Biology of Human Tumors

Clinical Cancer Research

Predicting the Grade of Dysplasia of Pancreatic Cystic Neoplasms Using Cyst Fluid DNA Methylation Markers

Tatsuo Hata[1], Marco Dal Molin[1], Seung-Mo Hong[2], Koji Tamura[1], Masaya Suenaga[1], Jun Yu[1], Hiraku Sedogawa[1], Matthew J. Weiss[3], Christopher L. Wolfgang[3], Anne Marie Lennon[3,4], Ralph H. Hruban[1,5], and Michael G. Goggins[1,4,5]

Abstract

Purpose: Pancreatic cysts are common and pose diagnostic and management challenges. Pancreatic cyst fluid markers have the potential to aid in the management of cysts with concerning imaging findings. Our aim was to evaluate cyst fluid methylated DNA markers for their accuracy for predicting the histologic grade of neoplastic pancreatic cysts.

Experimental Design: Pancreatic cyst fluid samples from 183 patients (29 discovery and 154 validation) aspirated after surgical resection were analyzed for methylated DNA at selected genes (*SOX17*, *BNIP3*, *FOXE1*, *PTCHD2*, *SLIT2*, *EYA4*, and *SFRP1*) using methylation-specific droplet-digital PCR (dd-QMSP). Methylated DNA levels were evaluated for their accuracy at predicting the grade of dysplasia of the pancreatic cyst.

Results: All six markers evaluated in the validation set could accurately distinguish high-risk cystic neoplasms (with high-grade dysplasia and/or associated invasive cancer) from low-risk cysts (lower grades of dysplasia) with accuracies from 79.8% to 83.6%. Methylated *SOX17* had the highest overall accuracy as a single marker (sensitivity, 78.4%; specificity, 85.6%; accuracy 83.6%, cutoff; 25 methylated DNA molecules/μL cyst fluid). The best four-gene combination had 84.3% sensitivity, 89.4% specificity, and 88.0% accuracy at distinguishing cysts with high-grade dysplasia and/or invasive cancer from those without. All six markers were independent predictors of having invasive cancer/high-grade dysplasia after adjusting for clinical/imaging factors known to be associated with grade of dysplasia. The combination of methylated *SOX17* with cytology better predicted neoplastic grade than cytology alone.

Conclusions: A panel of methylated gene markers quantified by dd-QMSP can be used to predict the grade of dysplasia of pancreatic cysts. *Clin Cancer Res; 23(14); 3935–44. ©2017 AACR.*

Introduction

Pancreatic cysts are common in the general population and frequently pose diagnostic and clinical management dilemmas (1). A number of different neoplasms with a wide spectrum of malignant potential can produce cysts in the pancreas. An accurate classification of cyst type is therefore critical for appropriate patient management (2, 3). Intraductal papillary mucinous neoplasms (IPMN) have a wide spectrum of malignant potential. Mucinous cystic neoplasms (MCN) arise in the pancreas body and tail of middle-aged women and are usually resected because of their malignant potential (2, 4). Serous cystic neoplasms (SCN) have extremely low malignant potential and most asymptomatic SCNs can safely be followed clinically (5). Solid-pseudopapillary neoplasms (SPN) have low malignant potential but require resection (6). Pancreatic cysts are currently evaluated using pancreatic imaging and any associated clinical features (7, 8). Recent guidelines, often referred to as the "modified Sendai or Tanaka guidelines," recommend that when an IPMN is diagnosed, only those suspected to have high-grade dysplasia or an associated invasive cancer need resection; IPMNs thought to have low- or intermediate-grade dysplasia are managed by surveillance (7). These guidelines have been developed for patients with incidentally detected pancreatic cysts and therefore may not apply as well to patients with a strong family history of pancreatic cancer or a predisposing inherited susceptibility gene mutation as they have an increased risk of developing invasive carcinoma (9–11). Subsequent studies evaluating the modified Sendai guidelines have generally found them to be valuable, although when strictly applied, many surgically resected cysts with "worrisome clinical features," have only low- or intermediate-grade dysplasia when resected, suggesting they could potentially have been managed with continued surveillance (12–16).

