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Biology of Human Tumors

Clinical Cancer Research

# Cyst Fluid Telomerase Activity Predicts the Histologic Grade of Cystic Neoplasms of the Pancreas

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## Abstract

**Purpose:** Pancreatic cysts frequently pose clinical dilemmas. On one hand, cysts with high-grade dysplasia offer opportunities for cure, on the other hand, those with low-grade dysplasia are easily over treated. Cyst fluid markers have the potential to improve the evaluation of these cysts. Because telomerase activity is commonly activated in malignant cells, we evaluated the diagnostic performance of cyst fluid telomerase activity measurements for predicting histologic grade.

**Experimental Design:** Telomerase activity was measured using telomerase repeat amplification with digital-droplet PCR in surgically aspirated cyst fluid samples from 219 patients who underwent pancreatic resection for a cystic lesion (184 discovery, 35 validation) and 36 patients who underwent endoscopic ultrasound fine-needle aspiration. Methodologic and clinical factors associated with telomerase activity were examined.

**Results:** Telomerase activity was reduced in samples that had undergone prior thawing. Among 119 samples not previously thawed, surgical cyst fluids from cystic neoplasms with high-grade dysplasia ± associated invasive cancer had higher telomerase activity [median (interquartile range), 1,158 (295.9–13,033)] copies/μL of cyst fluid than those without [19.74 (2.58–233.6) copies/μL; $P < 0.001$)]. Elevated cyst fluid telomerase activity had a diagnostic accuracy for invasive cancer/high-grade dysplasia of 88.1% (discovery), 88.6% (validation), and 88.2% (merged). Among cysts classified preoperatively as having "worrisome features," cyst fluid telomerase activity had high diagnostic performance (sensitivity 73.7%, specificity 90.6%, accuracy, 86.1%). In multivariate analysis, telomerase activity independently predicted the presence of invasive cancer/high-grade dysplasia.

**Conclusions:** Cyst fluid telomerase activity can be a useful predictor of the neoplastic grade of pancreatic cysts. *Clin Cancer Res; 22(20); 5141–51. ©2016 AACR.*

*See related commentary by Allen et al., p. 4966*

## Introduction

The evaluation and management of patients with pancreatic cysts can be a significant clinical challenge (1). A number of different pathologies with a wide spectrum of malignant potential can produce a cyst in the pancreas (1). Most neoplastic pancreatic cysts are intraductal papillary mucinous neoplasms (IPMN). Although the vast majority of IPMNs do not progress, some IPMNs do progress to invasive cancer (2). Patients with good performance status suspected to have an IPMN with low-grade dysplasia (LGD) can be managed with surveillance, whereas cysts with high-grade dysplasia (HGD) or an associated low-stage invasive cancer should be surgically resected (3, 4). Serous cystic neoplasms (SCN) are virtually always benign, and some SCNs can be difficult to distinguish from IPMNs and other cysts with malignant potential (5). Mucinous cystic neoplasms (MCN) have significant malignant potential and are usually resected without any surveillance (3).

Guidelines for the management of a patient with a pancreatic cyst currently are based on the patient's clinical symptoms and imaging findings. The most recent international consensus guidelines (ICG 2012) used clinical and imaging findings based on CT or MRI to classify pancreatic cysts into those with "high-risk stigmata," "worrisome features," or "low-risk" (3). These guidelines recommend surgical intervention for cases with "high-risk stigmata," whereas those with "worrisome features" should be further evaluated by endoscopic ultrasonography (EUS; ref. 6). These guidelines are effective, but still some patients who undergo pancreatic resection for a cyst with "worrisome features" (such as a history of pancreatitis, a cyst size of ≥30 mm, a thickened cyst wall, or a nonenhancing mural nodule) will have a lesion with little or no malignant potential (such as IPMNs with low-grade dysplasia or an SCN thought preoperatively to be mucinous neoplasm; refs. 7, 8). Indeed, recent evidence indicates that most patients with worrisome features who do not undergo pancreatic

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**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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## Translational Relevance

The surveillance of patients with pancreatic cysts represents an important opportunity to prevent the progression of pre-invasive cysts to pancreatic cancer. Pancreatic cyst evaluation relies on pancreatic imaging but cannot adequately evaluate the neoplastic nature of pancreatic cysts. Pancreatic cyst fluid analysis has the potential to improve the evaluation of pancreatic cysts, but better markers are needed. In this study, we evaluated the ability of cyst fluid telomerase activity to predict neoplastic grade of pancreatic cysts. We find cyst fluid telomerase activity can distinguish pancreatic cysts with high-grade dysplasia and/or invasive cancer from those with lower grades of dysplasia with high accuracy. Cyst fluid telomerase activity measurements were similarly useful when preoperative evaluation was worrisome, but lacked definitive evidence to suggest high-grade dysplasia or invasive cancer (sensitivity 73.7%, specificity 90.6%, and accuracy, 86.1%).

resection will not progress to death over the next 5 years (9). At the same time, once an invasive cancer arises progression may be rapid (10), and recurrence after resection and diminished survival is common even for patients with small cancers (11).

Cytologic assessment of cystic tumors of the pancreas and carcinoembryonic antigen (CEA) measurements have clinical utility, but they are both imperfect (12, 13). The detection of somatic mutations and copy number alterations in cyst fluid samples using next-generation sequencing has shown promise as an approach to classify pancreatic cysts (14, 15). Other pancreatic cyst fluid markers have been evaluated for their clinical utility, but to date none have been shown to reliably predict neoplastic grade (16–21). The development of additional cyst fluid markers that can predict the grade of neoplasia in a pancreatic cyst would have clinical utility. One marker that has been shown to indicate evidence of cancer in a variety of diagnostic settings is telomerase (22).

Telomerase is not expressed in most normal somatic cells, but is usually expressed during neoplastic development and plays an important role in the maintenance of telomere length and in the immortalization of neoplastic cells (23, 24). Telomerase activity can be measured directly using the telomerase repeat amplification protocol (TRAP) assay (25–28). In one study, telomerase activity, measured using gel-TRAP in fine-needle biopsies from the walls of 19 pancreatic cystic neoplasms and 10 pancreatic pseudocysts, was reported to be useful in distinguishing malignant cysts from benign cysts and pseudocysts (29). An alternative to measuring telomerase activity is to measure the expression of one of its RNA components, TERT, whose expression closely correlates with telomerase activity and can be more readily assayed in clinical samples (30). TERT RNA levels in pancreatic juice samples collected from the pancreatic duct can accurately differentiate benign from malignant pancreatic lesions (31–33). Studies have also measured TERT RNA in resected pancreatic cystic neoplasm specimens as a marker of telomerase activity (34).

The original TRAP assay has been modified to incorporate real-time quantitative PCR (27, 35–38), and more recently, using digital-droplet PCR (ddPCR) to quantify telomerase activity more reliably and to avoid gel-based methods (39). ddPCR involves isolating individual DNA molecules from a sample into nanoliter-sized droplets by emulsification so that thousands of individual PCR reactions can occur (40–42). Simple, precise, and reproducible quantification of the absolute amount of target DNA can be determined by counting the number of positive droplets after PCR.

In the current study, we evaluated the diagnostic accuracy of pancreatic cyst fluid telomerase measurements determined by ddPCR (dd-TRAP) as an approach to distinguishing pancreatic cysts with high-grade dysplasia and/or invasive cancer from those with benign features or lower grades of dysplasia.

## Materials and Methods

### Patients and specimens

Two hundred thirty-three patients (194 in a discovery set, 39 validation set) who had undergone surgical resection for a cystic pancreatic lesion at Johns Hopkins Hospital between 2008 and March 2016 and had pancreatic cyst fluid collected from their surgical resection specimen in the pathology laboratory as well as 36 subjects who had their pancreatic cyst fluid collected by fine-needle aspiration during endoscopic ultrasound were studied. Ten cases in the discovery set and four cases in the validation set were excluded because their cyst fluid sample was inadequate for telomerase analysis (insufficient volume of sample available or degraded protein). Patient information, including demographics, clinical symptoms, preoperative imaging findings, cyst fluid cytology, and CEA values were obtained from hospital records. Pancreatic imaging findings were classified on the basis of pancreatic CT and/or MRI results into "high-risk stigmata," "worrisome features," and "low-risk" following the ICG 2012 algorithm (3). The diagnostic accuracy of cyst fluid CEA was evaluated using the cut-off of 192 ng/mL as described previously (43). The decision to operate was based on the clinical estimated risk of cancer, or for symptoms thought to be caused by the cyst. Cases that underwent surgical resection and were classified as low-risk had a variety of indications for resection, including suspected MCN, elevated cyst fluid CEA, significant increase in cyst diameter but not 3 cm, symptoms suspected to be due to the cyst and resection before the development of the 2012 consensus guidelines.

