ can have imaging findings that are difficult to differentiate from PDAC using current US, CT, or MRI techniques.^{12, 13} Therefore, development of novel contrast agents targeted at markers differentially expressed in PDAC compared to benign diseases is critically needed.

The vasculature of cancer, termed neovasculature, differs from normal blood vessels at the molecular and protein level.¹⁴ Molecularly-targeted contrast-enhanced ultrasound (henceforth ultrasound molecular imaging) is a promising new imaging technique with the potential to detect molecular markers expressed on the neovasculature^{15, 16} and potentially increase the sensitivity and specificity of ultrasound in detecting early cancer. For ultrasound molecular imaging, micron-sized gas-filled contrast agents (microbubbles; MB), modified to bind to molecular markers, are intravenously injected. After a few minutes, the targeted MB accumulate at tissue sites expressing the molecular target. This results in a local increase in the ultrasound imaging signal and enhanced ability to visualize small lesions.¹⁷ Since contrast MB are several microns in size, they *remain in the intravascular compartment and exclusively detect* proteins expressed on the neovasculature of precursor lesions or early stage cancer. The challenge has been to discover and validate protein targets that are differentially expressed on the vasculature of PDAC versus normal pancreatic tissue and benign diseases, such as chronic pancreatitis, to maximize diagnostic .¹⁶

In this study, we exemplify a proof-of-principle approach from discovery of thymocyte differentiation antigen 1 (Thy1 or CD90)¹⁸ in human tissue samples as a target for PDAC neovascularity imaging to *in vivo* validation Thy-1 as a promising new ultrasound molecular imaging target in PDAC. We demonstrate that ultrasound molecular imaging can detect several mm cancers in a novel orthotopic mouse PDAC model expressing human Thy1 on its neovascularity. Finally, we validate the utility of using Thy1 ultrasound molecular imaging in a transgenic PDAC model that closely resembles human PDAC.

MATERIALS AND METHODS

The overall experimental set up of this study is summarized in Figure 1.

Thy1 Target Identification

Proteomic analysis was performed on whole tissues from patients with PDAC (n=5), chronic pancreatitis (n=5), and normal pancreas from donors (n=10) using a LTQ-Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific, Waltham, MA) coupled with a nano-flow HPLC (Eksigent Technologies, Dublin, CA) as previously described.¹⁹ A total of 118 proteins were over-expressed by a factor of 2.0 or more in PDAC compared to normal pancreas. These were searched against literature reports to identify proteins associated with tumor neovascularity. Putative neovascular proteins were triaged based on 1) the highest expression in cancer; 2) lack of or low expression in chronic pancreatitis tissue; 3) membrane association of the protein; and 4) assessment of protein expression in normal tissues using the Human Protein Atlas and/or published literature in PubMed, with lack of expression in normal organs being preferred. The highest rated candidate was the membrane protein Thy1.²⁰ The selection of Thy1 was further supported by its association with tumor vascular endothelium.^{21, 22}

Validation of Thy1 Protein Expression in Human Pancreatic Tissues

Samples used in these studies were collected with Human Subjects approval at the University of Washington and Stanford University. Furthermore, a pancreatic tissue microarray was purchased from USBioMax (Rockville, MD). Immunohistochemical (IHC) analysis of Thy1 expression was performed in pancreatic tissue obtained from 4 normal patients; 15 primary chronic pancreatitis tissues (defined as chronic pancreatitis not associated with PDAC); 21 PDAC; and in a commercial tissue microarray with 24 normal pancreatic tissues and 175 PDAC cases. Consecutive tissue sections were stained for the vascular endothelial cell marker CD31 and for human Thy1 using standard techniques (Supplementary Materials and Methods). All slides were reviewed and graded by a pathologist, experienced in pancreatic pathology. Vascular endothelial cell staining of Thy1 was scored with a semi-quantitative IHC score from 0 to 3+ as previously described.²³ In brief, cases with Thy1-staining of less than 5%, 5–32%, 33–67%, and greater than 67% of CD31 positive vessels were scored as 0, 1+, 2+, and 3+, respectively.

