In vivo diagnosis of murine pancreatic intraepithelial neoplasia and early-stage pancreatic cancer by molecular imaging

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Pancreatic ductal adenocarcinoma (PDAC) is a fatal disease with poor patient outcome often resulting from late diagnosis in advanced stages. To date methods to diagnose early-stage PDAC are limited and in vivo detection of pancreatic intraepithelial neoplasia (PanIN), a preinvasive precursor of PDAC, is impossible. Using a cathepsin-activatable near-infrared probe in combination with flexible confocal fluorescence laser microscopy (CFL) in a genetically defined mouse model of PDAC we were able to detect and grade murine PanIN lesions in real time in vivo. Our diagnostic approach is highly sensitive and specific and proved superior to clinically established fluorescein-enhanced imaging. Translation of this endoscopic technique into the clinic should tremendously improve detection of pancreatic neoplasia, thus reforming management of patients at risk for PDAC.

molecular in vivo imaging | early detection | carcinoma in situ | genetically engineered mouse model | cathepsin

Pancreatic cancer (pancreatic ductal adenocarcinoma, PDAC) is one of the deadliest human malignancies, with an extremely poor 5-y survival rate below 5% (1). Because patients generally present with symptoms relating to late stages of the disease, diagnosis of resectable PDAC is achieved in less than 15% of cases (1). However, investigators have reported that diagnosis and resection of early-stage PDAC (<2 cm in size) can result in a 4-y survival rate of up to 78% (2–5). These data suggest that early detection of PDAC can improve patient outcome. In addition, it has been shown that resection of PDAC combined with adjuvant chemotherapy can improve 5-y survival rates to 25%. Thus, increasing the chance of resection will lead to improved survival.

Of the more than 33,000 new cases of PDAC diagnosed in the United States every year, ~10% occur in families with a high prevalence of PDAC and are thus referred to as familial (6, 7). In a cohort study of twins, it was proposed that inherited factors may be responsible for up to 36% of pancreatic cancers, suggesting that the incidence of familial pancreatic cancer may be even higher than presently assumed (8). To date, several conditions associated with familial PDAC have been described and groups of individuals at low, moderate, or high risk for developing the disease have been defined (9, 10). Although clinical trials are currently testing screening protocols for the high-risk group, aimed at detecting curable precursors of PDAC such as intraductal papillary mucinous neoplasia, pancreatic intraepithelial neoplasia (PanIN), and early-stage PDAC (10–12), the results of these trials show that early lesions are often missed. Furthermore, false positive findings can lead to overtreatment of a significant fraction of the screened population (12). These findings show that better diagnostic tools for the detection of preneoplastic lesions and early-stage PDAC are urgently needed. Recent data demonstrate that preinvasive precursors progress slowly over many years to decades to invasive pancreatic cancer (13). The parental pancreatic cancer founder clone then requires more than 5 y to acquire the capacity to metastasize (13). Thus, there is a time frame of several years for the diagnosis of curable disease, implicating the need for sensitive diagnostics.

In the current study, we investigated a relevant genetically engineered KrasG12D-dependent endogenous mouse model of murine PanIN (mPanIN) development and progression to PDAC, which accurately recapitulates the human disease (14, 15). By using a cathepsin-activatable near-infrared (NIRF) probe in combination with confocal fluorescence laser microscopy (CFL) we were able to detect early-stage PDAC as well as mPanIN lesions in vivo on the cellular level. Furthermore, it was possible to differentiate between normal pancreatic tissue and low-grade mPanINs on the one hand and high grade mPanINs and early-stage PDAC on the other, illustrating the great potential of this technique in the diagnosis of curable precursors and early-stage PDAC.

