

## ORIGINAL ARTICLE

# Detection of pancreatic cancer tumours and precursor lesions by cathepsin E activity in mouse models

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## ABSTRACT

**Background and Aims** Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer death in the USA. Surgical resection is the only effective treatment; however, only 20% of patients are candidates for surgery. The ability to detect early PDAC would increase the availability of surgery and improve patient survival. This study assessed the feasibility of using the enzymatic activity of cathepsin E (Cath E), a protease highly and specifically expressed in PDAC, as a novel biomarker for the detection of pancreas-bearing pancreatic intraepithelial neoplasia (PanIN) lesions and PDAC.

**Methods** Pancreas from normal, chronic pancreatitis and PDAC patients was assessed for Cath E expression by quantitative real-time PCR and immunohistochemistry. Human PDAC xenografts and genetically engineered mouse models (GEMM) of PDAC were injected with a Cath E activity selective fluorescent probe and imaged using an optical imaging system.

**Results** The specificity of Cath E expression in PDAC patients and GEMM of pancreatic cancer was confirmed by quantitative real-time PCR and immunohistochemistry. The novel probe for Cath E activity specifically detected PDAC in both human xenografts and GEMM *in vivo*. The Cath E sensitive probe was also able to detect pancreas with PanIN lesions in GEMM before tumour formation.

**Conclusions** The elevated Cath E expression in PanIN and pancreatic tumours allowed *in-vivo* detection of human PDAC xenografts and imaging of pancreas with PanIN and PDAC tumours in GEMM. Our results support the usefulness of Cath E activity as a potential molecular target for PDAC and early detection imaging.

Despite great efforts to help patients with pancreatic ductal adenocarcinoma (PDAC) in the past few years, this disease remains devastating with the worst outcome of all major cancers. In the USA, PDAC ranks 10th in terms of incidence, but for both men and women, it is fourth in terms of cancer deaths.<sup>1</sup> The average survival for patients is less than 1 year from diagnosis<sup>1</sup> and to date surgery is the only curative treatment for this disease.<sup>2</sup> Unfortunately, only approximately 20% of patients are diagnosed early enough to benefit from surgery. For 80% of patients the disease is discovered when it is already unresectable due to the involvement of local blood vessels and nerves or metastasis to distant sites. These facts clearly emphasise the need for molecular biomarkers for PDAC that will enable earlier detection.

## Significance of this study

### What is already known about this subject?

- No highly specific and sensitive biomarkers are clinically available for the detection of PDAC at an early stage.
- Cath E is highly overexpressed in many cancers including PDAC.
- A Cath E selective peptide was recently identified that specifically detects its enzymatic activity.

### What are the new findings?

- We demonstrate that the elevated levels of Cath E expression in early pancreatic cancer lesions and pancreatic tumours could be exploited for PDAC detection.
- We illustrate that the detection and localisation of PDAC in mouse xenografts and GEMM was possible utilising the outstanding specificity of a novel Cath E-activatable imaging probe.

### How might it impact on clinical practice in the foreseeable future?

- The ability to detect and visualise pancreatic tumours and PanIN in PDAC by virtue of Cath E activity sensitive probes in preclinical mouse models suggests that modifications of this approach will be useful for the early detection and management of this deadly cancer in patients.
- The specificity of Cath E activity for PDAC suggests that this enzymatic activity will be useful in the future for the development of novel therapeutics or theranostics.

Although many molecular biomarker candidates of PDAC have been identified,<sup>3</sup> biomarkers with the necessary sensitivity and specificity for early detection are still lacking.<sup>4–6</sup> The most widely utilised blood-based biomarker is CA 19-9, which is not expressed in all patients, is not highly specific as it is elevated in other gastrointestinal cancers, and is not useful for the detection of early disease.<sup>7 8</sup> Furthermore, CA 19-9 levels do not provide information about the localisation of the disease nor the existence of metastases. The most sensitive diagnosis of PDAC currently requires invasive imaging procedures such as endoscopic ultrasonography, which

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can lead to pancreatic injury and the accuracy of which is highly operator dependent.<sup>9</sup>

In the current study, we investigated the utility of cathepsin E (Cath E) to act as an imageable biomarker for PDAC and tested the usefulness of a Cath E-activatable imaging probe<sup>10</sup> to selectively detect pancreas containing pancreatic intraepithelial neoplasia (PanIN) lesions and PDAC. Cath E is an intracellular aspartic protease that belongs to the pepsin family of proteases. In normal physiology Cath E is expressed primarily in immune cells, including antigen-presenting cells such as lymphocytes,<sup>11</sup> microglia,<sup>12</sup> dendritic cells<sup>13</sup> and human M cells.<sup>14</sup> Cath E has also been detected in gastric epithelial cells<sup>15</sup> and osteoclasts.<sup>16</sup> In the pancreas, Cath E is not expressed in normal healthy pancreas, but is present in PanIN lesions<sup>3 17</sup> and nearly all PDAC.<sup>3 18</sup> This specificity of Cath E for PDAC has been further demonstrated by evidence indicating that Cath E levels in pancreatic juice are diagnostic of the presence of PDAC.<sup>19</sup> Unfortunately, the collection of pancreatic juice is invasive and difficult rendering this approach to PDAC detection impractical. In contrast, the Cath E-activatable probe utilised in the current study can be modified for use in minimally invasive approaches to the detection and localisation of PDAC. Moreover, our data indicate that this probe can not only detect tumours and metastases but also identify pancreas containing precancerous lesions. The further development of this technology should provide clinically relevant progress for PDAC.