Human Thy1-expressing Vascular Endothelial Cells

Murine vascular endothelial (MS1) cells stably expressing human Thy1 on the cell surface were generated using standard protocols (Supplementary Materials and Methods). Stably-transfected cells (selected by incubation with 5 µg/ml puromycin; Sigma, St. Louis, MO) were confirmed for Thy1 expression by flow-cytometry analysis and by immunofluorescence staining (Supplementary Materials and Methods). For subsequent flow chamber experiments (see below), two endothelial cell clones with high (clone 1) versus low (clone 2) human Thy1 expression were selected. Clone 1 was also used for the generation of a novel human PDAC xenograft model in mice expressing human Thy1 on its neovascularity (see below).

Preparation of Microbubble Contrast Agents

Human and murine Thy1-targeted (MB_{Thy1}) and control (MB_{Control}) contrast MB were prepared by attaching anti-human or anti-murine Thy1 antibody or isotype-matched control IgG antibody onto the surface of perfluorocarbon-containing, lipid-shelled MB as described previously²⁴ (Supplementary Materials and Methods). The average number of attached antibodies per square micrometer of the MB surface was approximately 7,600 for all MB types per flow-cytometry analysis.

Flow Chamber Experiments

MB_{Thy1} binding to endothelial cell clones 1 and 2, as well as to wild-type negative control vascular endothelial cells was assessed in cell culture experiments under flow shear stress conditions, simulating flow in tumor capillaries (Supplementary Materials and Methods).^{25, 26}

Human Thy1-expression in Orthotopic PDAC Xenografts in Mice

The Administrative Panel on Laboratory Animal Care of Stanford University approved all procedures using laboratory animals. Human AsPC1 pancreatic ductal adenocarcinoma cells (ATCC, Manassas, VA) were cultured to 70–80% confluency. After midline laparotomy, the pancreas of nu/nu mice was exposed, and AsPC1 cells along with endothelial cell clone 1 cells at 1:5 ratio (total of 6×10^6 cells, dissolved in 25 μ l of Matrigel containing epidermal growth factor (0.7ng/mL), insulin-like growth factor (16ng/ml), and transforming growth factor-beta (2.3ng/ml); BD Biosciences, San Jose, CA) were co-injected into the body or tail of the pancreas in 25 female nude mice (6–8 weeks old; Charles River, Wilmington, MA). In negative control animals (n=14), AsPC1 cells alone (n=7) or combined with endothelial wild-type cells (n=7) were co-injected at the above-mentioned ratio. The abdomen was then closed by layers. Orthotopic xenografts were allowed to grow between 7 and 21 days to yield a spectrum of different tumor sizes with diameters ranging between 1.6 and 10.0 mm (mean, 4.5 mm) as measured by B-mode ultrasound.

Transgenic Mouse Model of PDAC

The transgenic pancreatic cancer mouse model, Pdx1-Cre^{tg/+}; KRas^{LSL G12D/+}; Ink4a/*Arf*^{-/-} was used (n=6) which spontaneously develop foci of pancreatic cancer within 4 to 7 weeks of age.²⁷ Tumor diameters ranged between 2.0 and 4.8 mm (mean, 3.1 mm). Age-matched littermates without KRas^{G12D} mutation were used as normal WT control (n=6).

Chronic Pancreatitis Model

Chronic pancreatitis was established using two subcutaneous injections of L-arginine dissolved in 0.9 % saline (6 g/kg) separated by one hour.²⁸ This was repeated weekly for 6 weeks after which the mice were allowed to recover for 14 days prior to US scanning.

In Vivo Ultrasound Molecular Imaging of PDAC Xenografts and PDAC in transgenic mice

All ultrasound molecular imaging was performed using a dedicated small animal ultrasound machine; the technical specifications are detailed in the Supplementary Materials and Methods.