Results

Identification of Cathepsins as Targets for Molecular Imaging of PanIN Lesions and Early-Stage PDAC. To identify targets for molecular imaging of PanIN lesions and early-stage PDAC we used genomewide pancreatic gene expression analyses of Ptf1aCre; LSL-KrasG12D+/ mice, littermate controls, as well as mice with acute caerulein-induced pancreatitis. Expression profiles of over 30 individual mice revealed high levels of cathepsins B, H, L, and S in all mice with mPanIN lesions or early-stage PDAC (Fig. 1A). In contrast, expression of these cathepsins was low in normal and inflamed pancreatic tissue (Fig. 1A). To validate expression of cathepsins and to examine the localization of the corresponding proteins, we performed immunohistochemistry. Strong staining for cathepsins B, H, L, and S was detected in preinvasive mPanIN lesions and PDAC (Fig. 1B). In line with the microarray mRNA expression data, minimal cathepsin B, H, L, and S staining was observed in normal and inflamed pancreatic tissue (Fig. 1B). Furthermore, immunohistochemical staining of human pancreatic tissue sections also revealed high expression of cathepsins B, H, L, and S in PanIN lesions and PDAC (Fig. S1 and Table S1).


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Together, these data suggest that cathepsins B, H, L, and S are highly expressed in human and murine PanINs and PDAC and thus represent suitable targets for a molecular imaging-based diagnostic approach.

Activation of the Cathepsin-Activatable NIRF Probe in mPanIN Lesions and Early-Stage PDAC. Cathepsins B, H, L, and S are activators of an established NIRF probe that is based on fluorescence resonance energy transfer (16–18). To test this cathepsin-activatable NIRF probe in vivo, we injected it into Ptf1aCre/+;LSL-KrasG12D/+ mice bearing mPanIN lesions or early-stage PDAC. Twenty-four hours after i.v. injection, cryosamples of the respective pancreata were prepared and sections were imaged on an Odyssey planar near-infrared scanner to detect the signal of the NIRF probe (Fig. 2A). Tissue sections showed a strong signal emitted by the cleaved NIRF probe in mPanINs and PDAC, proving specific activation of the probe (Fig. 2A). In contrast, normal pancreatic tissues from wild-type mice and pancreatic tissues from mice with acute pancreatitis showed only weak and focal activation of the NIRF probe (Fig. 2A and C). Hematoxylin and eosin (H&E) staining of the scanned cryosections revealed that the signal of the NIRF probe was emitted from neoplastic tissue (Fig. 2B). No NIRF-probe signal was detected in areas of tumor necrosis, demonstrating the specificity of the NIRF probe for vital tumor tissue (Fig. 2B).

To investigate activation of the cathepsin-activatable NIRF probe on a cellular level ex vivo, we performed confocal laser-scanning microscopy. Cryosections were stained for morphology using an anti-E-cadherin antibody and simultaneously imaged for the NIRF-probe signal. This signal was specifically present in neoplastic cells of mPanIN lesions and early-stage PDAC (Fig. 2D). Notably, signal intensity increased from low to high grade mPanIN lesions and was strongest in PDAC. In line with our previous findings only marginal activation of the NIRF probe was found in the controls (16) (Fig. 2D). Mice with pancreatitis showed presence of the signal in immune cells that could be clearly distinguished from neoplastic cells due to differences in cell morphology and staining pattern (Fig. 2D).

Early Diagnosis of Pancreatic Cancer and mPanIN Lesions by CFL in Vivo. To evaluate the NIRF probe for detection of mPanINs and PDAC in vivo, we used the genetically defined KrasG12D-dependent endogenous mouse model. In vivo CFL was performed after i.v. administration of the NIRF probe to 1, 3, 6, 9, and 12 mo old Ptf1aCre/+;LSL-KrasG12D/+ mice displaying the whole range of mPanIN grades and early-stage PDAC and wild-type littermate controls with no morphological changes (Fig. 3A). For CFL imaging the fiber optic miniprobe was placed in contact with the pancreas, which allows single-cell resolution imaging and thus grading of mPanIN lesions. The intensity of the NIRF-probe signal detected by CFL increased with mPanIN progression to PDAC and correlated well with histological alterations in corresponding matched H&E stained sections of imaged pancreata. Importantly, CFL allowed morphological characterization of the individual cells comprising the lesion and a clear differentiation between neoplastic cells and immune cells (Fig. 3A and Movies SI, S2, S3, S4, S5, S6, S7, and S8).