The pathologic features of each surgically resected neoplasm were reviewed by pancreatic pathologists (R.H. Hruban and M. Pittman). Surgical cyst fluid samples were aspirated from the resected cyst in the surgical pathology suite immediately after the surgical resection using a fine-needle sterile syringe and stored immediately at 4°C; aspirated endoscopic ultrasound fine-needle aspiration (EUS-FNA) samples placed immediately on ice. Samples were transferred on ice to the laboratory generally within 2 hours, aliquoted and stored at −80°C. All cyst fluid samples used in this study had been prospectively collected in our fluid and tissue bio-bank in a standardized fashion. All experiments and data analysis were conducted in a blinded fashion, without any prior knowledge of pathologic diagnosis. All elements of this study were approved by the institutional review board of Johns Hopkins Medical Institutions and written informed consent was obtained from all patients.

### Cell culture

Human pancreatic cancer cell lines MIA PaCa-2, BxPC-3, Hs 766T, PANC-1, AsPC-1, CFPAC-1, Capan-1, Capan-2, and

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SU.86.86 were obtained from the ATCC and A38-5 was obtained from the investigator who created the line (Dr. James Eshleman, Johns Hopkins University, Baltimore, MD). An HPV-E6/E7 immortalized human pancreatic duct epithelial cell line, HPDE, was kindly provided by Dr Ming-Sound Tsao (University of Toronto, Ontario, Canada). These cancer cell lines were tested for Mycoplasma and most recently authenticated using genetic markers by the Johns Hopkins Genetics Core facility. HPDE was authenticated by testing for genetic markers of HPV E6 and E7. The authentication was performed within a few weeks of completing this study's experiments. All cell lines, except for HPDE, were cultured in DMEM (Life Technologies, Inc.) supplemented with 10% FBS (Mediatech, Inc.) and 1% antibiotics (penicillin/streptomycin; Life Technologies) and incubated at 37°C in a humidified atmosphere of 5% $CO_2$ in air. HPDE cells were cultured in keratinocyte serum–free medium supplemented by bovine pituitary extract and EGF (Life Technologies).

### Protein extraction

Cyst fluid was inspected for turbidity to estimate the protein concentration before extraction and quantification. Cells were pelleted down by centrifugation at 5,000 rpm for 5 minutes. Supernatant was aspirated and discarded. Precipitated cells were lysed in NP-40 buffer (10 mmol/L Tris-HCl, pH 8.0, 1 mmol/L $MgCl_2$, 1 mmol/L EDTA, 1% (v/v) NP-40, 0.25 mmol/L sodium deoxycholate, 10% (v/v) glycerol, 150 mmol/L NaCl, 5 mmol/L β-mercaptoethanol, 0.1 mmol/L AEBSF (4-[2-aminoethyl] benzenesulfonyl fluoride hydrochloride) for a 30 minutes on ice (39) and the lysate was then centrifuged at 15,000 rpm for 20 minutes at 4°C and the supernatant collected as the cell extract. Protein concentration was measured by BCA method using Pierce BCA Protein Assay Kit—Reducing Agent Compatible (Thermo Fisher scientific) and stored at –80°C until ready for further analyses. To ensure that protein was sufficiently extracted from cell pellets, protein extraction procedures were repeated by adding equal volumes of NP-40 lysis buffer to any remaining pellet until there was no longer any lysed protein detectable. Total yield of lysed protein was then calculated per original cyst fluid volume. For cyst fluid samples, 1 to 5 μg of protein extract (depending on the yield of extracted protein from the original cyst fluid) was applied to further analysis.

### Gel-TRAP assay

The telomerase repeat amplification protocol (TRAP) assay measures endogenous telomerase within cell extracts by the detection of added TTAGGG hexameric repeats catalyzed onto the 3′ end of an oligo (the telomeric TS primer; refs. 26, 39). One μg of protein extract from cell pellets was applied and incubated with 50 μL of extension reaction mixture containing 1 × TRAP reaction buffer (10 × concentration: 200 nmol/L Tris-HCl, pH 8.3, 15 nmol/L $MgCl_2$), 0.4 mg/mL BSA, TS primer (200 nmol/L; 5′-AATCCGTCGAGCAGAGTT-3′), dNTP (2.5 mmol/L each, Thermo Fisher Scientific) at 25°C for 60 minutes.

The second step of the TRAP protocol, amplifying extension reaction products by PCR, was performed with a gel-TRAP PCR reaction mixture; 25 μL of this mixture contained 1 × Thermo-Pol Reaction Buffer (New England Biolabs), 12.5 ng of TS primer, 0.125 μL of TRAP primer mix (TS, ACX, and TSNT) as described previously (27). After PCR, reaction mixtures were analyzed by gel electrophoresis in 0.5 × Tris-borate-EDTA buffer on 12% polyacrylamide nondenaturing gels and visualized with ethidium bromide staining. The images were then processed and quantified by densitometry using ImageJ software (NIH, Bethesda, MD). Telomerase activity measured by the ratio of the intensity of 6-bp ladder to that of an internal control (IC) was calculated on the basis of the following formula: [(intensity of sample's 6-bp ladder)−(background intensity between the sample lanes)]/intensity of sample's IC band (27).

### Digital droplet TRAP assay

For ddPCR, each 20-μL reaction mixture contained 1 × ddPCR EvaGreen Supermix (Bio-Rad), 50 nmol/L of TS and ACX primer, 2 μL of extension reaction mixture (the same as for the gel-TRAP assay described above). The 20-μL droplet ddPCR reaction mixture was then loaded into the DG8 disposable droplet generator cartridge (Bio-Rad), and placed into the QX200 Droplet Generator (Bio-Rad). After droplet generation, the water-in-oil droplet emulsions were transferred to a 96-well polypropylene PCR plate (twin.tec PCR plate; Eppendorf). The plate was heat-sealed with foil using a PX1 PCR Plate Sealer (Bio-Rad) and placed in a Veriti Thermal Cycler (Applied Biosystems). PCR conditions were 95°C for 5 minutes (1 cycle), was followed by 37 cycles of 95°C for 30 seconds, 54°C for 30 seconds, 72°C for 30 seconds. A dye-stabilization step was also included at the end of PCR (4°C for 5 minutes then, 95°C for 5 minutes, and finally at 12°C indefinite hold). The temperature ramp rate was set to 2.5°C/second, and the lid was heated to 105°C, according to the manufacturer's recommendations. After thermal cycling, droplet reading was performed on a QX200 ddPCR Droplet Reader (Bio-Rad). Analysis was done using QuantaSoft 1.7.4 Analysis software (Bio-Rad) and the target concentration (copies/μL; PCR scale) was then computed using Poisson statistics. In the current study, to best reflect the nature of cyst fluid, telomerase products were calculated per microliter of cyst fluid. We evaluated the reproducibility of dd-TRAP measurements of cyst fluid using three patients' cyst fluid samples by performing multiple dd-TRAP on 4 aliquots of cyst fluid obtained each sample and found similar results across replicates (coefficient of variation ∼20%, see Supplementary Fig. S1).

### Statistical analysis

The nonparametric Mann–Whitney *U* test was used to compare continuous variables. The Fisher exact test was used to compare categorical variables. Correlations between two different variables were assessed by scatter plot and $R^2$ value. The diagnostic accuracy of telomerase activity for predicting the presence of either invasive cancer or high-grade dysplasia was assessed by receiver operating characteristics (ROC) curve analysis. The cutoff value was defined as the result with the highest sensitivity and specificity that lay closest to the top left corner of the curve. Separate analyses were performed for discovery and validation samples followed by combined analysis. A multivariate analysis using the logistic regression model was performed, including the variables with statistical significance by univariate analysis. Statistical analysis was performed using GraphPad Prism V6.0 (GraphPad Software) and JMP Pro 11.0.0 statistical software (SAS Institute Inc.). A *P* value of less than 0.05 was considered to statistically significant.