In all PDAC xenografts and tumors in transgenic mice, intra-animal comparisons of ultrasound imaging signals were performed following injection of both MB_{Thy1} and MB_{Control} in a randomized order during the same imaging session. Four minutes following tail vein injection of either MB_{Thy1} or MB_{Control} (5×10^7 ; bolus injected within 2 seconds through a tail vein catheter), 250 imaging frames were acquired of each tumor within 25 seconds.^{25, 26} This was followed by a one-second destruction pulse to destroy all MB in the

field-of-view (destruction pulse of 3.7 MPa; transmit power, 100%; mechanical index, 0.63). Nine seconds after the destruction pulse, another 250 imaging frames were acquired to capture the influx of freely circulating MB. After a waiting time of at least 30 minutes to allow MB to clear from previous injection,^{24, 29} this imaging sequence was repeated following intravenous injection of 5×10^7 MB of the second MB type of either MB_{Thy1} or MB_{Control}, with the transducer maintained at the same anatomical location. Furthermore, an *in vivo* competition assay was performed using anti-human Thy1 antibody in the xenografts (100 µg; eBioscience; San Diego, CA) as described previously.^{25, 26}

Imaging Analysis of *In Vivo* Ultrasound Molecular Imaging

Ultrasound molecular imaging was analyzed offline using dedicated software with motion compensation capabilities (VevoCQ, Visualsonics; Toronto, Canada). The imaging signal (expressed in arbitrary units, a.u.) from attached MB was defined as the difference between pre- and post-destruction imaging signals as described²⁶. Regions of interest were drawn over the different tumors and over adjacent normal pancreas tissue by one reader, blinded to the types of MB (MB_{Thy1} vs. MB_{Control}) and tumor (positive vs. control tumors).

Ex Vivo Analysis of PDAC Xenografts and PDAC in Transgenic Mice

To confirm expression of human and murine Thy1 on vascular endothelial cells of the tumor neovasculature in mice, all tumors were excised after ultrasound imaging and immunostained for either human Thy1 or murine Thy1, as well as for mouse CD31 according to standard protocols (Supplementary Materials and Methods).

Thy1 Staining of Normal Organs in Mice

Nu/nu mice were euthanized and the cerebral cortex, lungs, liver, spleen, pancreas, colon, uterus, skin, and skeletal muscle were analyzed for murine Thy1 expression using standard protocol (Supplementary Materials and Methods):

Statistical Analysis

All continuous measurements were expressed as means $\pm$ standard deviation. Differences in the Thy1 expression levels between groups of patient samples were tested for statistical significance using the Mann-Whitney test. Empirical receiver operating characteristic (ROC) curves were used to determine the sensitivity and specificity of Thy1 in separating PDAC from non-cancer controls. The two-sample Wilcoxon test was used for pair-wise comparisons of measurements in flow chamber experiments. For comparison between MB_{Thy1} vs. MB_{Control} in the same tumor and for competition assays, the one-sample paired Wilcoxon test was used. The two-sample t-test was used for comparisons in multiple groups (Thy1-positive and two control tumor types). For assessing associations between Thy1-expression levels and cell attachment, Spearman's correlation coefficient was estimated and the corresponding *P*-value was obtained based on Fisher's transformation. Statistical analyses of Thy1 IHC scores were performed using GraphPad Prism (La Jolla, CA); the remainder of the statistical analyses was performed with R 2.10.1 with a significance level of 0.05.

RESULTS

Validation of Thy1 Expression in Human Pancreatic Tissues

To verify the expression of Thy1 in PDAC-associated neovasculature in humans, IHC was performed on pancreatic tissue samples from normal pancreas (n=28), primary chronic pancreatitis (n=15) and PDAC patients (n=199). Thy1 expression was detected within the PDAC peritumoral compartment as well as within the tumor vasculature. Due to the

vascular restriction of the ultrasound molecular contrast agent, only vascular staining (guided by CD31) was evaluated. The expression of Thy1 was significantly increased in the pancreatic vasculature of PDAC patients (score, 2.1 ± 0.1) and was minimal in normal controls (0.5 ± 0.1 ; $P < 0.0001$) or primary chronic pancreatitis (0.6 ± 0.15 ; $P = 0.007$) (Figures 2A–C). Considering IHC scores of 2+ or 3+ as positive staining, 81% of PDAC cases stained positive for Thy1 while normal pancreas and chronic pancreatitis cases were positive in 11% and 7%, respectively. ROC analysis indicated that Thy1 neovascular immunostaining could distinguish PDAC from normal and chronic pancreatitis tissues with 90% specificity and 81% sensitivity [area under curve (AUC) value=0.89].