Molecular Imaging of mPanIN Lesions and Early-Stage PDAC in Vivo by Combined Dual Wavelength Imaging of Cathepsin Activity and Vascularity. Because the nonspecific vascular contrast agent fluorescein is approved for clinical use in humans including endoscopic flexible lasermicroscopic imaging of neoplasia in the gastrointestinal tract (19), we wished to compare and combine the diagnostic power of the specific cathepsin-activatable NIRF probe with that of fluorescein. By using fluorescein for in vivo CFL imaging, normal pancreatic tissue architecture could be visualized well on a cellular level (Fig. 4A and Movie S1). However, no clear

Fig. 1. Cathepsins are highly expressed in preinvasive mPanIN lesions and early-stage PDAC. (A) mRNA expression profiling of pancreatic tissues from wild-type controls (WT), mice with caerulein-induced pancreatitis, and Ptf1aCre/+;LSL-KrasG12D/+ mice bearing mPanINs and PDAC, respectively. RNA was isolated from tissue specimens, labeled, and hybridized onto a GeneChip Mouse Genome 430 2.0 array (Affymetrix). Expression levels of cathepsin proteases are shown as log-fold change in row-scale (ctsb, cathepsin B; ctsh, cathepsin H; ctsl, cathepsin L; ctss, cathepsin S). (B) Paraffin-embedded pancreatic tissue sections from wild-type mice, caerulein-treated mice with pancreatitis, and Ptf1aCre/+;LSL-KrasG12D/+ mice with mPanIN lesions and PDAC were stained for ctsb, ctsl, ctsh, and ctss.
discrimination between low- and high-grade mPanIN lesions and early-stage PDAC was possible. This lack of discrimination was mainly due to fibrosis, which is associated with all grades of mPanIN lesions as well as early-stage PDAC (Fig. 4A and Movies S1, S2, S3, and S4). In contrast, the specific cathepsin-activatable NIRF probe allows clear differentiation between normal pancreas, low-grade mPanIN lesion, high-grade mPanIN lesion, and early-stage PDAC on the cellular level (Figs. 3A and 4B and Movies S5, S6, S7, and S8). High-grade mPanIN lesions were specifically characterized and distinguished from low-grade mPanINs and normal or inflamed pancreatic tissue by increased signal intensity of the NIRF probe and a typical ductal-like pattern of activation produced by cells of differing shape and size (Figs. 3A and 4B and Movies S5, S6, S7, and S8). In early-stage PDAC, the strong intensity of the NIRF-probe signal together with disturbed tissue architecture and a heterogeneous activation pattern generated by aberrant neoplastic cells allowed the differentiation from normal pancreatic tissue, infiltrating immune cells and all grades of mPanIN (Figs. 3A and 4B and Movies S5, S6, S7, and S8). The single-cell resolution of CFL imaging enabled an in vivo histopathological diagnosis based on the cellular morphology of mPanINs and PDAC. The combination with the vascular contrast agent fluorescein provided additional morphological information about the pancreas, especially about fibrosis, but did not improve accuracy of diagnosing mPanINs and PDAC in vivo. These findings demonstrate that the use of CFL in combination with the highly specific cathepsin-activatable NIRF probe is superior to fluorescent imaging of blood vessels alone and allows the accurate diagnosis of neoplastic precursor lesions and early-stage PDAC by in vivo histopathology.