## MATERIALS AND METHODS

### Pancreatic tissues and cell lines

Paraffin-embedded tissue slides of human normal pancreas and pancreatic adenocarcinoma were obtained from the Department of Pathology, University of Texas, MD Anderson Cancer Center, Houston, Texas, USA. We used 37 human paraffin samples (10 of normal pancreas, 12 with PanIN1, 22 with PanIN2, 9 with PanIN3 and 13 with invasive carcinoma). A cell line derived from primary tumour grafts, MD Anderson pancreatic adenocarcinoma tumour cells 3 (MDA PATC-3) and an established pancreatic cancer cell line (Mpanc96)<sup>20</sup> (kindly provided by Dr Timothy J Eberlein, St Louis, Missouri, USA) were used and grown in RPMI-1640 supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 2 mM L-glutamine, 1X non-essential amino acids (Gibco, Carlsbad, California, USA), and 1X Pen/Strep (100 U/ml penicillin, 100 ug/ml streptomycin).

### Analysis of Cath E mRNA levels by real-time PCR

Total RNA from normal pancreas, chronic pancreatitis and pancreatic adenocarcinoma tissues were prepared using trizol reagent (Invitrogen, Carlsbad, California, USA) and further purified using the RNeasy kit (Qiagen Inc., Valencia, California, USA) with 15 min of DNase digestion. Reverse transcription was conducted using the AMV reverse transcriptase kit as per manufacturer's instructions (Promega, Madison, Wisconsin, USA). Briefly, 1 µg of total RNA was denatured for 5 min at 70°C, cooled for 5 min on ice, then reverse transcriptase (RT) was added to a total volume of 20 µl, and RT was conducted for 60 min at 42°C. RT-PCR was performed on a thermal cycler (Bio-Rad, Hercules, California, USA) for 40 cycles (denaturation, 20 s at 95°C; annealing and extension 1 min at 60°C) using specific primers for Cath E (NM\_001910, forward TCACCTT-CACCATTAACGGAGTC and reverse GCATTCCATCCAC-GAAGTCCA and a Taqman probe Hex-CCCTCAG CCCAACTGCCTACACCC-BHQ1. Ribosome protein S6

(NM\_001010 forward AAGGAGAGAAGGATATTCCTGGAC; reverse, AAGGGCTTTCTTACAACATACTGG) and a Taqman probe (FAM-TGATACTACAGTGCCTCGCCGCCT-BHQ-1) was used as internal controls for each sample.

### Immunohistochemical localisation of Cath E

Unstained 4 µm sections of human clinical specimens and mouse tissues were deparaffinised with xylene and rehydrated with ethanol. Antigen retrieval was performed with DAKO antigen retrieval solution (Dako, Carpinteria, California, USA) using a microwave at 98°C for 10 min. Endogenous peroxidase was blocked by hydrogen peroxide (3%) treatment for 10 min. For protein blocking 5% normal horse serum plus 1% normal goat serum in phosphate-buffered saline (PBS) was applied for 1 h. Primary antibodies were incubated overnight at 4°C in blocking solutions. The following antibodies were used: Cath E (sc-6508; Santa Cruz Biotechnology, Santa Cruz, California, USA) (1:50 dilution for human specimens and 1:200 dilution for mouse specimens) and a biotinylated rabbit anti-goat secondary (Vector Labs, Burlingame, California, USA) (1:100). Slides were then incubated with RTU horseradish peroxidase streptavidin solution (Vector Labs) for 30 min. Finally, slides were developed with 3,3-diaminobenzidine substrate (Vector Labs) and counterstained with haematoxylin, washed and dehydrated with ethanol, fixed with xylene and mounted.

### Cath E probe synthesis

Cath E peptide substrate, Ala-Gly-Phe-Ser-Leu-Pro-Ala-Gly-CysCONH<sub>2</sub> prepared by standard solid phase synthesis was labelled with Cy5.5 (GE, Piscataway, New Jersey, USA) and then used for probe synthesis as reported.<sup>10</sup> Peptide loadings on the polyethylene glycol protected grafted D-polylysine copolymer were calculated using the relative mole ratio of the imaging probes to the D-polyethylene glycol protected grafted copolymer. On average, each polymer carrier had approximately 23 of cy5.5 fluorochromes attached, and the probe showed approximately 95% of fluorescence quenching.

### Animal models

To assess whether Cath E activity will serve as a biomarker for in-vivo imaging, studies were performed in human pancreatic cancer xenografts in immunodeficient (acquired from the National Cancer Institute with ages ranging from 5 to 6 weeks) and transgenic mice. Animals were housed at the MD Anderson Cancer Center animal facility. All animal procedures were performed in accordance with the MD Anderson Cancer Center institutional guidelines using an approved animal protocol by the Institutional Animal Care and Use Committees of the University of Texas MD Anderson Cancer Center. Mice used for optical imaging studies were fed with a chlorophyll-free diet. Genetic PanIN and PDAC mouse models were developed by crossing cLGL-KRas<sup>G12V</sup> with Bac-Ela-CreER mice, as described previously.<sup>21</sup> These animals developed PanIN at 2 months and PDAC by approximately 6 months. In addition, LSL-KRas<sup>G12D</sup> mice<sup>22</sup> were crossed with floxed p53 mice<sup>23</sup> and pancreatic-specific cre (Pdx-1-Cre) mice<sup>24</sup> to yield mice that possessed conditional p53 deletion and endogenous levels of mutant KRas<sup>G12D</sup>.<sup>25</sup> PDAC in these mice developed in 6–8 weeks after birth.<sup>26</sup> Littermates without PDAC served as controls. LSL-KRas<sup>G12D</sup>, p53 floxed and Pdx-1-Cre genetic mice were obtained from the Mouse Models for Human Cancer Consortium Repository (Rockville, Maryland, USA).

For the immunohistochemical localisation of Cath E in mouse tissues, 46 samples from genetically engineered mouse

models (GEMM) were used. These included 31 samples of the cLGL-KRas<sup>G12V</sup> with Bac-Ela-CreER mice (27 contained PanIN1, 14 contained PanIN2, six contained PanIN3 and five contained invasive carcinoma) and five of the p53 conditional deletion/LSL-Kras<sup>G12D</sup>/Pdx1-Cre mice, which all contained invasive carcinoma. We also included 10 normal litter control mice, five per genetic strain.