Microbubble Binding in Flow Chamber Experiments

Mouse neovascular endothelial cells do not express human Thy1, thus to recapitulate the protein expression of Thy1 of human PDAC neovasculature, two mouse vascular endothelial cell lines with different expression levels of human Thy1 (clone 1 and clone 2) were created and the Thy1 expression levels were assessed by immunofluorescence microscopy (Figure 3A), and FACS analysis (Figure 3B, C). Supplementary Table 1 summarizes binding of MB_{Thy1} and MB_{Control} to endothelial cell clone 1 (high human Thy1 expression), clone 2 (low human Thy1 expression) and to wild-type vascular endothelial cells (no Thy1 expression) in flow chamber experiments. Binding of MB_{Thy1} to both clone 1 ($P = 0.008$) and clone 2 ($P = 0.01$) was significantly higher than binding to wild-type endothelial cells (Figure 4). Blocking of Thy1 receptors with anti-human Thy1 antibodies resulted in significantly decreased MB_{Thy1} attachment ($P = 0.03$), confirming binding specificity of MB_{Thy1} to human Thy1. In contrast, MB_{Control} only demonstrated low, non-specific attachment to all three cell types ($P = 0.5$). The number of attached MB_{Thy1} highly correlated with the expression levels of Thy1 on vascular endothelial cells ($\rho = 0.92$, $P < 0.001$).

In Vivo Ultrasound Molecular Imaging in PDAC Xenografts and in PDAC of Transgenic Mice

To validate binding of human Thy1-targeted MB_{Thy1} to human Thy1 *in vivo*, we developed a novel orthotopic PDAC xenograft mouse model by co-injecting human AsPC1 PDAC cells with mouse vascular endothelial cells expressing human Thy1. As negative control tumors, either AsPC1 cells only or AsPC1 cells mixed with human Thy1-negative endothelial cells were injected orthotopically into the mouse pancreas. Growth rates among the different tumor types were not significantly different ($P > 0.05$) and tumors over a broad size spectrum between 1.6 and 10.0 mm in diameter were imaged. Imaging enhancement of Thy1-positive tumors ($n = 25$) was significantly increased ($P < 0.001$) following injection of human MB_{Thy1} compared to MB_{Control} (Figure 5; Table 1). Control xenografts ($n = 14$) and normal pancreas tissue adjacent to the three different tumor types ($n = 39$) showed significantly lower imaging signal using human MB_{Thy1} (Table 1). *In vivo* competition assay ($n = 7$) using anti-human Thy1 antibodies resulted in a significant decrease ($P = 0.008$) of human Thy1-targeted imaging signal in Thy1-positive xenografts, which further confirmed binding specificity of MB_{Thy1} to human Thy1 *in vivo*.

Similarly, within tumors of a transgenic murine pancreatic cancer model, ultrasound molecular imaging signal obtained with murine Thy1-targeted MB was significantly higher than with MB_{Control} ($P = 0.01$) (Figure 6A; Supplementary Table 2). Also, imaging signal was significantly ($P = 0.04$) higher in pancreatic tumors compared to normal pancreas in WT control mice (~3-fold increase in imaging signal; Figure 6A). In contrast, in mice with chronic pancreatitis, there was no significant difference ($P = 0.92$) in imaging signal when using MB_{Thy1} versus MB_{Control} (Figure 6A).

Ex Vivo Analysis

Immunofluorescent imaging confirmed overexpression of human Thy1 on the neovasculature in Thy1- positive xenografts while there was no human Thy1 expression observed on the neovasculature of Thy1- negative and AsPC1-only xenografts (Figure 5, right panels). Similarly, the neovasculature of PDAC in transgenic mice stained positive for murine Thy1, whereas normal pancreas and chronic pancreatitis stained negative (Figure 6B).

Murine Thy1 expression was observed in neuronal tissue in the cerebral cortex of the brain, within dermal stroma of the skin, the mucosa of the colon and the myo- and endometrium of the uterus. Thy1 was also expressed on pericytes surrounding the arterioles of the pancreas, colon, kidney, liver, lung and uterus. Overall, there was no detectable murine Thy1 present on the vascular endothelial cells of any of the examined normal organs.