Detection and Grading of mPanIN Lesions and Early-Stage PDAC by in Vivo Imaging of Cathepsin Activity in a Double Blind Study. To investigate the sensitivity and specificity of CFL imaging of cathepsin activity to detect and grade mPanIN lesions and PDAC, we conducted a double blinded in vivo study. CFL was performed in a total of 24 mice with the investigators being unaware of the genotype and age of each individual animal and at least six representative films were recorded. The study group included 6 age-matched wild-type control mice and 18 Ptf1aCre+;LSL-KrasG12D/+ mice with ages varying from 43 to 389 d. On the basis of the laser microscopic sequences, two independent examiners blinded to the histological results, graded mPanIN lesions and PDAC. Finally, a histopathological diagnosis was made by a third independent blinded investigator. The third investigator evaluated and scored the corresponding serial histological sections unaware...
of the genotype and age of the animals and the CFL-based diagnosis. Blind analysis of the CFL recordings of the 24 individual mice by the two independent investigators correctly identified all ductal pancreatic adenocarcinomas (n = 2) and all cases showing normal pancreatic tissue (n = 6). An overall agreement of 95.8% was achieved for both investigators when comparing the diagnosis of the highest grade lesion seen on CFL recordings with grading based on histology. Interobserver agreement between the two investigators was 91.7%. Furthermore, CFL grading of the imaged pancreata correctly identified all cases that harbored mPanIN-3 lesions, demonstrating the value of the technique. For each investigator grading of mPanIN lesions on the basis of CFL recordings differed only in one case from histological findings, respectively. In each case, in vivo imaging overestimated a mPanIN-2 lesion as being mPanIN-3. However, in all cases it was possible to classify pancreata as either bearing low-grade mPanIN lesions (mPanIN-1A and -1B) versus high-grade mPanIN lesions (mPanIN-2 and -3) (Table 1).

Discussion

In addition to studying tumor biology, genetically engineered mouse models are now increasingly used to conduct translational investigations. Accordingly, it was recently demonstrated that the KrasG12D-dependent mouse model of PDAC accurately mimics the therapeutic response of human PDAC and thus offers the opportunity to develop novel treatments (20). Beyond testing therapeutic strategies, we now show that this model is applicable to defining novel diagnostics, which are urgently needed to detect curable lesions in the clinic. We demonstrate that CFL in combination with cathepsin-activatable NIRF probes detects premalignant pancreatic neoplasia and early-stage PDAC with high accuracy in an endogenous KrasG12D-dependent mouse model of PDAC. Using a cathepsin-activatable NIRF probe and the nonspecific vascular contrast agent fluorescein, we characterized the “histopathology” of low-grade and high-grade mPanINs and early-stage PDAC in vivo, combining the advantages of the standard section (i.e. promptness in diagnosis) and immunochemistry (i.e. high sensitivity) with real-time CFL in a single-examination.

Expression of oncogenic Kras from its endogenous promoter in the pancreas of Ptf1aCre/+;LSL-KrasG12D/+ mice induces the development of neoplastic changes (mPanINs and PDAC), which recapitulate the human disease in many aspects. These mice develop low-grade mPanIN lesions that progress to high-grade mPanINs and metastatic PDAC over a period of several months (14). Furthermore, desmoplastic changes similar to those seen in the human disease are regularly observed. Gene expression profiling and immunohistochemistry of mPanIN lesions and PDAC from these mice identified cathepsins as suitable targets for in vivo imaging. Cathepsins, which are known to be key factors in tumor progression and invasion (21), are also believed to play a critical role in tumor initiation (22). Consistent with the murine expression data, we detected high cathepsin expression in human preinvasive PanINs and invasive PDAC, which is in agreement with a study comprising 70 human PDAC cases. Here, 96% of primary pancreatic tumors stain positive for cathepsin B and 90% for cathepsin L (23). Therefore, our unique diagnostic strategy for the detection of high grade mPanINs can be translated directly into the clinic. Because cathepsin B and L have been shown to be independent prognostic factors of poor patient outcome in pancreatic cancer (23, 24), in vivo imaging of cathepsin activity may also provide additional information about the prognosis of the respective patient.

Using activatable NIRF probes for molecular in vivo imaging has several advantages: (i) in its native state the quenched probe is optically silent with no background fluorescence, minimizing false positive results; (ii) cleavage of multiple probes by each protease and accumulation of probes in tumors amplify the signal and increase sensitivity; (iii) protease recognition sites allow specific activation of probes; and (iv) probes can be designed to be activated by different proteases, permitting specific detection of tumors with different protease expression profiles (25). The advantages of the cathepsin-activatable NIRF probe were also observed in our study, when the molecular probe was compared with the nonspecific vascular contrast agent fluorescein, which has already been used...
for the detection of neoplastic precursor lesions and cancer in the gastrointestinal tract in humans (26–28). Morphological changes such as fibrosis, which are associated with pancreatic neoplasia, precluded the distinct detection and evaluation of mPanIN lesions and early-stage PDAC by CFL using fluorescein.