Because pancreatic imaging features are not optimal for predicting the malignant potential of a pancreatic cyst, cyst fluid markers are being evaluated for their potential to improve the clinical evaluation of pancreatic cysts. Although cyst fluid cytology is highly specific (17), cytology has a suboptimal sensitivity for

[1]Department of Pathology, The Sol Goldman Pancreatic Cancer Research Center, Johns Hopkins University School of Medicine, Baltimore, Maryland. [2]Department of Pathology, Asan Medical Center, University of Ulsan College of Medicine, Seoul, Korea. [3]Department of Surgery, The Sol Goldman Pancreatic Cancer Research Center, Johns Hopkins University School of Medicine, Baltimore, Maryland. [4]Department of Medicine, The Sol Goldman Pancreatic Cancer Research Center, Johns Hopkins University School of Medicine, Baltimore, Maryland. [5]Department of Oncology, The Sol Goldman Pancreatic Cancer Research Center, Johns Hopkins University School of Medicine, Baltimore, Maryland.

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

Corresponding Author: Michael G. Goggins, Johns Hopkins Medical Institutions, Department of Pathology, CRB2 351, 1550 Orleans Street, Baltimore, MD 21231. Phone: 410-955-3511; Fax: 410-614-0671; E-mail: mgoggins@jhmi.edu

doi: 10.1158/1078-0432.CCR-16-2244

Translational Relevance

New biomarkers are needed to predict the grade of dysplasia of pancreatic cysts. Aberrant DNA methylation is a hallmark of cancer and increases as precancerous lesions progress from normal to low-grade dysplasia to high-grade dysplasia and ultimately to invasive cancer. In this study, we evaluated the ability of digital-droplet quantitative methylation-specific PCR measurements of six aberrantly methylated genes to predict grade of dysplasia of pancreatic cysts. We find that levels of pancreatic cyst fluid methylated DNA biomarkers can distinguish pancreatic cysts with high-grade dysplasia and/or invasive cancer from those with lower grades of dysplasia with high accuracy.

identifying the presence of carcinoma (33.0%), largely because of difficulty obtaining satisfactory samples (18). Cyst fluid carcinoembryonic antigen (CEA) levels also have suboptimal diagnostic accuracy (19). Cyst fluid DNA markers have useful diagnostic characteristics (20, 21). *GNAS* mutations are highly specific for IPMN, but usually arise early in IPMN development and are not useful for predicting grade of dysplasia (22). Like *GNAS*, *KRAS* mutations are very helpful for identifying a cyst as mucin-producing, but its presence does not provide sufficient information about the grade of dysplasia (23). *VHL* mutations are characteristic of SCNs, and *CTNNB1* mutations are characteristic of SPNs (24). In one study, the combination of mutant *KRAS* and loss of chromosomes in cyst fluid DNA had high specificity (96%) but low sensitivity (37%) for predicting high-grade dysplasia with or without an associated invasive carcinoma (25). A combinatorial cyst fluid genetic marker panel may be useful for evaluating both pancreatic cyst type and grade of dysplasia (24, 26, 27).

Among other cyst fluid markers that have undergone evaluation (28–32), cyst fluid telomerase activity measured using droplet-digital PCR could distinguish cysts with high-grade dysplasia with or without an associated invasive carcinoma from those without with a 74.2% sensitivity and 93.2% specificity (33). While all of these approaches are promising, there remains a need for better tests than can more accurately predict the grade of dysplasia of pancreatic cysts.

One class of molecular alteration that becomes increasingly prevalent as precancerous lesions progress toward invasive cancer is aberrant DNA methylation (34). Aberrant methylation of numerous genes occurs during pancreatic tumorigenesis (35–41) including during the development and progression of IPMN (35, 42–45). In this study, we evaluated the diagnostic utility of pancreatic cyst fluid methylated gene markers for their ability to predict the presence of high-grade dysplasia/invasive carcinoma in pancreatic cystic neoplasms.

Materials and Methods

An overview of the study design is provided in Fig. 1.