Human cancer specimens used for transplantation as primary tumourgrafts were obtained from patients with pancreatic invasive ductal adenocarcinoma who had undergone initial surgical resection at UT MD Anderson Cancer Center and have been described previously.<sup>27</sup> Nude mice were anaesthetised with a single intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). After washing with sterile PBS, tumour fragments were placed in the subcutaneous space of immunodeficient mice.<sup>27</sup> When tumour was visible, it was dissected out and transplanted into male nude mice and allowed to grow for 30 days, then the Cath E-activatable fluorescent probe was injected and mice imaged 48 h after injection. At the end of the experiment tumour tissue was cut out, imaged, sectioned and stained with H&E stain.

To establish orthotopic tumours, mice were anaesthetised as described above, and then the pancreas was exposed through a left abdominal incision (laparotomy). Subconfluent MDA PATC-3 cells were detached with 0.25% trypsin-EDTA and cell viability was assessed by trypan blue exclusion. Cells ( $1 \times 10^6$ ) were resuspended in 50  $\mu$ l of Hanks balanced salt solution and directly injected into the pancreas (caudal). After tumour implantation, the pancreas was carefully returned to the peritoneal cavity and the abdomen was closed. Two months after tumour implantation, fluorescent probe was injected via the tail vein and imaged 48 h after injection.

### In-vivo and ex-vivo imaging

Subcutaneous models (human material PDAC tumour grafts with mice bearing two tumours each  $n=4$ , MDA PATC-3 orthotopic model  $n=3$ , MPanc96 expressing low levels of Cath E (MPanc96-FG30)  $n=10$ , and MPanc96 expressing high levels of Cath E (Mpanc96-Cath E)  $n=10$ ) and two transgenic models with litter controls ( $n=5$  each) were used to assess the usefulness of the Cath E-activatable imaging probe. Animals were injected with a single dose of the optical imaging probe (1 nmol/100  $\mu$ l PBS) or saline (controls) intravenously through the tail vein using a 30 gauge syringe needle. The anaesthetised mice were placed in the heated imaging platform of IVIS-100/Spectrum optical imaging systems (Xenogen/Caliper, Mountain View, California, USA). White light and near infrared fluorescence images were acquired sequentially using Cy5.5 fluorescence filters (615–665 nm excitation filter and 695–770 nm emission filter). Mice were imaged 48 h post-probe intravenous injection. Mice were killed and organs/tissues were excised and rinsed with PBS then imaged for their associated near infrared fluorescence. Fluorescence variations between different organs/tissues were corrected by subtracting the autofluorescence signals obtained from imaging organs/tissues of mice without any probe injected. General illumination setting and image acquisition parameters were: epi-illumination; 0.5 s exposure time; f/stop 2; binning (HR) 4; field of view (12.9 cm or 6.5 cm width and height). The mean fluorescence flux from each image was defined as photons per second per centimetre squared per steradian (p/s/cm<sup>2</sup>/sr). Acquired images were analysed using Living Image 3.1 software (Xenogen/Caliper, Alameda, California, USA). Fluorescence contrast, defined as radiance, was quantified using identical size regions of interest.

### Statistical analysis

Statistically significant differences were determined by two-tailed unpaired Student's *t* test ( $p < 0.05$  was taken as significant) with Graph Pad Prism five software (GraphPad Software, La Jolla, California, USA).

## RESULTS

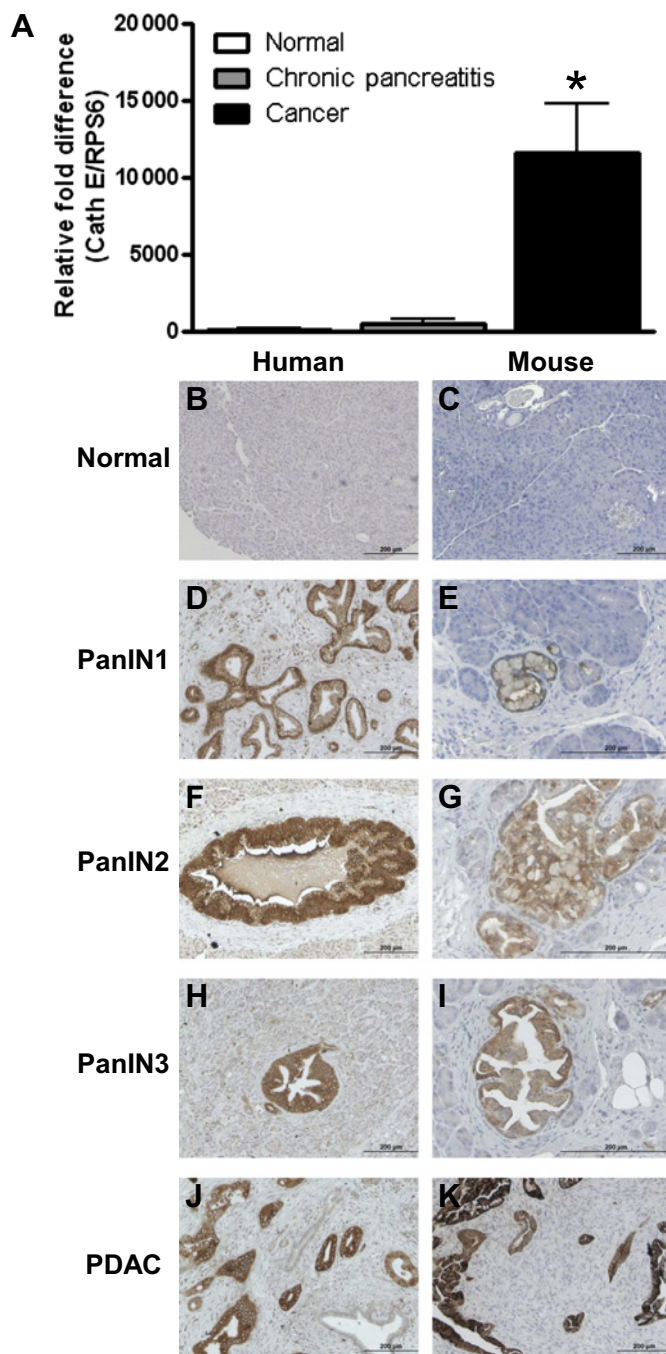
### Cath E is highly upregulated in human and mouse PDAC and PanIN