Given the poor survival rates of PDAC patients even after resection, detecting preinvasive pancreatic neoplasia and early-stage PDAC could tremendously improve survival. This is underscored by recent data showing that PanIN progression, from the initiating mutation to the emergence of the parental founder cell of the invasive carcinoma within the high-grade PanIN lesion, takes at least one decade and that another 5 to 6 y pass before cancer cells acquire metastatic capacity (13). These observations argue for a broad diagnostic window to detect curable neoplastic lesions in the pancreas and demonstrate the need for diagnostic approaches such as ours. Especially in the context of surveillance programs for familial pancreatic cancer, this window may be exploited to detect false positive findings minimized, using our unique diagnostic imaging approach (29).

Preinvasive PanIN lesions are the most common precursors to invasive PDAC, rendering them promising targets for diagnosis and intervention, especially in the high-risk population (30). Using CFL in combination with an established cathepsin-activatable NIRF probe allowed us to distinguish between different grades of mPanIN lesions by signal intensity, cellular morphology, and pattern of activation. Thus, it was possible to differentiate on the cellular level between low-grade mPanINs, which on the basis of current knowledge do not require treatment and high-grade mPanIN lesions and early-stage PDAC, requiring therapeutic intervention (11, 31). This differentiation is essential in the management of high-risk individuals who show suspicious changes within the pancreas in endoscopic ultrasound (EUS) or other imaging studies. Because many high-risk individuals show pancreatitis-like changes within the pancreas, differentiation between these irregularities and high-grade PanINs and PDAC is important (12, 32). In the future, these lesions can be evaluated by in vivo CFL using targeted probes to improve diagnostic accuracy. This improvement may significantly decrease overtreatment of persons at risk for PDAC.

The approach we have demonstrated in mice has the capacity to be translated to clinical medicine. First, the confocal laser-microscopic imaging system is US Food and Drug Administration (FDA) approved and Conformité Européenne (CE) marked. Second, the cathepsin-activatable NIRF probe shows no toxic side effects when applied i.v. and is currently being reviewed for FDA approval (18). Given the compatibility of the flexible confocal laser microscope with any conventional endoscope, our approach can be incorporated into a regular endoscopic examination. However, the translation from our model to clinical practice faces the problem of accessibility of the pancreas in humans, which needs to be resolved. However, this problem can be overcome by advancing the confocal laser microscope through a 19-G fine needle used for EUS guided puncture of the pancreas. This procedure allows the examiner to obtain an “in vivo histopathology” of nondisrupted tissue and subsequently obtain targeted biopsies. The feasibility of this EUS guided approach was recently shown by our group in a porcine large-animal model using fluorescein as a vascular contrast agent (33). Additionally, the small size of the confocal laser microscope allows the intubation of the main pancreatic duct during endoscopic retrograde pancreatoscopy as has already been shown in humans by our group (34). Furthermore, the diagnostic approach we describe here may also help to refine laparoscopic staging and surgical resection of PanINs and PDAC by improving intraoperative localization and evaluation of suspicious lesions and assessment of resection margins on a cellular level in real time during surgery (35). Finally, CFL in combination with the established activatable NIRF probe should be used to image other gastrointestinal neoplasias, many of which are more easily accessible than those of the pancreas (36–38).