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## Results

### Evaluation of the dd-TRAP assay

Abundant telomerase activity was detected in protein extracts of the pancreatic cancer cell line MIAPaCa-2 and was almost completely lost when cell extracts were treated with RNase A (37°C for 20 minutes) or heat (95°C for 5 minutes) before PCR (Supplementary Fig. S2A). Supplementary Figure S2B shows the number of droplets in each lane (16,000–18,000 droplets). Supplementary Fig. S2C shows the concentration of telomerase products corresponding to Supplementary Fig. S2C after the computational analysis using Poisson statistics. After ddPCR, DNA from the droplets was extracted and analyzed by gel electrophoresis to confirm the presence of a DNA ladder generated by telomerase (Supplementary Fig. S2D).

We next detected the dynamic range and limit of detection of the dd-TRAP assay and compared it with the conventional gel-TRAP assay using serial dilutions of MIAPaCa-2 cell extracts. The dd-TRAP assay had a greater dynamic range and lower limit of detection than the gel-TRAP assay. The gel-TRAP assay had a dynamic range from 8 ng to 500 ng (gel electrophoresis and densitometry, Supplementary Fig. S3A and S3B), whereas the dd-TRAP could detect telomerase activity between 0.8 ng and 2,500 ng of MIAPaCa-2 protein extract (Supplementary Fig. S3C and S3D). The limit of detection of the dd-TRAP assay was estimated to be approximately 9 telomerase-positive copies (Supplementary Fig. S3E) and the dynamic range of the assay to be between 10 and 34,000 copies with high linearity ($R^2 = 0.99657$, Supplementary Fig. S3F).

We compared telomerase activity measured by gel-TRAP and dd-TRAP in 10 pancreatic cancer cell lines and one immortalized pancreatic ductal epithelial cell (HPDE) with varying levels of telomerase activity (44), using levels in MIAPaCa-2 cell as a reference (Fig. 1A and B). All cell lines, including HPDE, had telomerase activity as has been previously reported (Fig. 1B and C; ref. 45). There was a close correlation between the telomerase activity measured by gel-TRAP and dd-TRAP ($R^2 = 0.91498$, Fig. 1D) with a wider detectable range again noted for the dd-TRAP assay. Further description of the telomerase activity assay is provided in Supplementary Materials (and Supplementary Figs. S4–S6).

### Pancreatic cystic fluid telomerase activity

We next evaluated the diagnostic performance of the dd-TRAP assay in pancreatic cyst fluid in 219 patients (Supplementary Table S1). First, 184 patients in the discovery set were studied; their cyst fluid characteristics, imaging findings, and final pathologies are provided in Supplementary Table S2. Because the dd-TRAP assay measures enzymatic activity and therefore needs samples of optimal quality, we determined whether cyst telomerase activity was affected by length of storage at −80°C or by the effects of freeze/thawing (some samples from each diagnostic group had previously undergone one or more freeze/thaws; see Supplementary Results in Supplementary Materials). We found evidence that prior freeze/thawing, but not length of storage, was associated with reduced telomerase activity (Supplementary Fig. S7). Therefore, we stratified cyst fluid sample analyses according to their freeze/thaw status and analyzed the difference of telomerase activity across diagnostic groups. Table 1 describes the characteristics of 119 patients (84 discovery, 35 validation) whose samples had not undergone prior freeze/thawing.

Among the 84 patients in the discovery set, higher levels of telomerase activity were detected in the cyst fluid of IPMN cases with HGD compared with IPMN cases with intermediate-grade dysplasia (IGD), cases with SCN, or cases with a pseudocyst ($P < 0.001$; Supplementary Fig. S8A). We also compared cyst fluid telomerase activity in the most relevant diagnostic groups; cases with IPMN and HGD ± an associated invasive cancer versus those with IPMN and either IGD or low-grade dysplasia (LGD; $P < 0.001$), and versus SCN ($P < 0.022$), respectively (Supplementary Fig. S8B). There was no significant difference in telomerase activity between cyst fluids from IPMNs with an associated invasive cancer and those from IPMNs with HGD only ($P = 0.280$, Supplementary Fig. S8A). The median value and interquartile range of telomerase activity levels in each group are shown in Supplementary Fig. S8C. The telomerase activity level (cutoff value; 730 copies/μL of cyst fluid) with the best overall accuracy for distinguishing cyst fluids with invasive cancer/HGD versus those from IGD/LGD among all 84 cases had an area under the ROC curve (AUC) of 0.890; for the 58 IPMN cases the AUC was 0.853 (Supplementary Fig. S8D and S8E). In this series, telomerase activity had higher diagnostic sensitivity (83.3%) than other clinical parameters for distinguishing IPMNs with invasive cancer or HGD from IPMNs with lower grade dysplasia (Supplementary Table S3). We also measured telomerase activity in a validation set in surgically aspirated samples from 35 additional patients. In the validation set, telomerase activity had similarly high diagnostic performance; (AUC of 0.929, Supplementary Fig. S9) for distinguishing samples from patients with invasive cancer or HGD from samples from all other cases with lower grades of dysplasia and samples from the IPMN cases alone (AUC of 0.889, Supplementary Fig. S9; and results in Supplementary Materials).

Among discovery set cases whose cyst fluid samples had undergone multiple (2 or 3) rounds of thawing and re-freezing, we found the differences in telomerase activity between the IPMN-invasive cancer/HGD group versus the IPMN IGD/LGD group were still statistically significant and had similar diagnostic accuracy to samples not previously thawed (Supplementary Fig. S10). Furthermore, the diagnostic performance of cyst fluid telomerase activity in the discovery set analyzed without regard for sample thawing was very similar to subset of cyst fluids without multiple freeze/thaws: telomerase activity was significantly higher in the IPMN HGD/invasive cancer cases compared with IPMN cases with IGD, cases with LGD, and cases with SCN, and diagnostic accuracy was only slightly less (AUC 0.83 for IPMN HGD/cancer vs. IPMN IGD/LGD; Supplementary Fig. S11).

Among the discovery set cases, the diagnostic accuracy of telomerase activity was not increased significantly by combining this measurement with clinical parameters that predict neoplastic progression of pancreatic cysts (Supplementary Table S4).

We also determined whether there was evidence for inhibitors of telomerase activity in cyst fluid samples with HGD yet low telomerase activity by mixing extracts from two such cyst fluid samples with various dilutions of telomerase-positive pancreatic cancer cell line extract. There was no evidence of inhibition of telomerase activity found when cell line extracts