DISCUSSION

There is a great need for inexpensive and highly accurate imaging of lethal cancers such as PDAC. Earlier detection of cancer is limited, in part, by the availability and cost of the diagnostic test. Ultrasound fulfills many prerequisites for becoming a promising imaging tool for early cancer detection: It is noninvasive and inexpensive compared to other imaging modalities; does not use ionizing irradiation; has a very high spatial and temporal (real-time exam) resolution;^{30–32} provides deep tissue penetration (e.g., compared to optical based approaches); and is routinely available in almost all clinical imaging departments worldwide. Several morphological imaging criteria of pre-invasive or early PDAC have been described on endoscopic ultrasound, including parenchymal heterogeneity, echogenic foci, and hypoechogenic nodules.³³ However, these findings are subtle, require interpretation by expert endosonographers, and are often nonspecific compared to chronic pancreatitis, and therefore must be interpreted with caution.³³ Indeed, in a recent study the inter-observer agreement of 17 expert endosonographers interpreting endoscopic US scans in high risk patients for PDAC was only fair to poor and did not improve even on consensus interpretations.³⁴ Adding molecular imaging capability to clinical ultrasound may substantially increase the diagnostic accuracy while adding little cost to the procedure.¹⁵ Molecular imaging targets that can differentiate normal and benign disease from malignant pancreatic tissue are necessary to minimize false-positive results, thereby decreasing the number of unnecessary biopsies or surgical interventions.

The goal of this proof-of-principle study was to discover a PDAC-associated neovascular protein that could be used as an imaging target for ultrasound molecular imaging of the pancreas to increase diagnostic accuracy. Since most current ultrasound contrast agents remain exclusively within the vascular compartment, molecular imaging targets for ultrasound need to be expressed on the luminal site of vascular endothelial cells of the pancreatic neovasculature. Through quantitative proteomic analysis and several prioritizing steps, Thy1, a membrane protein, was identified as a promising new marker for ultrasound molecular imaging of PDAC.

Thy1, also known as cluster of differentiation 90 (CD90), is a cell-surface glycoprotein that belongs to the immunoglobulin-like supergene family.³⁵ Thy1 was originally described as a mouse thymocyte differentiation marker³⁶ and subsequently it was found to be expressed on other tissues¹⁸ including the surface of newly formed blood vessels³⁷ of colon cancer,²² glioblastoma,³⁸ hepatocellular carcinoma,³⁹ and ovarian cancer tissues⁴⁰ compared to normal or benign lesions, respectively. Our immunohistochemical validation study demonstrated positive Thy1 expression on the neovasculature of 81% of human pancreatic

adenocarcinoma samples while there was minimal staining in normal pancreatic tissue or chronic pancreatitis in humans or mice.

After identification and validation of Thy1 as a promising molecular imaging target for PDAC, we designed a new contrast MB targeted to human Thy1. In flow chamber experiments that simulated shear stress flow in tumor capillaries, we confirmed binding specificity of MB_{Thy1} to Thy1-positive vascular endothelial cells and showed correlation between the number of cell-attaching contrast MB and the number of Thy1 receptors expressed on the cell surface of vascular endothelial cells.

To further assess the performance of MB_{Thy1} to detect human Thy1-expressing vasculature *in vivo*, an animal model expressing human Thy1 on the tumor vasculature was needed. Since the sprouting neoangiogenic vessels in animal xenograft models are derived from the host (e.g., murine vessels in xenografts in mice), we expected that human Thy1 would not be expressed on tumor vasculature after injection of human PDAC cells into the mice. Immunohistochemical analysis of several subcutaneous human PDAC xenografts (using human AsPC1, CaPan2, and PANC1 pancreatic adenocarcinoma cells) confirmed no human Thy1 expression on the tumor vasculature (only data for AsPC1 tumors is shown; Figure 5). This necessitated the design of a novel mouse PDAC model with tumor vessels expressing the human Thy1 receptor on the vascular cell surface. Embedding spheroids containing human umbilical vein endothelial cells in a Matrigel-fibrin matrix has been previously shown to generate a functional three-dimensional human vascular network in severe combined immunodeficiency mice.^{41, 42} We modified this approach by directly co-injecting mouse vascular endothelial cells stably expressing human Thy1 along with human PDAC cells embedded in a Matrigel matrix enriched with growth factors. MS1 cells are murine pancreatic microvasculature cells transformed with a temperature-sensitive simian virus 40 large T antigen⁴³. When injected into immunocompromised mice, these cells form benign hemangiomas with a maximum tumor volume of 2 mm.⁴³ In our study, co-injection of AsPC1 cells along with the modified MS1 cells resulted in a neovasculature compartment with human Thy1-expression that was directly involved with the tumor circulation. This was confirmed by *ex vivo* immunofluorescence staining of Thy1-positive tumors which showed co-localization of Thy1 with the vascular endothelial cell marker CD31. Negative control tumors had no vascular staining of human Thy1.