Table 1. Assessment of neoplastic changes within the pancreas by CFL in a double blind study

<table>
<thead>
<tr>
<th>Histological grading</th>
<th>Sensitivity of CFL, %</th>
<th>Specificity of CFL, %</th>
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</thead>
<tbody>
<tr>
<td>Normal pancreas</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>PanIN-1</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>PanIN-2</td>
<td>75.0</td>
<td>100</td>
</tr>
<tr>
<td>PanIN-3</td>
<td>100</td>
<td>95.4</td>
</tr>
<tr>
<td>PDAC</td>
<td>100</td>
<td>100</td>
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</table>

Accuracy of detecting and grading neoplastic changes within the pancreas on the basis of in vivo CFL studies with the cathepsin-activatable NIRF probe was determined in a total of 24 mice in a double blind study (Materials and Methods for study design). Overall agreement of 95.8% was achieved by each of two independent blinded investigators when comparing CFL-based diagnosis of highest grade lesion observed (i.e., no lesion, mPanIN-1, -2, or -3, and PDAC) with diagnosis on the basis of serial histological sections. Interobserver agreement over all cases was 91.7%. Sensitivity and specificity of CFL-based histopathological diagnoses compared with gold standard H&E diagnoses are shown for each grade of PanIN, PDAC, and normal pancreatic tissue.
on which individuals at risk for PDAC can be monitored and treated successfully.

**Materials and Methods**

**Mouse Strains and Tumor Models.** The LSL-KrasG12D (39) and Ptf1aCre+/– (40) mice were previously described. The strains were interbred to obtain Ptf1aCre+/–;LSL-KrasG12D mice. Acute pancreatitis was induced as described (16, 41). All animal studies were conducted in compliance with European guidelines for the care and use of laboratory animals and were approved by the local authorities.

**Gene Expression Profiling.** mRNA expression profiles were generated using GeneChip Mouse Genome 430 2.0 arrays (Affymetrix) as recently described (16, 42) (SI Materials and Methods).

**Histology and Immunohistochemistry.** Formalin-fixed paraffin-embedded tissue sections were H&E stained or stained with anti-cathepsin B (1:50), anti-cathepsin H (1:40), anti-cathepsin L (1:40), or anti-cathepsin S (1:40) (R&D Systems) as described (33, 44) (SI Materials and Methods).

**Confocal Laser Scanning Microscopy.** Confocal microscopy was performed on cryosections (SI Materials and Methods), stained with an anti-E-cadherin antibody (diluted 1:80, AF-748; R&D Systems), followed by an Alexa Fluor Gene Expression Pro guidelines for the care and use of laboratory animals and were approved by

**Application of NIFIR Probes and Fluorescein.** Mice were injected i.v. with either 150 μL (2 nm) of a cathepsin BrHLS sensitive probe (ProSense 680) (ViSEn Medical) as described (16) or 100 μL 0.05% fluorescein.

**In Vivo CFL.** CFL was performed as described (16) using the Cellvizio488 and 660 Laser Scanning units (Mauna Kea Technologies) for fluorescein and cathepsin NIFIR-probe imaging, respectively (SI Materials and Methods).

**Ex Vivo Fluorescence Imaging.** Ex vivo imaging was performed as described (16, 44) on an Odyssey near-infrared reader (Li-Cor) at 680 nm for probe-specific fluorescence and at 800 nm for bright field imaging (SI Materials and Methods).

**Animal Study Design.** The potential of in vivo CFL imaging to detect and grade neoplastic changes within the pancreas was evaluated in a blind study. Mice of varying age, bearing neoplastic changes spanning the whole spectrum from mPanIN-1A to PDAC (n = 18) or normal littermate controls (n = 6), were imaged in vivo using the cathepsin-activatable NIFIR probe. Imaging-based diagnoses were compared with histological diagnoses (SI Materials and Methods).

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Supporting Information

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SI Materials and Methods

Gene Expression Profiling. Total RNA from homogenized tissue samples was prepared using the RNaseasy kit (Qiagen). Labeled cRNA was synthesized and hybridized onto GeneChip Mouse Genome 430 2.0 arrays (Affymetrix) as recently described (1, 2).

Human Pancreatic Tissue Samples. This study conformed to the Declaration of Helsinki and was approved by the ethics committee of the Technische Universität München. Informed consent was obtained from all patients included in the study.