Cath E has been previously reported to be expressed in PDAC<sup>3 18 19</sup> and PanIN lesions.<sup>3 17</sup> Nonetheless, we confirmed the specificity of Cath E expression in human and animal GEMM PDAC samples including the different stages of PanIN lesions. For this purpose, levels of Cath E messenger RNA were compared in human samples of normal pancreas, chronic pancreatitis (a benign inflammatory disease of the pancreas) and PDAC using quantitative real-time PCR ( $n=5$  each; figure 1A). Cath E expression was confirmed to be upregulated in PDAC approximately 117-fold when compared with normal pancreas ( $p=0.0072$ ) and an approximately 22-fold increase when compared with chronic pancreatitis ( $p=0.0089$ ), therefore confirming its previously shown specificity for PDAC. To verify the cell type expressing Cath E, we next examined human and mouse tissues by immunohistochemistry. We observed that Cath E was absent in normal pancreas of both human and GEMM (figure 1B,C). In contrast, Cath E expression was found to be highly upregulated in PanIN lesions (figure 1D,I and table 1) and PDAC (figure 1J,K and table 1) in both humans and GEMM.

### PDAC in xenografts and genetic mouse models can be selectively detected by Cath E activity

We then explored the potential usefulness of detecting PDAC by measuring Cath E activity using a Cath E-activatable imaging probe.<sup>10</sup> This Cath E-activatable imaging probe was able to detect Cath E activity in lysates from PDAC cells engineered to express Cath E enzyme at different levels (supplementary figure S1A, available online only) and the signal correlated with the Cath E expression in vitro (supplementary figure S1B–D, available online only) and in vivo (supplementary figure S2, available online only). Therefore, we tested the Cath E-activatable imaging probe in various clinically relevant animal tumour models. Initially, tumours were formed in immunodeficient mice by subcutaneous transplantation of PDAC tumour material from a patient (tumour graft). The animals were then injected intravenously with the Cath E-activatable imaging probe. Animals injected with the probe, but not uninjected animals, developed a strong specific fluorescence signal localised to the implanted tumour grafts after 48 h ( $n=4$ ; figure 2A). Next, for a more clinically relevant model, primary pancreatic cancer cells isolated from patient tumour grafts were injected orthotopically into the pancreas of immunodeficient mice. The Cath E-activatable imaging probe was then administered to these animals with orthotopic human primary cells from tumour grafts. Because of the limited penetration of the fluorescence signals through the deep internal organs and biological components of the animal tissue, organs were excised and examined ectopically ( $n=3$ ; figure 2B). We observed a strong signal specifically localised to the tumours in the pancreas. These human primary tumour cell models develop a prominent stroma similar to that in the tumour grafts (figure 2C,D) or observed in human PDAC. High Cath E expression was seen in the tumours localised specifically to the cancer cells





**Figure 1** Cathepsin E (Cath E) is overexpressed in pancreatic human and mouse pancreatic intraepithelial neoplasia (PanIN) and pancreatic ductal adenocarcinoma (PDAC). (A) Taq-Man real-time PCR relative expression levels of Cath E mRNA in normal human pancreas tissue, chronic pancreatitis and pancreatic cancer samples (n=5) that were normalised to RPS6 mRNA expression (\*p=0.0072 when compared with normal pancreas). Error bars represent SD. (B, D, F, H, J) Representative images of immunohistochemical localisation of Cath E in human pancreas tissue sections of (B) normal pancreas, (D) PanIN1, (F) PanIN 2, (H) PanIN3 and (J) PDAC, showing strong cytoplasmic Cath E expression on PanIN and cancer cells but not on normal pancreas. (C, E, G, I, K) Representative images of Cath E immunohistochemical localisation of mouse pancreas tissue sections from (C) wild-type mouse, (E) genetically engineered mouse models (GEMM) with PanIN 1, (G) GEMM with PanIN 2, (I) GEMM with PanIN 3 and (K) GEMM PDAC. Scale bar as shown in  $\mu\text{m}$ .

**Table 1** Percentage of human and mouse pancreas tissues containing different grade PanIN and tumours that were positive for Cath E expression

	Normal	PanIN1	PanIN2	PanIN3	PDAC
Human	0/10 0%	12/19 63%	22/22 100%	9/10 90%	13/14 93%
Mouse	0/10 0%	27/27 100%	14/14 100%	6/6 100%	10/10 100%

Cath E, cathepsin E; PanIN, pancreatic intraepithelial neoplasia; PDAC, pancreatic ductal adenocarcinoma.

(figure 2F) and no Cath E expression was seen in the normal pancreas (figure 2E).

To verify our results in an autochthonous model of PDAC we also imaged tumours developed in well established GEMM. We found that the Cath E activity sensitive probe readily enabled the imaging of PDAC tumours from both GEMM models used (n=5 each) (figure 3A,B, and supplementary figure S3 (available online only); signal increased approximately 15-fold, p=0.0002) and also a tumour metastasis in the lung (figure 3C) in these models. The characteristics of the GEMM PDAC tumours were confirmed by histology and Cath E expression was assessed (figure 3D–G), which confirmed that the cancer cells are the source of the Cath E expression in the tissues imaged. Outside the pancreas signals were limited except for the liver (supplementary figure S3, available online only), probably due to high non-specific enzymatic activity. To examine the relative signals directly, different tissues were subjected to side-by-side imaging comparisons and quantitative fluorescence signal analysis (supplementary figure S3D, available online only). The tumour to non-tumour signal ratios were as follows: tumour/normal pancreas 2.2; tumour/kidney 1.9; tumour/muscle 5.5; tumour/bone 2.6; tumour/small intestine 1.7; tumour/lung 2.9; tumour/spleen 3.9, which clearly suggest the tumour specificity of the probe.