Patients and specimens

The study included 164 patients (29 discovery and 135 validation) who had undergone surgical resection for a pancreatic cyst at the Johns Hopkins Hospital between 2008 and March 2016, as well as 28 patients from Asan Medical Center (Seoul, South Korea) who had cyst fluid collected from their surgical resection specimen (typically ~0.5 mL or more). These 28 patients were included in the validation set, for 163 validation cases. Nine cases in the validation set were excluded because of inadequate cyst fluid volume. The study population was selected to evaluate cyst fluid tests in the most common diagnostic scenario, i.e., distinguishing cases with IPMN with high-grade dysplasia or with an associated invasive cancer versus lower grade IPMNs or from serous cystadenomas. As described in Fig. 1, we first tested the diagnostic performance of the seven methylated gene markers in 29 discovery cyst fluid samples. The six markers with a diagnostic accuracy (AUC) of > 0.85 were carried forward into the validation set analysis of 154 independent samples (Fig. 1). Patient information including demographics, clinical symptoms, preoperative imaging findings, and cyst fluid cytology and CEA values were obtained from hospital records (summarized in Table 1). Cyst fluid consistency (serous or mucinous) was judged by cyst fluid viscosity during aspiration and aliquoting. The diagnostic accuracy of cyst fluid CEA was evaluated using the cutoff of 192 ng/mL as previously described (46). Surgical resection was performed for clinical indications that evolved during the study period as new guidelines emerged.

Pancreatic pathologists at each center (R.H. Hruban and S.-M. Hong) reviewed the pathologic features of each surgically resected neoplasms. The collection and processing of surgical cyst fluid samples have been described previously (33). For cases with high-grade dysplasia, the extent of dysplasia was determined from the pathology report. All cyst fluid experiments and data analyses were conducted without prior knowledge of the cyst pathology or other diagnostic information. Excess normal pancreas tissues from patients who had undergone pancreatic resection for nonmalignant disease were used to determine the DNA methylation levels of our gene marker panel in normal pancreas. All elements of this study were approved by the institutional review boards of Johns Hopkins Medical Institutions and Asan Medical Center and written informed consent was obtained from all patients.

Marker gene selection

We previously reported the results of a study aimed at identifying differentially methylated genes in IPMN versus normal pancreas (35). This previous study utilized methylated CpG island amplification and Agilent 244K Human promoter chip-on-chip microarrays and identified 245 commonly hypermethylated genes in IPMN with HGD including *SOX17*, *BNIP3*, *PTCHD2*, *SLIT2*, *EYA4*, and *SFRP1* (35). In prior studies, we have also identified other differentially methylated genes in pancreatic cancers versus normal pancreatic duct, including *FOXE1* (40, 47, 48).

Cell culture

Human pancreatic cancer cell lines MIA PaCa-2, BxPC-3, Hs 766T, PANC-1, AsPC-1, CFPAC-1, Capan-1, Capan-2, SU.86.86, Panc 05.04, and Panc 08.13 were obtained from the American Type Culture Collection. 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 recently authenticated using genetic markers by the Johns Hopkins Genetics Core facility. HPDE was authenticated by testing it for genetic markers of HPV, E6 and E7.