Histology and Immunohistochemistry. Formalin-fixed paraffin-embedded murine and human tissue sections were dewaxed and H&E stained or microwaved (15 min, 600 W) to recover antigens before immunohistochemical staining. The following antibodies were used: anti-cathepsin B (1:50), anti-cathepsin H (1:40), anti-cathepsin L (1:40), and anti-cathepsin S (1:40) (R&D Systems), followed by secondary biotin-conjugated antibodies. Peroxidase-conjugated streptavidin was used with 3,3′-diaminobenzidine tetrahydrochloride (Sigma-Aldrich) as a chromogen for detection as described (3, 4).

Confocal Laserscanning Microscopy. Tissue samples were fixed in 4% PFA for 2 h at 4 °C and dehydrated in 15% sucrose for 4 h and 30% sucrose overnight. Subsequently, tissues were embedded in TissueTech (Sakura Finetek), frozen in a dry ice/ethanol bath, and stored at −80 °C. Cryosections of 30–50 μm were cut, rehydrated with PBS, blocked with 3% BSA, and stained with an anti–E-cadherin antibody (diluted 1:80, AF-748; R&D Systems), followed by an Alexa Fluor 555-labeled secondary antibody (Invitrogen). Stained sections were analyzed with a confocal laserscanning microscope (Leica; SP5).

In Vivo Confocal Fluorescence Lasermicroscopy (CFL). Mice were anesthetized as described (1, 4). A small incision was made in the ventral skin and the peritoneum, and the fiber optics miniprobe connected to a Cellvizio® or 660 Laser Scanning unit (Mauna Kea Technologies) was introduced. The fiber optics miniprobe was brought in contact with the pancreas to obtain single-cell resolution images for the grading of mPanIN lesions. Dynamic observations of the pancreas were made by visualizing the cathepsin-activatable NIRF-probe signal using an excitation wavelength of 660 nm or the fluorescein signal with an excitation wavelength of 488 nm. Real-time video sequences (frame rate, 12 images per second) were recorded and stored digitally. Images were recorded in grayscale and displayed in pseudocolors using Image Cell software (Mauna Kea Technologies).

Ex Vivo Fluorescence Imaging. Mice were killed immediately after in vivo flexible confocal lasermicroscopy and organs were explanted and placed on an Odyssey near-infrared reader (Li-Cor) and scanned with 680 nm, intensity 1, focus offset 3 mm, for probe-specific fluorescence (red color) and with 800 nm, intensity level 5, focus offset 3 mm, for bright field imaging (green color). Pictures were merged using SimplePCI software (Hamamatsu).

Animal Study Design. The potential of in vivo CFL imaging to detect and grade neoplastic changes within the pancreas was evaluated in a double blind study. Mice of varying age, bearing neoplastic changes spanning the whole spectrum from mPanIN-1A to PDAC (n = 18) or normal littermate controls (n = 6), were imaged in vivo using the cathepsin-activatable NIRF probe. At least six representative films were recorded per animal. After in vivo imaging, pancreata were processed for standard histology. Recordings were then blinded and randomized and viewed independently by two investigators with expertise in CFL imaging (D.S. and M.B.). The investigators graded all changes depicted in the films as being normal, mPanIN-1 through -3 or PDAC. This grading was then compared with histological grading of blinded serial H&E stained sections of the imaged pancreata performed according to the established nomenclature for the grading of mPanIN lesions in mice (5). Agreement between CFL recordings and histology in regard to the highest grade lesion seen was assessed for the diagnoses of each investigator and overall agreement for all 24 cases as well as interobserver agreement was calculated. Sensitivity and specificity of CFL for the detection and identification of each grade of mPanIN lesion as well as for normal pancreatic tissue and PDAC were determined for both investigators.