This mouse model showed that a human neovasculature marker can be expressed in a murine tumor model by using a co-injection technique of modified vascular endothelial and tumor cells along with tissue matrix and growth factors. In the future, this mouse model may provide versatility for *in vivo* testing of neovasculature-targeted ultrasound contrast agents in mice by simply replacing the human *Thy1* gene by any other human gene. In our study, we validated the feasibility of visualizing human Thy1 *in vivo* with ultrasound imaging in this new model system. In orthotopically implanted human Thy1-positive tumors in the pancreas, injection of MB_{Thy1} resulted in an about 4–5.5-fold higher imaging signal compared to control conditions. While the goal of our study was not to determine the smallest detectable size of PDAC, a specific MB_{Thy1} signal could be detected in tumors with a diameter as small as 1.6 mm with ultrasound molecular imaging.

To further assess the performance of Thy1-targeted ultrasound molecular imaging in a clinically relevant *in vivo* model that recapitulates the dense stromal environment of PDAC, murine Thy1-targeted MB were tested on tumors in transgenic mice and in normal pancreas of control WT littermates. Tumors with a diameter as small as 2 mm could be visualized with an about 3-fold higher signal compared to the ultrasound molecular imaging signal in normal pancreas or chronic pancreatitis. Furthermore, vascular expression of murine Thy1

was not observed in any of the normal organs assessed by immunofluorescence analysis in our study, which is consistent with previous studies in humans.⁴⁴

In conclusion, our proof-of-principle study illustrates the development and testing process of vascular protein target discovery and validation in human pancreatic tissues to testing of *in vivo* ultrasound molecular imaging properties of new human Thy1-targeted microbubble in a novel orthotopic human PDAC model in mice that expresses human Thy1 on the neovasculature. Our study also shows substantially increased ultrasonic imaging signal in tumors developed in transgenic mice that mimic human PDAC. Since ultrasound is free of radiation, relatively inexpensive, and widely available, ultrasonic molecular imaging may eventually become useful for screening strategies in high risk patients to detect early PDAC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AUC	Area under curve
BSA	Bovine serum albumin
DAPI	4'6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
FACS	Fluorescence-activated cell sorting
IHC	Immunohistochemistry
MB	Microbubble
PBS	Phosphate buffered serum
ROC	Receiver operating characteristic
Thy1	Thymocyte differentiation antigen 1

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Figure 1.

Overview of study design, from identification of novel human PDAC-neovasculature-associated imaging biomarker Thy1, to target validation, creation of human Thy1-targeted ultrasound contrast agent, generation of a novel orthotopic PDAC xenograft model expressing human Thy1 on its neovasculature, and *in vivo* testing of imaging properties of Thy1-targeted ultrasound contrast agent.

Figure 2. Immunohistochemistry (IHC) analysis of Thy1 staining in human pancreatic tissue samples. Examples of normal pancreas (**A**) and primary chronic pancreatitis (**B**) with no Thy1 staining on vasculature. (**C**). Example of positive Thy1-staining on vessels associated with PDAC. **D**. Summary of IHC scores on Thy1 stained tissues from normal pancreas (normal), chronic pancreatitis (CP), and pancreatic cancer (PC).

Figure 3.