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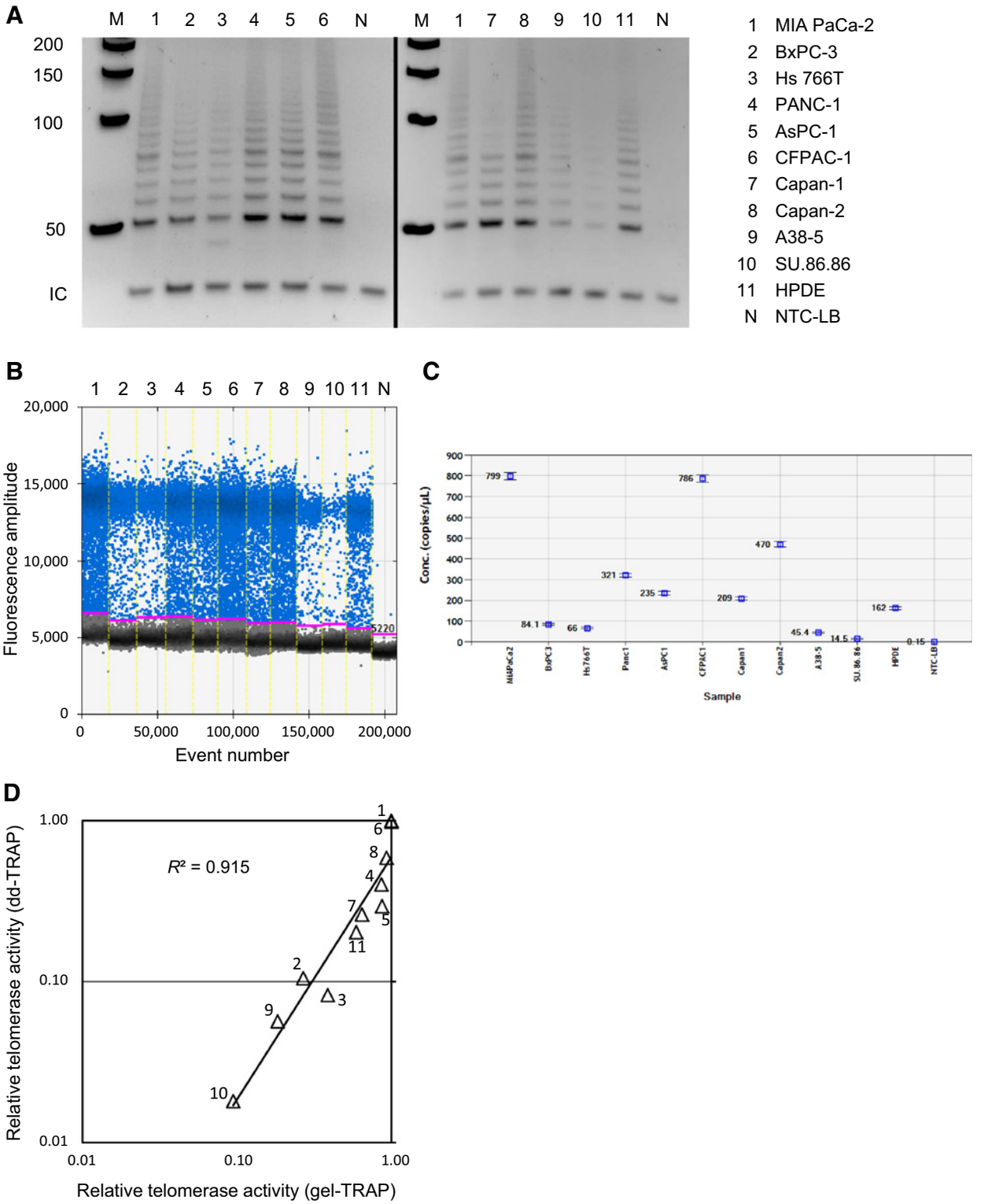


**Figure 1.**

**A** and **B,** representative gel image and 1-D plot graph of gel-TRAP assay (**A**) and dd-TRAP assay (**B**) using pancreatic cancer cell lines. **C,** concentration of telomerase products determined by dd-TRAP assay. Error bar, Poisson 95% confidence limits. **D,** correlation of telomerase activity level by gel-TRAP and dd-TRAP assays in 10 pancreatic cancer cell lines and HPDE-immortalized pancreatic ductal epithelial cell line. Relative telomerase activity was calculated using the level in MIA PaCa-2 cells set at 1.0 for reference. $R^2$ means coefficient of determination. N, nontemplate control lysis buffer.

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**Table 1.** Patient and cyst characteristics (patients whose cyst fluid samples had not undergone prior thawing)

| Characteristics | Total ($n = 119$) | IPMN ($n = 74$) | MCN ($n = 10$) | SCN ($n = 25$) | PanNET ($n = 4$) | Pseudocyst ($n = 5$) | Simple cyst ($n = 1$) |
|---|---|---|---|---|---|---|---|
| Male/female (*n*) | 55/64 | 42/32 | 0/10 | 6/19 | 3/1 | 4/1 | 0/1 |
| Age (median, range), y | 65 (29–87) | 69 (42–87) | 52 (30–61) | 63 (29–77) | 57 (43–75) | 73 (54–82) | 64 |
| Symptoms (*n*) | | | | | | | |
| Abdominal pain | 20 | 11 | 2 | 5 | 0 | 2 | 0 |
| Pancreatitis | 14 | 12 | 1 | 1 | 0 | 0 | 0 |
| Jaundice | 1 | 1 | 0 | 0 | 0 | 0 | 0 |
| Cyst location (*n*) | | | | | | | |
| Head and uncinate/body and tail | 54/65 | 40/34 | 0/10 | 11/14 | 1/3 | 2/3 | 0/1 |
| Cyst size, median (range), cm | 2.5 (0.5–11.5) | 2.0 (0.5–9.0) | 2.6 (1.6–7.0) | 2.5 (1.5–10.0) | 5.5 (2.5–7.6) | 3.8 (2.5–11.5) | 1.5 |
| Mural nodule (*n*)[a] | | | | | | | |
| Absent/present | 76/43 | 45/29 | 10/0 | 16/9 | 0/4 | 4/1 | 1/0 |
| Communication with MPD (*n*)[a] | | | | | | | |
| Absent/present | 73/46 | 33/41 | 10/0 | 20/5 | 4/0 | 5/0 | 1/0 |
| Dilatation of MPD ≥10 mm (*n*)[a] | | | | | | | |
| Absent/present | 105/14 | 60/14 | 10/0 | 25/0 | 4/0 | 5/0 | 1/0 |
| Dilatation of MPD ≥5 mm (*n*)[a] | | | | | | | |
| Absent/present | 84/35 | 42/32 | 10/0 | 24/1 | 3/1 | 4/1 | 1/0 |
| CT/MRI findings (*n*) | | | | | | | |
| Worrisome features | 72 | 42 | 5 | 16 | 4 | 5 | 0 |
| High-risk stigmata | 21 | 19 | 0 | 2 | 0 | 0 | 0 |
| No concerning features | 26 | 13 | 5 | 7 | 0 | 0 | 1 |
| Cyst fluid color (*n*) | | | | | | | |
| Bloody/sero-bloody/brown/straw/clear | 26/51/13/5/24 | 17/35/4/3/15 | 0/2/2/0/6 | 7/12/4/0/2 | 2/1/1/0/0 | 0/1/2/2/0 | 0/0/0/0/1 |
| Cyst fluid appearance (*n*) | | | | | | | |
| Serous/mucinous | 63/56 | 23/51 | 9/1 | 23/2 | 3/1 | 4/1 | 1/0 |
| Original cyst volume (median, range), μL | 139 (10–1,000) | 92.5 (10–800) | 250 (50–600) | 250 (120–1,000) | 125 (80–200) | 50 (50–200) | 400 |
| EUS cytology ($n = 45$), *n* | | | | | | | |
| Nondiagnostic | 6 | 2 | 1 | 3 | 0 | 0 | 0 |
| Benign/atypia/cancer | 20/9/10 | 12/8/10 | 3/0/0 | 4/1/0 | 1/0/0 | 0/0/0 | 0/0/0 |
| Cyst fluid CEA ($n = 23$), *n* | | | | | | | |
| <192 ng/mL/≥192 ng/mL | 16/7 | 7/5 | 2/2 | 6/0 | 1/0 | 0/0 | 0/0 |
| Operative procedure (*n*) | | | | | | | |
| PD/DP/TP/MP | 56/58/3/2 | 42/28/2/2 | 0/10/0/0 | 12/13/0/0 | 0/3/1/0 | 2/3/0/0 | 0/1/0/0 |
| Morphologic duct type (*n*)[a] | | | | | | | |
| Main duct/mixed/branch duct | | 14/18/42 | | | | | |
| Grade of dysplasia (*n*)[b] | | | | | NET G1 $n = 3$ | | |
| LGD/IGD/HGD/Cancer | | 7/36/20/11 | 7/3/0/0 | | NET G2 $n = 1$ | | |

Abbreviations: CEA, carcinoembryonic antigen; DP, distal pancreatectomy; HGD, high-grade dysplasia; IGD, intermediate grade dysplasia; IPMN, intraductal papillary mucinous neoplasm; LGD, low grade dysplasia; MCN, mucinous cystic neoplasm; MP, middle pancreatectomy; MPD, main pancreatic duct; PanNET, pancreatic neuroendocrine tumor; PD, pancreaticoduodenectomy; SCN, serous cystic neoplasm; TP, total pancreatectomy.