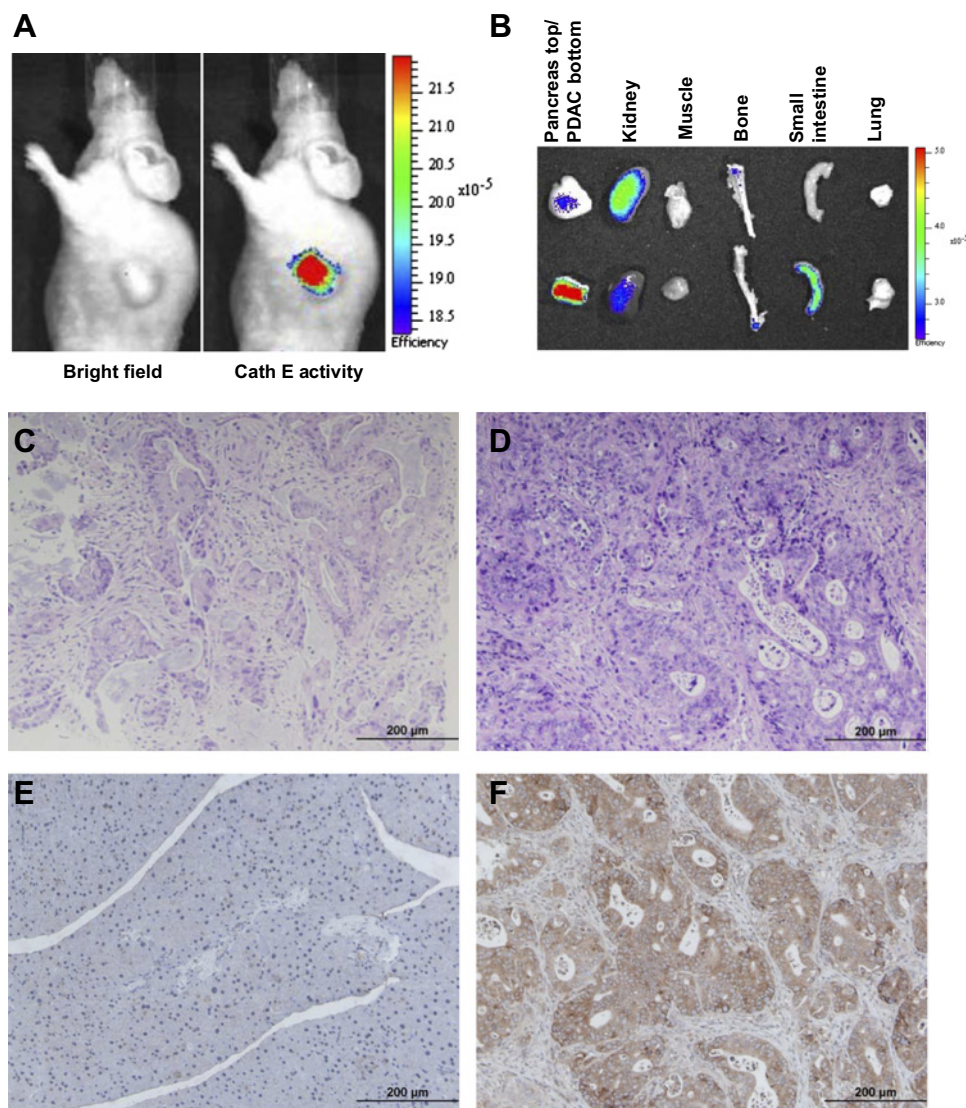
### Pancreas with PanIN lesions can be detected using Cath E activity

Histological immunohistochemical analysis showed high Cath E expression in human and mouse PanIN lesions (figure 1D–I). Therefore, we wished to establish whether the Cath E-activatable imaging probe could detect pancreas with precancerous lesions. For this purpose, we utilised a transgenic pancreatic cancer mouse model that form abundant PanIN at early times and then progress to invasive and metastatic disease over several months.<sup>21–24</sup> We injected mice with the Cath E-activatable imaging probe at times during which only PanIN (2 months), but no tumours, were present (cLGL-KRas<sup>G12V</sup> with Bac-Ela-CreER mice). We observed that the Cath E probe generated a strong specific signal (approximately threefold, p=0.0058) in the pancreas of these mice bearing PanIN lesions when compared with normal pancreas (n=5; figure 4A,E) and this signal was significantly lower than that of PDAC tumours (figure 4A,E). The presence of PanIN lesions and lack of tumours was verified by histological analysis (figure 4C), and Cath E expression of the pancreas containing PanIN was confirmed to be specifically in the PanIN lesions (figure 4D). Pancreas containing PanIN and tumours from both GEMM gave similar results and the data were pooled for both models in the quantitative analysis of fluorescence (figure 4E).

### DISCUSSION

The expression of Cath E in PDAC and PanIN observed in this and earlier studies<sup>3</sup> suggested the potential of Cath E to serve as

**Figure 2** Cathepsin E (Cath E) activity can be detected efficiently in human pancreatic cancer xenografts in mouse. (A) Representative in-vivo image of human pancreatic cancer primary patient tumour grafts in mice showing brightfield (left) and fluorescence (Cath E activity) (right) signal at tumour location (n=4). (B) Representative ex-vivo image of mouse orthotopic tumour formed from cells (MDA PATC-3) isolated from human pancreatic cancer tumour grafts (A) showing fluorescence signal from pancreas with tumour (bottom) compared with a pancreas without a tumour (top) as well as kidney, muscle, bone, small intestine and lung (n=3). (C) Representative H&E staining of primary patient tumour grafts from (A) showing the presence of human tumour cells surrounded by the stromal components that characterise human pancreatic tumour. (D) Representative H&E staining of orthotopic pancreas tumour from (B) which shows the similar characteristics to human pancreatic ductal adenocarcinoma. Representative images of immunohistochemical localisation of Cath E of normal pancreas (E) from pancreas imaged in (B) and tumour (F) from tumour imaged in (B). Scale bar as shown in  $\mu\text{m}$ .