Evaluation of Thy1 expression on vascular endothelial cells. (A) Stably transfected cells and wild-type cells were assessed for human Thy1 expression by immunofluorescence staining. Clone 1 showed strong Thy1 staining, clone 2 showed low staining, and wild-type cells showed no Thy1 staining. (B) Human Thy1 expression levels on different cell types were quantitatively assessed by FACS analysis. Histogram overlay of signals from cells with different levels of Thy1 expression is shown. (C) Mean fluorescence intensity values are shown in bar graph. Error bars are $\pm$ standard deviations.

Figure 4.

Dynamic cell culture binding assay of microbubbles (MB) in a parallel plate flow chamber setting. Phase-contrast bright-field micrographs show binding of MB_{Thy1} and $MB_{Control}$ (white spheres, arrowheads) to different cell types; binding could be substantially blocked by incubation of cells beforehand with an anti-human Thy1 antibody (quantitative data in Table 1).

Figure 5.

In vivo ultrasound molecular imaging of orthotopic PDAC xenografts in mice and corresponding *ex vivo* immunofluorescence analysis. Transverse ultrasound images obtained in contrast mode following intravenous injection of MB_{Thy1} and MB_{Control} show strong imaging signal in human Thy1-positive tumor and background signal in both types of control tumors (scale bar = 5 mm; color coded scale is shown for ultrasound molecular imaging signal in arbitrary units, a.u.). Note low imaging signal following MB_{Control} in all tumor types (green circles, region of interest). Corresponding immunofluorescence micrographs of merged double-stained sections (murine CD31, red; human thy1, green) confirm human Thy1 expression on neovasculature in Thy1-positive tumors (yellow) while both negative control tumors did not show human Thy1 staining on the neovasculature (scale bar = 50 μm).

Figure 6.

In vivo ultrasound molecular imaging of a transgenic pancreatic tumor, chronic pancreatitis and normal pancreas tissue and corresponding *ex vivo* immunofluorescence analysis. **A)** Transverse US images obtained in contrast mode following intravenous injection of murine Thy1-targeted MB show strong imaging signal in pancreatic tumor and a low signal following MB_{Control}. Note low imaging signal following MB_{Thy1} or MB_{Control} in chronic pancreatitis and normal pancreas tissue (scale bar = 5mm; color coded scale is shown for ultrasound molecular imaging signal in arbitrary units, a.u.). **B)** Tiled confocal micrographs of murine Thy1 expression in a transgenic pancreatic tumor (top), chronic pancreatitis (middle), and WT pancreas (bottom) with murine Thy1 in red and CD31 in green show expression of murine Thy1 on the tumor vasculature but no expression on the vasculature in chronic pancreatitis and normal WT tumor.

Table 1

Summary of US Molecular Imaging Signals in Orthotopic Pancreatic Ductal Adenocarcinoma Xenografts in Mice using Human Thy1-targeted Microbubbles (MB_{Thy1}) and Control Microbubbles (MB_{Control}).

	MB _{Thy1}	MB _{Control}	<i>P</i> (MB _{Thy1} vs. MB _{Control})
Thy1-positive tumors (n=25)	7.7 ± 2.3	1.4 ± 2.2	<0.001
Thy1-negative control tumors (n=7)	1.9 ± 1.8	1.9 ± 1.7	1.0
AsPC1 control tumors (n=7)	1.7 ± 1.6	1.4 ± 0.6	0.68
Adjacent normal pancreas tissue (n=39)	1.7 ± 1.6	1.6 ± 2.2	0.37
<i>P</i> (Thy1-positive vs. Thy1-negative tumors)	0.001	0.54	
<i>P</i> (Thy1-positive vs. AsPC1 tumors)	<0.001	0.06	
<i>P</i> (Thy1-negative vs. AsPC1 tumors)	0.82	0.56	
<i>P</i> (Thy1-positive vs. normal pancreas)	<0.001	0.18	
<i>P</i> (Thy1-negative vs. normal pancreas)	0.81	0.74	
<i>P</i> (AsPC1 tumors vs. normal pancreas)	0.98	0.69	

Data are means ± standard deviation of ultrasound molecular imaging signal in arbitrary units. AsPC1 is a human pancreatic ductal adenocarcinoma cell line.