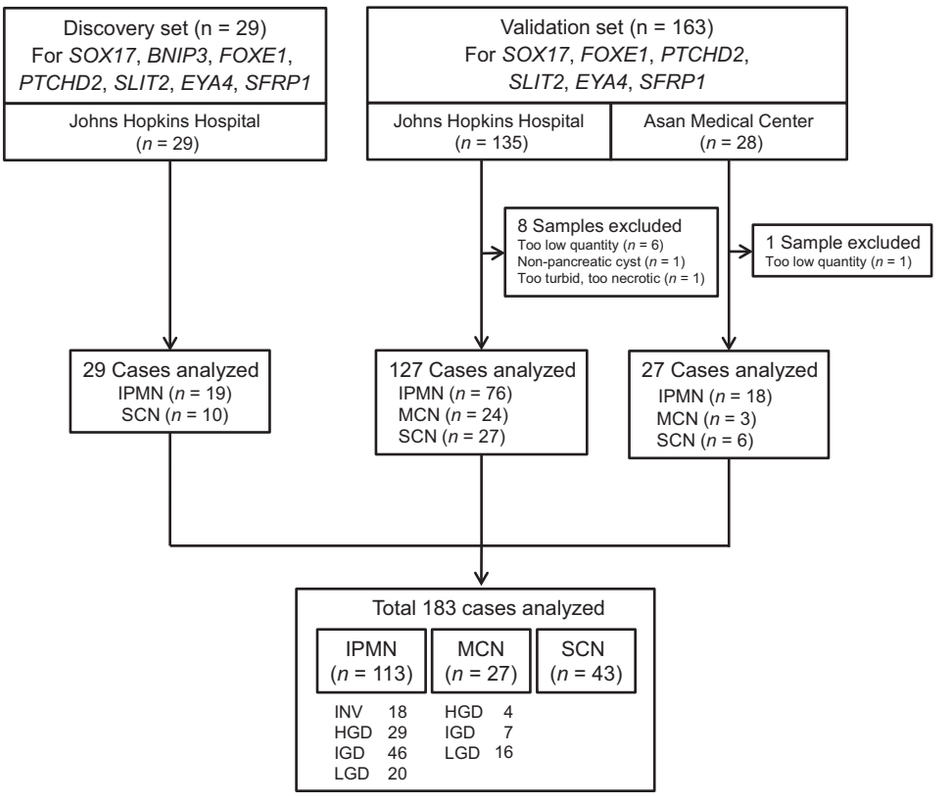

Figure 1.
An overview of study workflow and sample selection process. IPMN, intraductal papillary mucinous neoplasm; SCN, serous cystic neoplasm; MCN, mucinous cystic neoplasm; INV, invasive cancer; LGD, low-grade dysplasia; IGD, intermediate-grade dysplasia; HGD, high-grade dysplasia.

All cell lines were cultured as recommended as previously described (33).

DNA isolation and bisulfite conversion

DNA was extracted from 10 to 600 μL of surgically aspirated cyst fluid using DNeasy blood and tissue kit (QIAGEN). For cyst fluid samples with mucus, mucin was dissolved by increasing the length of proteinase K digestion. Extracted DNA was eluted with 50 μL of EB buffer (10 mmol/L Tris-Cl, pH 8.5) and quantified using the Quantifiler Human DNA Quantification kit (Applied Biosystems). Using 20 to 200 ng of cyst fluid DNA and 500 ng of DNA from pancreatic cancer cell lines and normal pancreas tissues, bisulfite conversion was performed using the Zymo EZ DNA Methylation Kit (Zymo Research) according to the manufacturer's protocol. Bisulfite-modified DNA was eluted in 20 μL of EB buffer and stored at −80 °C until needed.

Methylation-specific PCR (MSP)

For each locus, MSP primer pairs were designed to differentiate between methylated and unmethylated DNA. Primer sequences and annealing temperatures are provided in Supplementary Table S1. PCR was carried out with Platinum *Taq* DNA polymerase (Invitrogen) in 20 μL. PCR assays included nontemplate control wells, positive control (100% methylated DNA, EpiTect Control DNA, QIAGEN), and negative control (100% unmethylated DNA, EpiTect Control DNA, QIAGEN). One microliter of bisulfite-modified DNA was used for each MSP assay. After amplification, 5 μL of each PCR product was electrophoresed on 3% agarose gels, stained with ethidium bromide, and visualized under UV illumination in ChemiDoc Touch Imaging system (Bio-Rad).

Bisulfite sequencing

DNA sequencing of cloned bisulfite sequencing was performed to confirm the methylation status of 5′ CpG sites amplified by MSP. Purified PCR products (using QIA quick Gel Extraction Kit, QIAGEN) were cloned into pCR 2.1-TOPO TA vector using the TOPO TA Cloning Kit (ThermoFisher Scientific) following the manufacturer's instructions. Twelve clones were chosen randomly for plasmid DNA extraction with a QIAprep Spin Mini kit (QIAGEN) and sequenced using M13 F and R primer. Sequence analysis was carried out at The Johns Hopkins Synthesis and Sequencing Facility using automated DNA sequencers (Applied Biosystems).

5-Aza-2′-deoxycytidine and trichostatin A treatment

Cells were seeded at a density of 2×10^6 cells/mL in 100-mm dishes and grown. After 24 hours, cells were treated with 5-Aza-2′-deoxycytidine (5-Aza-dC, Sigma-Aldrich) and trichostatin A (TSA, Sigma-Aldrich) either alone or in combination. Cells were exposed to 5-Aza-dC (10 μmol/L) for 4 days (culture medium with 5-Aza-dC was exchanged in day 3) and TSA (1 μmol/L) for 24 hours. For combined treatment, cells were cultured with 5-Aza-dC for 3 days, then the culture medium was replaced with 5-Aza-dC plus TSA incubated for another 24 hours and collected for analysis.