[a]Determined by CT/MRI.

[b]Histologic grade of PanNET was diagnosed on the basis of WHO 2010 criteria.

were mixed 1:1 with cyst fluid. Minimal reductions in telomerase activity were found when cell line extracts were mixed with greater amounts of cyst fluid, not sufficient to explain the lack of detectable telomerase activity in these samples (Supplementary Fig. S12). Telomerase activity in SCN samples was minimal (median 7 copies/μL) and was not associated with differences in sample freeze/thaws). Because some SCN cyst fluid samples are bloody and as there is modest level of telomerase activity in inflammatory cells, we also investigated whether the bloody cyst fluid samples from SCN cases had higher telomerase measurements. We did find that SCN cyst fluid samples with a bloody appearance had slightly higher levels of telomerase activity than those with a serosanguinous or clear appearance (Supplementary Fig. S13).

To evaluate the diagnostic accuracy of cyst fluid telomerase activity measurements in relation to the preoperative imaging evaluation of patients, we classified the study population by their clinical features into those with "high-risk stigmata," "worrisome features," and "low-risk" as defined by the revised Sendai criteria (ref. 3; some patients in this study underwent pancreatic resection before these criteria were developed). Among the 84 discovery set cases, 11 (57.9%) of 19 cases with "high-risk stigmata" and 13 (26.0%) of 50 cases with "worrisome features" had invasive cancer or HGD (Supplementary Fig. S14). Among the 50 discovery set cases with "worrisome features," telomerase measurements had a diagnostic sensitivity for distinguishing those with invasive cancer or HGD from those with lower grades of dysplasia of 92.3% (cutoff, 730 copies/μL) and an AUC of 0.927 (Supplementary Table S5). Among the 58 discovery set IPMN cases, 31 had worrisome features and in this group telomerase activity had similarly high diagnostic performance (AUC of 0.876) for distinguishing those with invasive cancer or HGD from those with lower grades of dysplasia (using the cutoff level of 730 copies/μL).

We then analyzed the discovery and validation sets of patients whose samples had not undergone prior thawing in a combined analysis (Fig. 2), compared these results with other clinical parameters (Table 2), performed multivariate analysis on this combined set (Table 3), and examined telomerase activity diagnostic performance according to the preoperative risk classification (Table 4). Using the 730 copies/μL cyst fluid

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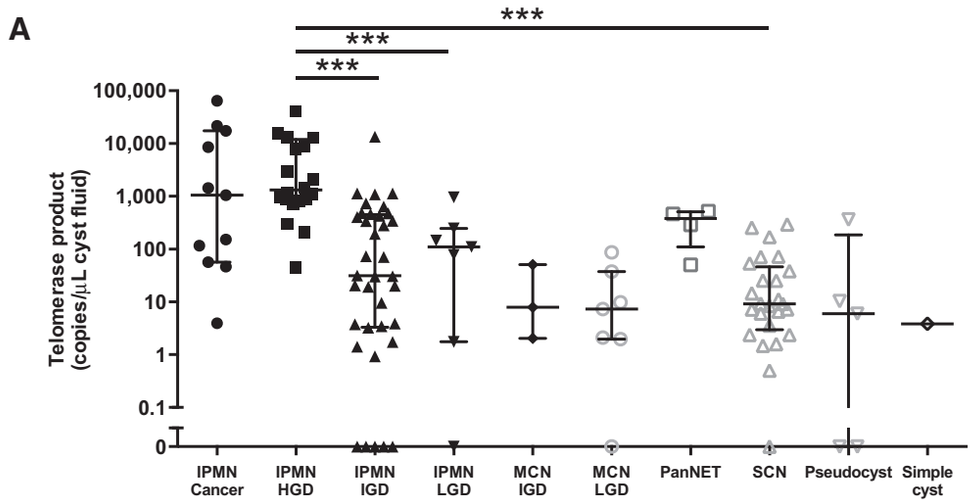
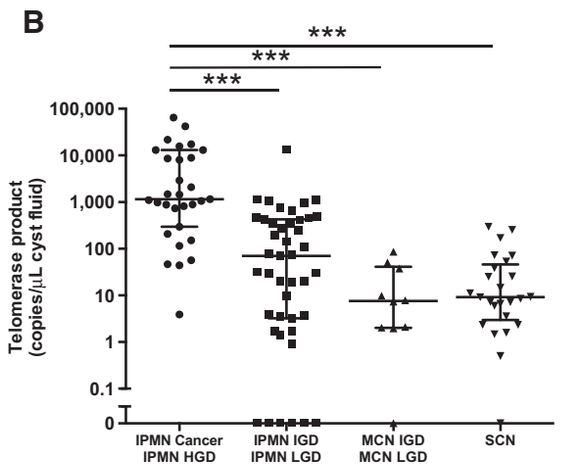
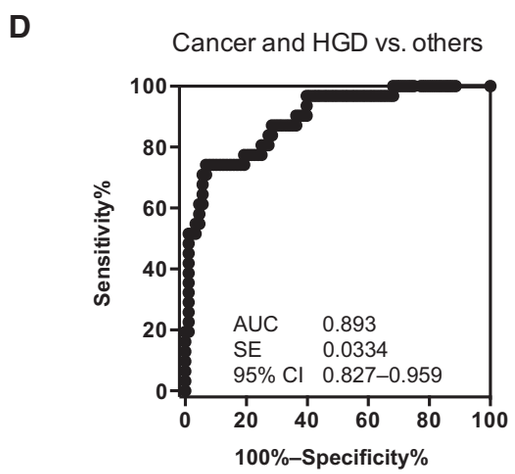



C

| | n | Median | IQR |
|---|---|---|---|
| IPMN | | | |
| Cancer | 11 | 1055 | (56.7–17345) |
| HGD | 20 | 1315 | (830.2–11980) |
| IGD | 36 | 31.19 | (3.303–452.2) |
| LGD | 7 | 109.8 | (1.76–246.5) |
| MCN | | | |
| IGD | 3 | 7.9 | (2.04–50.68) |
| LGD | 7 | 7.36 | (1.98–37.59) |
| PanNET | 4 | 377.3 | (110.2–511) |
| SCN | 25 | 9.2 | (2.97–46.03) |
| Pseudocyst | 5 | 5.97 | (0–186) |
| Simple cyst | 1 | 3.84 | |
| IPMN Cancer, HGD | 31 | 1158 | (295.9–13033) |
| IPMN IGD, LGD | 43 | 70.68 | (3.25–425.9) |
| MCN IGD, LGD | 10 | 7.63 | (2.025–40.86) |



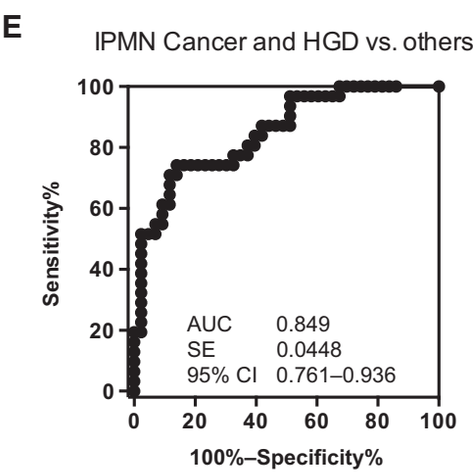




**Figure 2.**

**A,** absolute quantification of telomerase activity per microliter of original cyst fluid samples among 119 samples from the discovery and validation set that had not undergone any prior thawing. The longer horizontal bar represents the median value and shorter ones represent values of the 75th and 25th percentiles, respectively. **B,** comparison of telomerase activity of IPMN cases classified by their surgical indication (invasive cancer and HGD vs. IGD and LGD) and MCN and SCN cases. **C,** telomerase activity levels per microliter of cyst fluid samples in each group. **D** and **E,** ROC curve analysis for the diagnostic accuracy of telomerase activity in predicting malignancy among all 119 cases (**D**) and 74 IPMN cases (**E**). HGD, high-grade dysplasia; IGD, intermediate-grade dysplasia; LGD, low-grade dysplasia; MCN, mucinous cystic neoplasm; SCN, serous cystic neoplasm; PanNET, pancreatic neuroendocrine tumor; IQR, interquartile range; AUC, area under the curve; SE, standard error; CI, confidence interval. N.S., not significant; ***, $P < 0.001$.