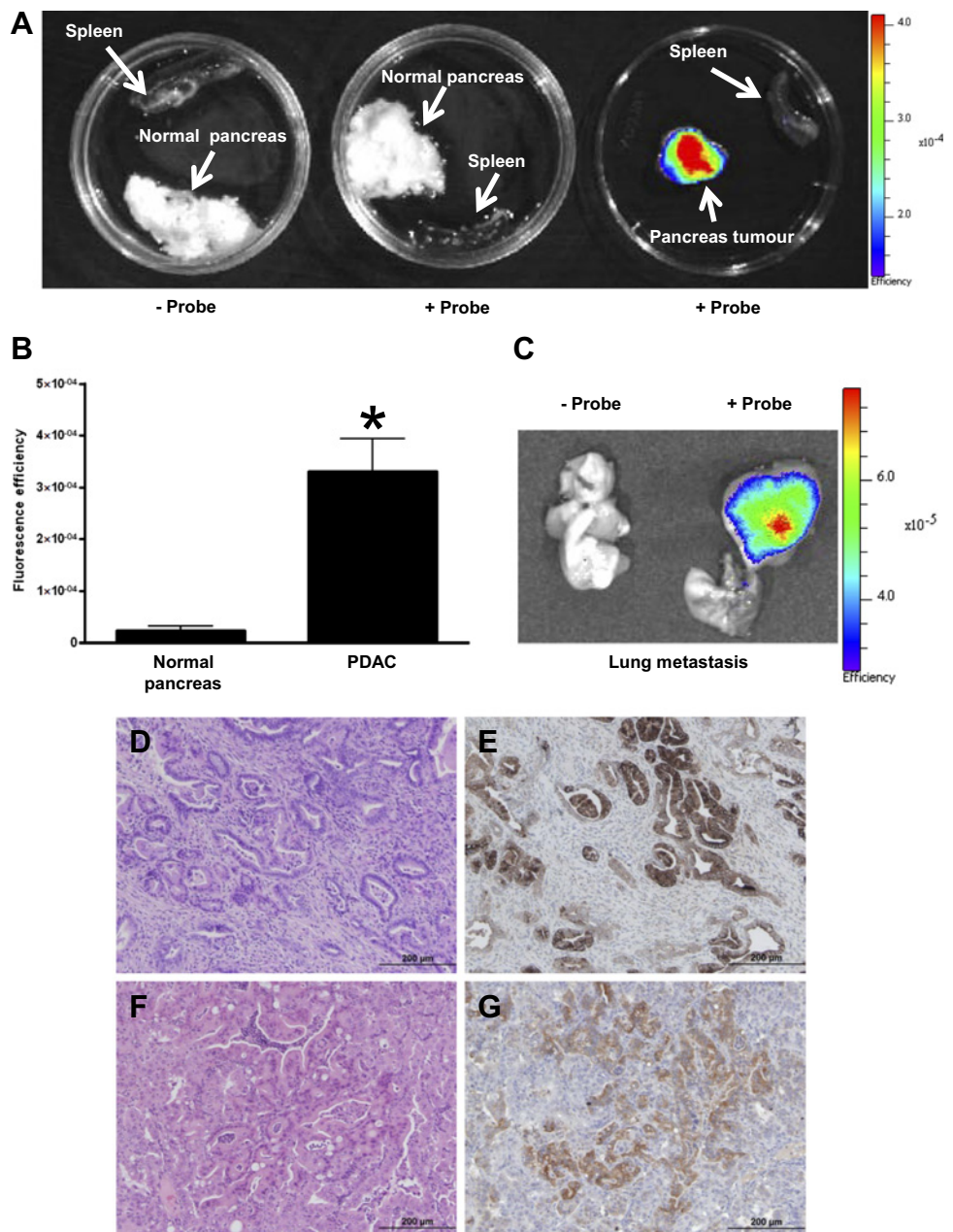
an early biomarker of pancreatic cancer. Therefore, in order to utilise Cath E as a biomarker for detecting and localising PDAC a Cath E-activatable imaging probe was developed<sup>10</sup> using a previously established platform technology for molecular imaging of protease activity.<sup>28</sup> The Cath E-activatable imaging probe consisted of an inert polymeric template and multiple copies of a fluorescently labelled Cath E selective peptide sequence.<sup>29</sup> This selective peptide sequence was recently proved to be specific for the detection of Cath E when compared with other reported substrates using various proteases.<sup>29</sup> Due to the close proximity of the attached fluorochromes in the imaging probe, the probe remains optically quenched, until the Cath E enzyme acts on the peptide linkers and releases the fluorescent reporters. Our study describes the effectiveness of using this specific Cath E-activatable imaging probe in detecting Cath E activity for localising PDAC (figure 2A,B, figure 3A,B, figure 4A and supplementary figures S2 and S3, available online only), metastases (figure 3C) and pancreas with early preneoplastic lesions (figure 4). This is the first direct demonstration of a biomarker imaging agent specific for Cath E that can localise and detect PDAC tumours and pancreas containing PanIN lesions. These data support the further development of clinically relevant imaging and therapeutic agents based on the enzymatic activity of Cath E in PDAC.