RT-PCR

Total RNA was isolated from cell line pellets using a miRNeasy mini kit (QIAGEN) according to the manufacturer's instructions. For each RT-PCR, 1 μg of total RNA was converted to cDNA using SuperScript VILO cDNA Synthesis Kit (Invitrogen) according to the manufacturer's instructions. Quantitative

Table 1. Patient and cyst characteristics

Characteristics	Total ($n = 183$)	IPMN ($n = 113$)	MCN ($n = 27$)	SCN ($n = 43$)
Male/female (*n*)	69/114	57/56	0/27	12/31
Age (median, range), year	67 (23–88)	69 (43–88)	51 (23–68)	59 (29–85)
Institution				
JHH/AMC	156/27	95/18	24/3	37/6
Race/ethnicity (*n*)				
AA/C/Asian/Others, unknown	13/124/31/15	5/82/20/6	4/16/4/3	4/26/7/6
Symptoms (*n*)				
Abdominal pain	37	19	8	10
Pancreatitis	12	10	1	1
Jaundice	3	3	0	0
Cyst location (*n*)				
Head and uncinate/body and tail	82/101	66/47	1/26	15/28
Cyst size, median (range), cm	3.0 (0.6–25.0)	2.9 (0.6–10.0)	4.0 (1.0–25.0)	4.4 (1.5–13.5)
Mural nodule (*n*)[a]				
Absent/present	137/46	79/34	24/3	34/9
Communication with MPD (*n*)[a]				
Absent/present	88/95	53/60	1/26	34/9
Dilatation of MPD $\geq$10 mm (*n*)[a]				
Absent/present	159/24	89/24	27/0	43/0
Dilatation of MPD $\geq$5 mm (*n*)[a]				
Absent/present	135/48	68/45	27/0	40/3
Cyst fluid appearance (*n*)				
Serous/mucinous	144/39	79/34	22/5	43/0
CT/MRI findings				
High-risk stigmata	38	38	0	0
Worrisome features	116	58	22	36
No concerning features	29	17	5	7
Original cyst volume (median, range), μL	200 (10–600)	200 (10–450)	250 (150–600)	250 (20–450)
EUS-FNA ($n = 63$), *n*				
Non-diagnostic	16	8	3	5
Benign/Atypia/Cancer	26/10/11	16/8/11	4/1/0	6/1/0
Cyst fluid CEA ($n = 34$), *n*				
<192 ng/mL/$\geq$192 ng/mL	22/12	10/8	4/4	8/0
Operative procedure (*n*)				
PD/DP/TP/MP	82/96/4/1	66/42/4/1	1/26/0/0	15/28/0/0
Morphological duct type (*n*) [a]				
Main duct/mixed/branch duct		24/21/68		
Grade of dysplasia (*n*)				
LGD/IGD/HGD/INV		20/46/29/18	16/7/4/0	

Abbreviations: AA, African American; AMC, Asan Medical Center; C, Caucasian; CEA, carcinoembryonic antigen; DP, distal pancreatectomy; EUS-FNA, endoscopic ultrasound-fine needle aspiration; HGD, high-grade dysplasia; IGD, intermediate-grade dysplasia; INV, invasive cancer; IPMN, intraductal papillary mucinous neoplasm; JHH, Johns Hopkins Hospital; LGD, low-grade dysplasia; MCN, mucinous cystic neoplasm; MP, middle pancreatectomy; MPD, main pancreatic duct; PD, pancreaticoduodenectomy; SCN, serous cystic neoplasm; TP, total pancreatectomy.
[a]Determined by CT/MRI.

PCR was performed using Power SYBR Green PCR master mix (Life Technologies) using a 7900HT thermocycler (Applied Biosystems). PCR conditions were as follows: initial denaturation at 95 °C for 10 minutes followed by 40 cycles of denaturation at 95 °C for 10 seconds, and annealing at 60°C for 60 seconds. RQ value (relative level of mRNA expression) was calculated by the comparative $2^{-\Delta\Delta Ct}$ method with *GAPDH* as endogenous controls with SDS 2.4 and RQ Manager 1.2 (Applied Biosystems). Primer were designed to span multiple exons except for *FOXE1*. All primer sequences and PCR conditions are listed in Supplementary Table S1.