Table S1. Distribution of cathepsin-positive PanIN lesions and PDAC in human pancreatic tissue sections

<table>
<thead>
<tr>
<th>Histological grading</th>
<th>Cathepsin B positive lesions, %</th>
<th>Cathepsin H positive lesions, %</th>
<th>Cathepsin L positive lesions, %</th>
<th>Cathepsin S positive lesions, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>PanIN-1 ( (n = 53) )</td>
<td>44</td>
<td>47</td>
<td>34</td>
<td>32</td>
</tr>
<tr>
<td>PanIN-2 ( (n = 11) )</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>PanIN-3 ( (n = 9) )</td>
<td>78</td>
<td>100</td>
<td>100</td>
<td>89</td>
</tr>
<tr>
<td>PDAC ( (n = 5) )</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>80</td>
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</table>

Paraffin-embedded tissue sections from pancreata of five patients harboring different grade PanIN lesions and PDAC were evaluated for staining patterns of cathepsin B, H, L, and S by immunohistochemistry. Percentages of PDAC and PanIN lesions from the five patients that stained positive for the different cathepsins are shown. Of note, each individual high-grade PanIN lesion (PanIN-2 and -3) stained positive for at least two of the four cathepsins.

Movie S1. In vivo CFL imaging of the pancreas using the nonspecific vascular contrast agent fluorescein (green color) in Movies S1, S2, S3, and S4. Imaging of normal pancreatic tissue on a microscopic level. Blood flow within the undisturbed pancreatic tissue can be visualized. (Scale bar, 50 \( \mu \)m.)

Fig. S1. High expression of cathepsins in human PanINs and PDAC. Paraffin-embedded human pancreatic tissue sections from five patients harboring all grades of PanIN lesions (PanIN-1–3) and PDAC were stained for cathepsin B (ctsb), cathepsin H (ctsh), cathepsin L (ctsl), and cathepsin S (ctss). Representative pictures of normal pancreas, PanIN-1–3 lesions and PDAC are shown (magnification, 200×).
Movie S2. CFL imaging of pancreatic tissue harboring low-grade mPanIN lesions and associated fibrosis using the nonspecific vascular contrast agent fluorescein. From 26′ to 28′30″, normal pancreatic acini surrounded by strands of fibrosis with an intense fluorescence signal are shown. From 29′30″ to 32′00″, mPanIN lesions of indeterminate grade in the Upper part of the picture with a string of fibrosis (Lower) are shown.

Movie S3. CFL imaging of low- and high-grade mPanIN lesions with extensive fibrosis using the nonspecific vascular contrast agent fluorescein. No clear distinction between low- and high-grade mPanIN lesions is possible (15′ to 23′). From 22′50″ to 23′96″, normal pancreatic tissue is shown in the Left part of the picture.

Movie S4. PDAC with intense fibrosis is shown that cannot be distinguished from low- or high-grade mPanIN lesions using CFL imaging with fluorescein. (Scale bar, 50 µm.)
Movie S5. **In vivo CFL imaging of the pancreas using the specific cathepsin-activatable NIRF probe (red color) in Movies S5, S6, S7, and S8.** Representative pattern of normal pancreatic tissue as seen by CFL imaging. Only individual ductal cells show weak fluorescent signals (32′′′). (Scale bar, 20 μm.)

Movie S6. **CFL imaging of pancreatic tissue bearing only low-grade mPanIN lesions surrounded by normal pancreatic tissue using the specific cathepsin-activatable NIRF probe.** From 0′ to 7′, normal pancreatic tissue is shown. A coherent fluorescent signal of intermediate intensity in ductal structures characteristic of mPanIN-1 lesions is seen from 8′35′′ to 12′10′′.

Movie S7. **A high-grade mPanIN-3 lesion shown from the beginning of the video at 2′62′′ to 5′60′′ can be clearly distinguished from surrounding fibrosis with no fluorescent signal (8′01′′) and lower-grade mPanIN lesions (e.g., 6′64′′) by signal intensity and morphology using the specific cathepsin-activatable NIRF probe.** At 9′11′′, individual infiltrating immune cells with segmented nuclei can be seen.
Movie S8. CFL imaging of PDAC. The strong signal intensity of the specific cathepsin-activatable NIRF probe, the disturbed and heterogeneous tissue architecture and the typical cellular morphology allow the diagnosis of PDAC. At 9:37’, typical ductal structures are visible. PDAC can be clearly differentiated from low-grade mPanIN lesions and normal pancreatic tissue shown in Movies S5 and S6. (Scale bar, 20 μm.)