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**Table 2.** The diagnostic accuracy of telomerase activity, imaging, and clinical factors for predicting high-grade dysplasia/invasive cancer

| Findings | Cutoff | Sensitivity (%; 95% CI) | Specificity (%; 95% CI) | Accuracy (%) | PPV (%) | NPV (%) |
|---|---|---|---|---|---|---|
| All cases ($n = 119$)[a] | | | | | | |
| Cyst appearance | Mucinous | 83.9 (66.3–94.6) | 63.6 (52.7–73.6) | 68.9 | 44.8 | 91.8 |
| Cyst size | ≥30 mm | 32.3 (16.7–51.4) | 64.8 (53.9–74.7) | 56.3 | 24.4 | 73.1 |
| MPD dilatation | ≥10 mm | 25.8 (11.9–44.6) | 93.2 (85.8–97.5) | 84.0 | 57.1 | 78.1 |
| MPD dilatation | ≥5 mm | 58.1 (39.1–75.5) | 80.7 (70.9–88.3) | 74.8 | 51.4 | 84.5 |
| Mural nodule | Present | 58.1 (39.1–75.5) | 71.6 (61.0–80.7) | 68.1 | 41.9 | 82.9 |
| Telomerase activity | ≥730 copies/μL cyst fluid | 74.2 (55.4–82.1) | 93.2 (85.8–97.5) | 88.2 | 79.3 | 91.1 |
| IPMN cases ($n = 74$) | | | | | | |
| Cyst appearance | Mucinous | 83.9 (66.3–94.6) | 41.9 (27.0–57.9) | 59.5 | 51.0 | 78.3 |
| Cyst size | ≥30 mm | 32.3 (16.7–51.4) | 24.4 (58.8–86.5) | 56.8 | 47.6 | 60.4 |
| MPD dilatation | ≥10 mm | 25.8 (11.9–44.6) | 86.1 (72.1–94.7) | 60.8 | 57.1 | 61.7 |
| MPD dilatation | ≥5 mm | 58.1 (39.1–75.5) | 67.4 (51.5–80.9) | 63.5 | 56.3 | 69.1 |
| Mural nodule | Present | 58.6 (39.1–75.5) | 74.4 (58.8–86.5) | 67.6 | 62.1 | 71.1 |
| Telomerase activity | ≥730 copies/μL cyst fluid | 74.2 (55.4–88.1) | 86.1 (72.1–94.7) | 81.1 | 79.3 | 82.2 |

Abbreviations: CI, confidence interval; MPD, main pancreatic duct; NPV, negative predictive value; PPV, positive predictive value.
[a]Discovery and validation sets combined (samples without prior thawing).

cut-off value, determined by the discovery set results, the diagnostic performance for predicting the presence of high-grade dysplasia/invasive cancer was somewhat higher when all 119 cases were considered (AUC, 0.893; sensitivity, 74.2%; specificity, 93.2%, true-positives 23, false-positives 6, false-negatives 8, true-negatives 82; Fig. 2D; Table 2) than among the 74 cases with IPMN (AUC, 0.849; sensitivity, 74.2%; specificity, 86.1%, true-positives 23, false-positives 6, false-negatives 8, true-negatives, 37; Fig. 2E; Table 2). Table 3 shows that the level of telomerase activity as a predictor of malignancy (invasive cancer/HGD) was independent of other factors associated with malignancy by multivariate analysis (which in this series were factors associated with having an IPMN). Table 4 shows that the higher diagnostic performance in the subgroup of patients whose preoperative evaluation was "worrisome features" was similar in the combined (discovery and validation) set (AUC, 0.900; sensitivity, 73.7; specificity, 90.6%; accuracy, 86.1%) as it was in the discovery set (AUC, 0.927; Supplementary Table S5).

We also performed telomerase activity analysis on pancreatic samples obtained by EUS-FNA preoperatively from 36 cases who underwent subsequent pancreatic resection. A summary of the endoscopic findings, cyst fluid analyses, and pathologic diagnoses for these cases are provided in Supplementary Table

**Table 3.** Univariate and multivariate analyses of factors predictive of invasive cancer or high-grade dysplasia in pancreatic cysts among the combined discovery and validation sets[a]

| Variable | Cancer, HGD ($n = 31$) | Others ($n = 88$) | Univariate | Multivariate | |
|---|---|---|---|---|---|
| | | | *P* | OR (95% CI) | *P* |
| Age | | | | | |
| <65 | 8 | 47 | **0.011** | 1.000 | |
| ≥65 | 23 | 41 | | 4.218 (1.104–19.889) | **0.035** |
| Sex | | | | | |
| Female | 10 | 54 | **0.007** | 1.000 | |
| Male | 21 | 34 | | 1.702 (0.478–6.199) | 0.408 |
| Fluid appearance | | | | | |
| Serous | 5 | 54 | <**0.0001** | 1.000 | |
| Mucinous | 26 | 34 | | 1.668 (0.292–8.502) | 0.544 |
| Cyst size | | | | | |
| <30 mm | 21 | 57 | 0.829 | | |
| ≥30 mm | 10 | 31 | | | |
| Cyst location | | | | | |
| Body and tail | 11 | 54 | **0.020** | 1.000 | |
| Head | 20 | 34 | | 0.713 (0.148–2.937) | 0.647 |
| MPD dilatation | | | | | |
| <5 mm | 13 | 71 | **0.0001** | 1.000 | |
| ≥5 mm | 18 | 17 | | 0.957 (0.204–3.858) | 0.952 |
| MPD communication | | | | | |
| Absent | 9 | 64 | <**0.0001** | 1.000 | |
| Present | 22 | 24 | | 2.776 (0.705–11.070) | 0.142 |
| Mural nodule | | | | | |
| Absent | 13 | 63 | **0.005** | 1.000 | |
| Present | 18 | 25 | | 2.131 (0.570–8.052) | 0.255 |
| Telomerase activity | | | | | |
| <730 copies/μL (cyst fluid) | 8 | 82 | <**0.0001** | 1.000 | |
| ≥730 copies/μL (cyst fluid) | 23 | 6 | | 20.698 (4.195–140.94) | <**0.0001** |

Abbreviations: CI, confidence interval; HGD, high-grade dysplasia; MPD, main pancreatic duct.
[a]Cases whose cyst fluid samples had not undergone prior thawing.

**Table 4.** Subgroup analysis of diagnostic performance of telomerase activity among 119 merged cases without prior thawing

| Subgroup | *n* | AUC | Cutoff[a] | TP | FN | FP | TN | Sensitivity (%; 95% CI) | Specificity (%; 95% CI) | Accuracy (%) | PPV (%) | NPV (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| All cases ($n = 119$) | | | | | | | | | | | | |
| High-risk stigmata | 21 | 0.796 | 730 | 9 | 3 | 1 | 8 | 75.0 (42.8–94.5) | 88.9 (51.8–99.7) | 81.0 | 90.0 | 72.7 |
| Worrisome features | 72 | 0.900 | 730 | 14 | 5 | 5 | 48 | 73.7 (48.8–90.9) | 90.6 (79.3–96.9) | 86.1 | 73.7 | 90.6 |
| IPMN cases ($n = 74$) | | | | | | | | | | | | |
| High-risk stigmata | 19 | 0.774 | 730 | 9 | 3 | 1 | 6 | 75.0 (42.8–94.5) | 85.7 (42.1–99.6) | 78.9 | 90.0 | 66.7 |
| Worrisome features | 42 | 0.842 | 730 | 14 | 5 | 5 | 18 | 73.7 (48.8–90.9) | 78.3 (56.3–92.5) | 76.2 | 73.7 | 78.3 |

Abbreviations: AUC, area under the curve; FN, false negative; FP, false positive; NPV, negative predictive value; PPV, positive predictive value; TN, true negative; TP, true positive.

[a]Value of telomerase activity in pancreatic cyst fluid (copies/μL of cyst fluid).