Methylation specific droplet-digital PCR (dd-QMSP)

We performed methylation-specific droplet-digital PCR (dd-QMSP) using a gene-specific probe with a 5′ reporter dye (FAM) for the marker genes and *ACTB* as the reference (HEX). To reduce background noise and increase assay sensitivity, all probes were quenched with double quenchers, a 3′ Iowa Black dark quencher (IABkFQ) combined with an internal ZEN quencher. All probes were synthesized by IDT (Integrated DNA Technologies, Inc.). The dd-QMSP reaction mix consisted of 2 × ddPCR Supermix for Probes (no-dUTP), 750 nmol/L of each primer and 250 nmol/L of the corresponding probe in a final volume of 20 μL. Digital-droplet PCR was carried out as previously described (33). Thermocycling conditions were as follows: 95°C for 5 minutes (1 cycle), followed by 40 cycles of 95 °C for 15 seconds, 60 °C for 1 minutes (ramp rate: 2.5 °C/seconds). Analysis was performed with QuantaSoft 1.7.4 Analysis software (Bio-Rad) and droplet concentrations (copies/μL; PCR scale) were computed using Poisson statistics. Only tests providing more than 10,000 droplets were used for analysis. In the present study, to best reflect the nature of original cyst fluid, the number of methylated target molecules per 20 μL PCR reaction was extrapolated to calculate molecules per microliter of original cyst fluid using the following equation:

Molecules/μL of cyst fluid = $(C_{20}/Qp) \times (Qc/Vc)$
C_{20}: copies/20 μL of PCR scale (data derived from Quantasoft)
Qp: Quantity of DNA applied to PCR reaction (20 μL scale)
Qc: Quantity of DNA extracted from cyst fluid
Vc: Volume of cyst fluid used for DNA extraction

We also calculated the methylated molecules per nanogram of extracted cyst fluid DNA and the ratio of methylated target molecules and *ACTB* molecules.

The threshold for calling positive and negative droplets was determined by visual inspection of serial dilutions of positive control DNA. Based on these results, one positive droplet was considered a "true positive" (Supplementary Fig. S1). A positive droplet was set as 6,000 fluorescence units for *SOX17*, 5,000 for *BNIP3*, 3,500 for *ACTB*, and 4,000 for *FOXE1*, *PTCHD2*, *SLIT2*, *EYA4*, and *SFRP1* (Supplementary Fig. S1). No droplets were detected above this threshold in unmethylated template and nontemplate samples.

The correlation between increasing concentrations of bisulfite-modified input DNA and quantification by dd-QMSP is shown in Supplementary Fig. S2A and S2B. Interassay reproducibility of replicates performed on different days is provided in Supplementary Fig. S2C and S2D.

Next-generation sequencing (NGS)

We performed targeted NGS to detect *KRAS* (codons 12 and 13) and *GNAS* (codon 201) mutations using the Ion Torrent PGM (Life Technologies) platform (11, 49). Cyst fluid DNA was assayed in duplicate. Amplicon libraries were generated from 2 ng under the thermocycling conditions: 94°C for 3 minutes (1 cycle), followed by 40 cycles of 94°C for 30 seconds, 58°C for 30 seconds, 68°C for 1 minutes. The libraries were cleaned, quantified, loaded into a OneTouch2 for emulsion PCR, enriched, and sequenced on the Ion Torrent PGM using 316v2 Chip according to the manufacturer's protocols. DNA samples with low or absent mutant allele fractions (<5.0%) were also analyzed for *KRAS* and *GNAS* mutations using droplet-digital PCR as described previously (11).

Statistical analysis

The nonparametric Mann–Whitney *U* test was used to compare continuous variables. The χ^2 test was used to compare categorical variables. Correlations between variables were assessed by scatter plot and R^2 value. The diagnostic accuracy 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 left upper corner of the curve. A multivariate analysis using the logistic regression model was performed, including potential confounding factors. All statistical analysis was performed using the JMP Pro 12.2.0 statistical software (SAS Institute Inc.) and GraphPad Prism V7.0 (GraphPad Software). A *P* value of less than 0.05 was considered to indicate statistical significance.