Previously, there was a report using a broad cathepsin-activatable fluorescent imaging probe for pancreatic cancer detection in vivo,<sup>30</sup> which was also recently used to detect murine PanIN lesions.<sup>31</sup> Both studies used a commercially available fluorescence probe (Prosense 680) that is sensitive to cathepsins B/H/L/S and plasmin. In the study murine PanIN lesions were detected using a flexible confocal fluorescence laser microscope to analyse probe distribution in a GEMM of pancreatic cancer.<sup>31</sup> The results appeared to be quite promising and support the further development of the fluorescence laser microscope. However, the expression levels of cathepsins B/H/L/S in human pancreatic tumours are only elevated approximately two to threefold, compared with a 28-fold increase in the expression of cathepsin E.<sup>5</sup> The study also attempted to examine the specificity of the commercial broad cathepsin-activatable fluorescent imaging probe using caerulein-induced pancreatitis as a control. Caerulein treatments are known to be a reasonable model for acute pancreatitis, but they do not recapitulate human chronic pancreatitis and to date there are no good GEMM that can recapitulate human chronic pancreatitis. In addition, this broad cathepsin probe has been studied previously in different tumour models and was reported to be activated mostly by tumour-associated macrophages, not tumour cells.<sup>32</sup> Live imaging of cysteine-cathepsin activity reveals dynamics of focal inflammation, angiogenesis and polyp growth.<sup>33</sup> In addition,



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**Figure 3** Cathepsin E (Cath E) activity can be detected efficiently in genetically engineered mouse model (GEMM) tissues. (A) Representative ex-vivo image of mouse normal pancreas with spleen (left) in which no Cath E-activatable imaging probe was injected, mouse normal pancreas with spleen (middle) after 48 h of Cath E-activatable imaging probe injection, and GEMM (cLGL-KRas<sup>G12V</sup> with Bac-Ela-CreER mice) human pancreatic ductal adenocarcinoma (PDAC) tumour with spleen (right) after 48 h of Cath E-activatable imaging probe injection showing fluorescence signal specific to Cath E activity from the tumour and (B) fluorescence quantification of ex-vivo normal pancreas and GEMM (pooled data from both GEMM used) PDAC tumours showing approximately 15-fold increase in fluorescence in the pancreas tumours when compared with normal pancreas (n=5) (\*p=0.0002). (C) Representative ex-vivo image of lung metastasis from mice with PDAC (p53 conditional deletion/LSL-Kras<sup>G12D</sup>/Pdx1-Cre mice) without probe injection (left) and mice injected with Cath E-activatable imaging probe (right) showing fluorescence signal in lung metastasis when Cath E-activatable imaging probe is added (n=2). (D) Representative H&E staining and (E) representative image of immunohistochemical localisation of Cath E from tumour imaged in (A). (F) Representative H&E staining and (G) representative image of immunohistochemical localisation of Cath E from lung metastasis imaged in (C). Scale bar as shown in  $\mu\text{m}$ .



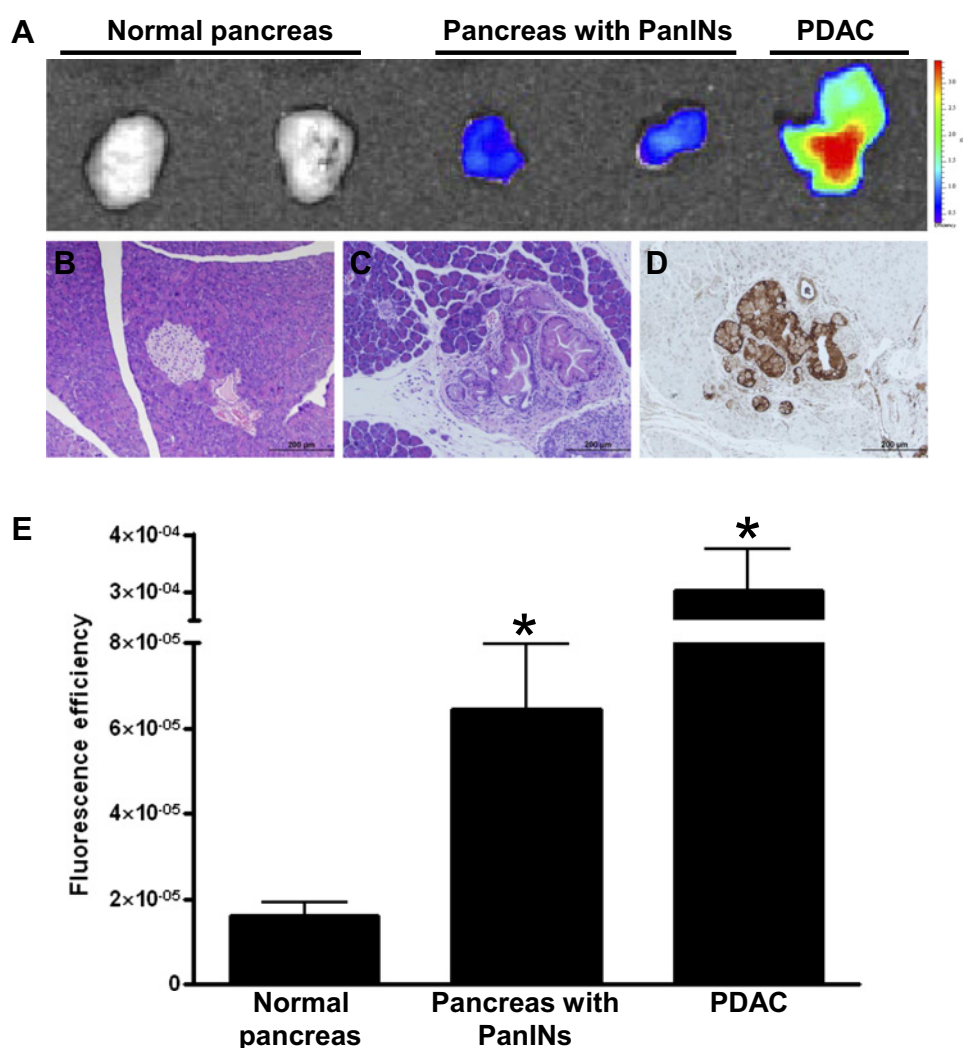
cellular activation of the self-quenched fluorescent reporter probe in a tumour microenvironment<sup>33</sup> suggests that this probe might not be ideal to distinguish chronic pancreatitis from PDAC. In contrast, we show that Cath E expression levels in tumours are greatly elevated compared with human chronic pancreatitis (figure 1A) as previously reported.<sup>3</sup> Recent comparisons of gene expression in human and mouse PDAC also find that cathepsins B/H/L/S are more highly expressed in GEMM than in the human disease (unpublished observation). From these considerations it seems likely that the cathepsin E probe will have advantages to identify human PDAC and pancreas containing PanIN lesions compared with the less specific broad cathepsin probe previously described.