S6. In these samples, the telomerase activity levels correlated well with those found in the surgical cyst fluid samples (Supplementary Fig. S15).

## Discussion

In the current study, we demonstrate that measurement of telomerase activity in pancreatic cyst fluid samples using dd-TRAP adds diagnostic utility for predicting whether or not a pancreatic cystic lesion harbors high-grade dysplasia and/or an associated invasive cancer. The need to determine the neoplastic grade of a pancreatic cyst is usually the most important question clinicians need to answer when evaluating patients with pancreatic cysts and a pancreatic cyst fluid test that could do this reliably would likely have high clinical utility.

The diagnostic utility of cyst fluid telomerase activity levels for distinguishing samples obtained from cystic neoplasms with HGD ± invasive cancer versus cysts with LGD compares favorably to other clinically used measures such as cyst fluid cytology and cyst fluid CEA, which have lower diagnostic accuracy (14). We recently evaluated a panel of genetic biomarkers in cyst fluid and found that a combination of molecular markers had excellent accuracy for classifying pancreatic cysts and for predicting their grade of dysplasia (15, 46). Telomerase activity measured by dd-TRAP had similar accuracy ($AUC_{combined} = 0.849$) to our recently reported molecular marker model for the predicting the histologic grade of IPMNs (invasive cancer/high-grade dysplasia vs. intermediate-grade dysplasia or low-grade dysplasia). Given the high prevalence of asymptomatic cysts in the general population and in high-risk groups, the ability to reliably stratify pancreatic cysts into high-risk cysts (requiring surgery or close surveillance) versus low-risk cysts (less frequent surveillance) is vital for optimal patient management (47–50).

The diagnostic performance of telomerase activity was similarly high in cases whose pancreatic cyst evaluation was classified as having "worrisome features" ($AUC_{combined} = 0.842$). This is the group that most needs pancreatic cyst fluid markers since cysts considered low-risk are not recommended to have FNA and cysts with high-risk stigmata are generally recommended for surgical resection without further examination (3).

Although this study had the advantage that all patients underwent surgical resection and therefore had defined histology, most of the samples analyzed in this study were obtained from surgical resection specimens. Prospective validation studies measuring telomerase activity in larger numbers of EUS-FNA cyst fluid samples, particularly those from patients whose cysts pose diagnostic and management dilemmas, are needed to further evaluate its diagnostic performance. Our study population consisted of patients who underwent resection of their pancreatic cysts and the most important clinical setting in which pancreatic cyst fluid markers such as telomerase activity should be evaluated is when surgical resection is being considered, but it will also be valuable to know if cyst fluid markers can help evaluate the risk of future neoplastic progression of pancreatic cysts that do not currently meet indications for surgical resection or in patients whose pancreatic cysts do meet criteria for surgical resection but elect to undergo surveillance. The diagnostic utility of telomerase activity might also be strengthened by combining the results of cyst fluid analysis obtained at multiple time points during patient surveillance.

In conclusion, quantification of pancreatic cyst fluid telomerase activity using dd-TRAP can accurately predict the presence or absence of invasive cancer/high-grade dysplasia.

### Disclosure of Potential Conflicts of Interest

A.M. Lennon is a consultant/advisory board member for NovoNordisc and Olympus. R.H. Hruban is an employee of MiDiagnostics and reports receiving royalty payments from Myriad Genetics for the PALB2 invention in a relationship supervised by Johns Hopkins University. No potential conflicts of interest were disclosed by the other authors.

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**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** T. Hata, M.D. Molin, M.I. Canto, C. Wolfgang, M. Goggins
**Writing, review, and/or revision of the manuscript:** T. Hata, M.D. Molin, J. Yu, M. Weiss, M.I. Canto, C. Wolfgang, A. Marie Lennon, R.H. Hruban, M. Goggins
**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** M.D. Molin, R.H. Hruban
**Study supervision:** M.I. Canto, M. Goggins