Non-invasive molecular imaging with a highly specific probe based on Cath E activity, as described in this study, would be simpler, more specific and less risky than endoscopic ultrasound. It will also have the additional advantage of the localisation of tumours, guiding surgical procedures, and the monitoring of disease recurrence and treatment effectiveness over time. The

sensitivity of Cath E imaging used in the clinic will depend on the specific imaging technology utilised. The current study utilised fluorescence imaging as a proof of principle technique to examine the specificity and ability of the Cath E-activatable probe to localise pancreatic tumours and pancreas with PanIN lesions. However, fluorescence may not be the most suitable modality or the best for use in patients at this time. Fortunately, the technology behind the fluorescence-based probe for Cath E described here can be adapted to many other imaging modalities including positron emission tomography and MRI.<sup>34</sup> The catalytic activity of Cath E may also be adapted to develop control released therapeutics.

A few other molecular imaging probes with specificity for pancreatic cancer have recently been reported. For example, plectin-1 targeting peptides were demonstrated to localise to primary orthotopic PDAC tumours as well as metastatic foci in mice and could be imaged using micro single-photon emission CT/CT.<sup>35 36</sup> Plectin is ubiquitously expressed, but in PDAC its

**Figure 4** Cathepsin E (Cath E) activity can be detected efficiently in mouse pancreas with pancreatic intraepithelial neoplasia (PanIN) lesions. (A) Representative ex-vivo Cath E-activatable imaging probe image of pancreas from genetically engineered mouse models (GEMM) with normal pancreas and pancreas with PanIN lesions (cLGL-KRas<sup>G12V</sup> with Bac-Ela-CreER mice) and pancreatic ductal adenocarcinoma (p53 conditional deletion/LSL-Kras<sup>G12D</sup>/Pdx1-Cre mice) injected with Cath E-activatable imaging probe. (B, C) Representative H&E staining of transgenic animals with normal pancreas and pancreas with PanIN but no tumours from (A). (D) Representative image of immunohistochemical localisation of Cath E from pancreas with PanIN but no tumours from (A). (E) Fluorescence quantification of ex-vivo pancreas showing approximately threefold increase in fluorescence in the pancreas with PanIN lesions when compared with normal pancreas (n=4) (\*p=0.0058), and a significant increase in tumour fluorescence signal compared with the fluorescence signal from pancreas containing PanIN lesions but no tumour. Scale bar as shown in  $\mu\text{m}$ .



cellular localisation is altered such that it is found on the cell surface where it is available to an affinity agent. Therefore, plectin has great promise as an imaging target for PDAC. Furthermore, micro single-photon emission CT/CT is already used in the clinic, so that this plectin probe may be quickly adapted for human use. In another approach, it was shown that it is possible to image increased levels of pancreatitis-associated protein (PAP), which is expressed in pancreatic acinar cells surrounding foci of pancreatic cancer or pancreatic inflammation, using (<sup>18</sup>F)-fluoroethyl-deoxylactose (FDG) positron emission tomography.<sup>37</sup> The localisation of PAP has the advantage that the area occupied by cells expressing this molecule is much greater than the volume of the cancer cells themselves. However, PAP is also induced in chronic pancreatitis and other inflammatory diseases so it is not specific for cancer. It was also recently reported that pancreases with PanIN can be imaged using standard FDG uptake in a mouse model.<sup>38</sup> Several difficulties with applying FDG in clinical pancreatic cancer imaging have been identified<sup>39</sup> and will probably limit the usefulness of this approach. In addition, while it was reported that urokinase plasminogen activator receptor could be used for targeting PDAC, amino-terminal fragment composed of 135 amino acid residue was needed to achieve efficient binding.<sup>40</sup> The current lack of short endogenous peptide might hinder the utilisation of urokinase plasminogen activator receptor as a promising target.

It is unclear how all these new imaging approaches would be applicable in a patient whose pancreas is much larger than those of a mouse model but which would be likely to possess focal PanIN lesions. It is also uncertain whether fluorescent probes detected by new endoscopic techniques, or other imaging modalities, will be most useful in the clinic. Whether the probes are sufficiently sensitive, whether non-specific signals originating in the liver will be an obstacle, whether the probes can accurately differentiate different grades of PanIN and many other practical questions cannot be answered before human studies are conducted. Nonetheless, these new probes provide promise and improvements in pancreatic cancer diagnosis, which is currently a great challenge.

In summary, this work provides fundamental preclinical evidence using various mouse models that a new multiplex molecular enzymatic imaging probe based on Cath E activity will be useful for the clinical detection and management of pancreatic cancer. The data indicate the specificity of Cath E expression in PDAC. The full utility of this approach will not be known until clinical trials are performed. However, our demonstration of the ability of this approach to visualise tumours and pancreas with PanIN in PDAC GEMM that mimic the human disease suggests that we may finally be near to a useful means for early detection of this deadly cancer. The use of this probe for surveillance of PDAC will help improve survival as it will be able to detect most tumours at a resectable stage.

## Pancreatic cancer

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## Detection of pancreatic cancer tumours and precursor lesions by cathepsin E activity in mouse models

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