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## References


1. Brugge WR. Diagnosis and management of cystic lesions of the pancreas. J Gastrointest Oncol 2015;6:375–88.
2. Lennon AM, Manos LL, Hruban RH, Ali SZ, Fishman EK, Kamel IR, et al. Role of a multidisciplinary clinic in the management of patients with pancreatic cysts: a single-center cohort study. Ann Surg Oncol 2014;21: 3668–74.
3. Tanaka M, Fernandez-del Castillo C, Adsay V, Chari S, Falconi M, Jang JY, et al. International consensus guidelines 2012 for the management of IPMN and MCN of the pancreas. Pancreatology 2012;12:183–97.
4. Lennon AM, Wolfgang C. Cystic neoplasms of the pancreas. J Gastrointest Surg 2013;17:645–53.
5. Jais B, Rebours V, Malleo G, Salvia R, Fontana M, Maggino L, et al. Serous cystic neoplasm of the pancreas: a multinational study of 2622 patients under the auspices of the international association of pancreatology and european pancreatic club (european study group on cystic tumors of the pancreas). Gut 2016;65:305–12.
6. Ridtitid W, DeWitt JM, Schmidt CM, Roch A, Stuart JS, Sherman S, et al. Management of branch-duct intraductal papillary mucinous neoplasms: a large single-center study to assess predictors of malignancy and long-term outcomes. Gastrointest Endosc 2016 Feb 18. [Epub ahead of print].
7. Valsangkar NP, Morales-Oyarvide V, Thayer SP, Ferrone CR, Wargo JA, Warshaw AL, et al. 851 resected cystic tumors of the pancreas: a 33-year experience at the Massachusetts General Hospital. Surgery 2012;152: S4–12.
8. Sahora K, Mino-Kenudson M, Brugge W, Thayer SP, Ferrone CR, Sahani D, et al. Branch duct intraductal papillary mucinous neoplasms: does cyst size change the tip of the scale? A critical analysis of the revised international consensus guidelines in a large single-institutional series. Ann Surg 2013;258:466–75.
9. Crippa S, Bassi C, Salvia R, Malleo G, Marchegiani G, Rebours V, et al. Low progression of intraductal papillary mucinous neoplasms with worrisome features and high-risk stigmata undergoing non-operative management: a mid-term follow-up analysis. Gut 2016 Jan 7. [Epub ahead of print].
10. Yu J, Blackford A, Dal Molin M, Wolfgang C, Goggins M. Time to progression of pancreatic ductal adenocarcinoma from low-to-high tumour stages. Gut 2015;64:1783–89.
11. Winter JM, Jiang W, Basturk O, Mino-Kenudson M, Fong ZV, Tan WP, et al. Recurrence and survival after resection of small intraductal papillary mucinous neoplasm-associated carcinomas (less than or equal to 20 mm invasive component): a multi-institutional analysis. Ann Surg 2016;263: 793–801.
12. Woolf KM, Liang H, Sletten ZJ, Russell DK, Bonfiglio TA, Zhou Z. False-negative rate of endoscopic ultrasound-guided fine-needle aspiration for pancreatic solid and cystic lesions with matched surgical resections as the gold standard: one institution's experience. Cancer Cytopathol 2013;121:449–58.
13. Thornton GD, McPhail MJ, Nayagam S, Hewitt MJ, Vlavianos P, Monahan KJ. Endoscopic ultrasound guided fine needle aspiration for the diagnosis of pancreatic cystic neoplasms: a meta-analysis. Pancreatology 2013;13: 48–57.
14. Maker AV, Carrara S, Jamieson NB, Pelaez-Luna M, Lennon AM, Dal Molin M, et al. Cyst fluid biomarkers for intraductal papillary mucinous neoplasms of the pancreas: a critical review from the international expert meeting on pancreatic branch-duct-intraductal papillary mucinous neoplasms. J Am Coll Surg 2015;220:243–53.
15. Springer S, Wang Y, Molin MD, Masica DL, Jiao Y, Kinde I, et al. A combination of molecular markers and clinical features improve the classification of pancreatic cysts. Gastroenterology 2015;4:01067–7.
16. DiMaio CJ, Weis-Garcia F, Bagiella E, Tang LH, Allen PJ. Pancreatic cyst fluid concentration of high-mobility group A2 protein acts as a differential biomarker of dysplasia in intraductal papillary mucinous neoplasm. Gastrointest Endosc. 2016;83:1205–9.
17. Yip-Schneider MT, Wu H, Dumas RP, Hancock BA, Agaram N, Radovich M, et al. Vascular endothelial growth factor, a novel and highly accurate pancreatic fluid biomarker for serous pancreatic cysts. J Am Coll Surg 2014;218:608–17.
18. Jabbar KS, Verbeke C, Hyltander AG, Sjovall H, Hansson GC, Sadik R. Proteomic mucin profiling for the identification of cystic precursors of pancreatic cancer. J Natl Cancer Inst 2014;106:djt439.
19. Cao Z, Maupin K, Curnutte B, Fallon B, Feasley CL, Brouhard E, et al. Specific glycoforms of MUC5AC and endorepellin accurately distinguish mucinous from nonmucinous pancreatic cysts. Mol Cell Proteomics 2013;12:2724–34.
20. Hong SM, Omura N, Vincent A, Li A, Knight S, Yu J, et al. Genome-wide CpG island profiling of intraductal papillary mucinous neoplasms of the pancreas. Clin Cancer Res 2012;18:700–12.
21. Das KK, Xiao H, Geng X, Fernandez-Del-Castillo C, Morales-Oyarvide V, Daglilar E, et al. mAb Das-1 is specific for high-risk and malignant intraductal papillary mucinous neoplasm (IPMN). Gut 2014;63:1626–34.
22. Balcom JHt, Keck T, Warshaw AL, Antoniu B, Graeme-Cook F, Fernandez-del Castillo C. Telomerase activity in periampullary tumors correlates with aggressive malignancy. Ann Surg 2001;234:344–50.
23. Maser RS, DePinho RA. Connecting chromosomes, crisis, and cancer. Science 2002;297:565–9.
24. Shay JW. Role of telomeres and telomerase in aging and cancer. Cancer Discov. 2016Mar 30. [Epub ahead of print].
25. Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, et al. Specific association of human telomerase activity with immortal cells and cancer. Science 1994;266:2011–5.
26. Kim NW, Wu F. Advances in quantification and characterization of telomerase activity by the telomeric repeat amplification protocol (TRAP). Nucleic Acids Res 1997;25:2595–7.
27. Herbert BS, Hochreiter AE, Wright WE, Shay JW. Nonradioactive detection of telomerase activity using the telomeric repeat amplification protocol. Nat Protoc 2006;1:1583–90.
28. Krupp G, Kuhne K, Tamm S, Klapper W, Heidorn K, Rott A, et al. Molecular basis of artifacts in the detection of telomerase activity and a modified primer for a more robust `TRAP' assay. Nucleic Acids Res 1997;25:919–21.
29. Yeh TS, Cheng AJ, Chen TC, Jan YY, Hwang TL, Jeng LB, et al. Telomerase activity is a useful marker to distinguish malignant pancreatic cystic tumors from benign neoplasms and pseudocysts. J Surg Res 1999;87:171–7.
30. Saji M, Xydas S, Westra WH, Liang CK, Clark DP, Udelsman R, et al. Human telomerase reverse transcriptase (hTERT) gene expression in thyroid neoplasms [In Process Citation]. Clin Cancer Res 1999;5:1483–9.
31. Ohuchida K, Mizumoto K, Ogura Y, Ishikawa N, Nagai E, Yamaguchi K, et al. Quantitative assessment of telomerase activity and human telomerase reverse transcriptase messenger RNA levels in pancreatic juice samples for the diagnosis of pancreatic cancer. Clin Cancer Res 2005;11:2285–92.
32. Hashimoto Y, Murakami Y, Uemura K, Hayashidani Y, Sudo T, Ohge H, et al. Detection of human telomerase reverse transcriptase (hTERT) expression in tissue and pancreatic juice from pancreatic cancer. Surgery 2008;143:113–25.
33. Nakashima A, Murakami Y, Uemura K, Hayashidani Y, Sudo T, Hashimoto Y, et al. Usefulness of human telomerase reverse transcriptase in pancreatic juice as a biomarker of pancreatic malignancy. Pancreas 2009;38:527–33.
34. Hashimoto Y, Murakami Y, Uemura K, Hayashidani Y, Sudo T, Ohge H, et al. Telomere shortening and telomerase expression during multistage carcinogenesis of intraductal papillary mucinous neoplasms of the pancreas. J Gastrointest Surg 2008;12:17–28.
35. Jakupciak JP.Real-time telomerase activity measurements for detection of cancer. Expert Rev Mol Diagn 2005;5:745–53.
36. Shim WY, Park KH, Jeung HC, Kim YT, Kim TS, Hyung WJ, et al. Quantitative detection of telomerase activity by real-time TRAP assay in the body fluids of cancer patients. Int J Mol Med 2005;16:857–63.
37. Hou M, Xu D, Bjorkholm M, Gruber A. Real-time quantitative telomeric repeat amplification protocol assay for the detection of telomerase activity. Clin Chem 2001;47:519–24.
38. Wege H, Chui MS, Le HT, Tran JM, Zern MA. SYBR Green real-time telomeric repeat amplification protocol for the rapid quantification of telomerase activity. Nucleic Acids Res 2003;31:E3.
39. Ludlow AT, Robin JD, Sayed M, Litterst CM, Shelton DN, Shay JW, et al. Quantitative telomerase enzyme activity determination using droplet digital PCR with single cell resolution. Nucleic Acids Res 2014;42:e104.
40. Pinheiro LB, Coleman VA, Hindson CM, Herrmann J, Hindson BJ, Bhat S, et al. Evaluation of a droplet digital polymerase chain reaction format for DNA copy number quantification. Anal Chem 2012;84:1003–11.
41. Hindson BJ, Ness KD, Masquelier DA, Belgrader P, Heredia NJ, Makarewicz AJ, et al. High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. Anal Chem 2011;83:8604–10.

42. Dube S, Qin J, Ramakrishnan R. Mathematical analysis of copy number variation in a DNA sample using digital PCR on a nanofluidic device. PLoS ONE 2008;3:e2876.
43. Brugge WR, Lewandrowski K, Lee-Lewandrowski E, Centeno BA, Szydlo T, Regan S, et al. Diagnosis of pancreatic cystic neoplasms: a report of the cooperative pancreatic cyst study. Gastroenterology 2004;126: 1330–6.
44. Sato N, Maehara N, Mizumoto K, Nagai E, Yasoshima T, Hirata K, et al. Telomerase activity of cultured human pancreatic carcinoma cell lines correlates with their potential for migration and invasion. Cancer 2001;91:496–504.
45. Ouyang H, Mou L, Luk C, Liu N, Karaskova J, Squire J, et al. Immortal human pancreatic duct epithelial cell lines with near normal genotype and phenotype. Am J Pathol 2000;157:1623–31.
46. Amato E, Molin MD, Mafficini A, Yu J, Malleo G, Rusev B, et al. Targeted next-generation sequencing of cancer genes dissects the molecular profiles of intraductal papillary neoplasms of the pancreas. J Pathol 2014;233:217–27.
47. Canto MI, Hruban RH, Fishman EK, Kamel IR, Schulick R, Zhang Z, et al. Frequent detection of pancreatic lesions in asymptomatic high-risk individuals. Gastroenterology 2012;142:796–804.
48. de Jong K, Nio CY, Hermans JJ, Dijkgraaf MG, Gouma DJ, van Eijck CH, et al. High prevalence of pancreatic cysts detected by screening magnetic resonance imaging examinations. Clin Gastroenterol Hepatol 2010; 8:806–11.
49. Vege SS, Ziring B, Jain R, Moayyedi P. American gastroenterological association institute guideline on the diagnosis and management of asymptomatic neoplastic pancreatic cysts. Gastroenterology 2015;148: 819–22.
50. Scheiman JM, Hwang JH, Moayyedi P. American gastroenterological association technical review on the diagnosis and management of asymptomatic neoplastic pancreatic cysts. Gastroenterology 2015;148:824–48.

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Clinical Cancer Research

# Cyst Fluid Telomerase Activity Predicts the Histologic Grade of Cystic Neoplasms of the Pancreas

Tatsuo Hata, Marco Dal Molin, Masaya Suenaga, et al.

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