Results

Biomarker assay evaluation using pancreatic cell lines and tissues

We selected 7 genes (*SOX17*, *PTCHD2*, *BNIP3*, *FOXE1*, *SLIT2*, *EYA4*, and *SFRP1*) from our previously published list of differentially methylated genes in IPMN (35) and pancreatic cancer tissues (40, 47). Prior to cyst fluid analysis, MSP was performed to detect differential methylation of these 7 genes in pancreatic cancer cell lines compared with normal pancreas samples. As expected, pancreatic cancer cell lines had a high prevalence of methylation for all 7 genes compared with the nonneoplastic pancreatic ductal epithelial cell line, HPDE, and normal pancreatic tissues (Supplementary Fig. S3). We also performed bisulfite sequencing of 40 CpG sites within the *SOX17* CpG island which included CpGs amplified by dd-QMSP (Supplementary Fig. S4A) in pancreatic cancer cell lines, HPDE, and three representative normal pancreas tissues (Supplementary Fig. S4B). This analysis found concordance between the DNA methylation determined by MSP and bisulfite sequencing. We then performed the dd-QMSP using the pancreatic cell lines (Supplementary Fig. S4C) and found a close correlation between methylation levels quantified by dd-QMSP and bisulfite sequencing ($R^2 = 0.52$, $P = 0.008$; Supplementary Fig. S4D).

We examined the expression levels of these genes and the relationship of expression to DNA methylation. As expected, expression for all 7 genes was low or absent in pancreatic cancer cell lines with promoter hypermethylation (Supplementary Fig. S5). The 5-Aza-dC and/or TSA treatment of pancreatic cancer cell lines (MIA PaCa-2, AsPC-1, and Capan-1) confirmed epigenetic silencing of gene expression (Supplementary Fig. S6).

Diagnostic performance of methylated markers in pancreatic cyst fluid

We developed dd-QMSP assays for each methylated DNA marker assay and compared the diagnostic performance of the 7 markers using 29 discovery set cases. Six of the 7 markers (all but *BNIP3)* had very good ability to discriminate between cases with invasive cancer/high-grade dysplasia versus all other cysts (AUC values higher than 0.85; Supplementary Fig. S7). The AUC value for the *BNIP3* assay in the discovery set was 0.781.

We next evaluated the best six markers in an independent validation set ($n = 154$) that included cyst fluids from IPMN, MCN, and SCN. A summary of the patient and cyst characteristics are found in Supplementary Table S2. The distribution of the neoplastic grade of the pancreatic cysts was similar in the discovery and validation sets. A representative example of results of the dd-QMSP assay is shown in Supplementary Fig. S8. The diagnostic performance of 4 of the 6 markers (*SOX17*, *SLIT2*, *EYA4*, *SFRP1*), as measured by AUC was very similar in the discovery and validation sets while the AUCs of *FOXE1* and *PTCHD2* were a little lower in the validation set (Supplementary Fig. S7). We next merged the discovery and validation set results (Fig. 2). For each marker, the number of methylated target molecules was higher in cyst fluids from IPMNs with high-grade compared with intermediate-grade or low-grade dysplasia. For 4 of the 6 genes *(PTCHD2*, *SLIT2*, *EYA4*, and *SFRP1)*, there was also a significantly higher number of methylated target molecules among cases with IPMN and intermediate-grade compared with those with low-grade dysplasia. The level of methylated DNA did not significantly differ in cyst fluids from IPMN with high-grade dysplasia compared with IPMN with associated invasive cancer (Fig. 2). To determine if the extent of high-grade dysplasia influenced the results, we stratified IPMNs with high-grade dysplasia (without invasive cancer) into those with focal versus extensive dysplasia. In addition, we also subdivided IPMNs with invasive cancer by their extent of invasion (depth of invasion $>$ or $\leq$ 1.0 cm). There was no evidence of any difference in methylation level by extent of high-grade dysplasia or extent of invasion (Supplementary Fig. S9).

We examined the diagnostic performance of the marker panel for predicting grade of dysplasia both in the whole study population and for just IPMN cases as the question of grade of dysplasia is most relevant for this population. Methylated *SOX17* had the highest diagnostic performance of the 6 markers

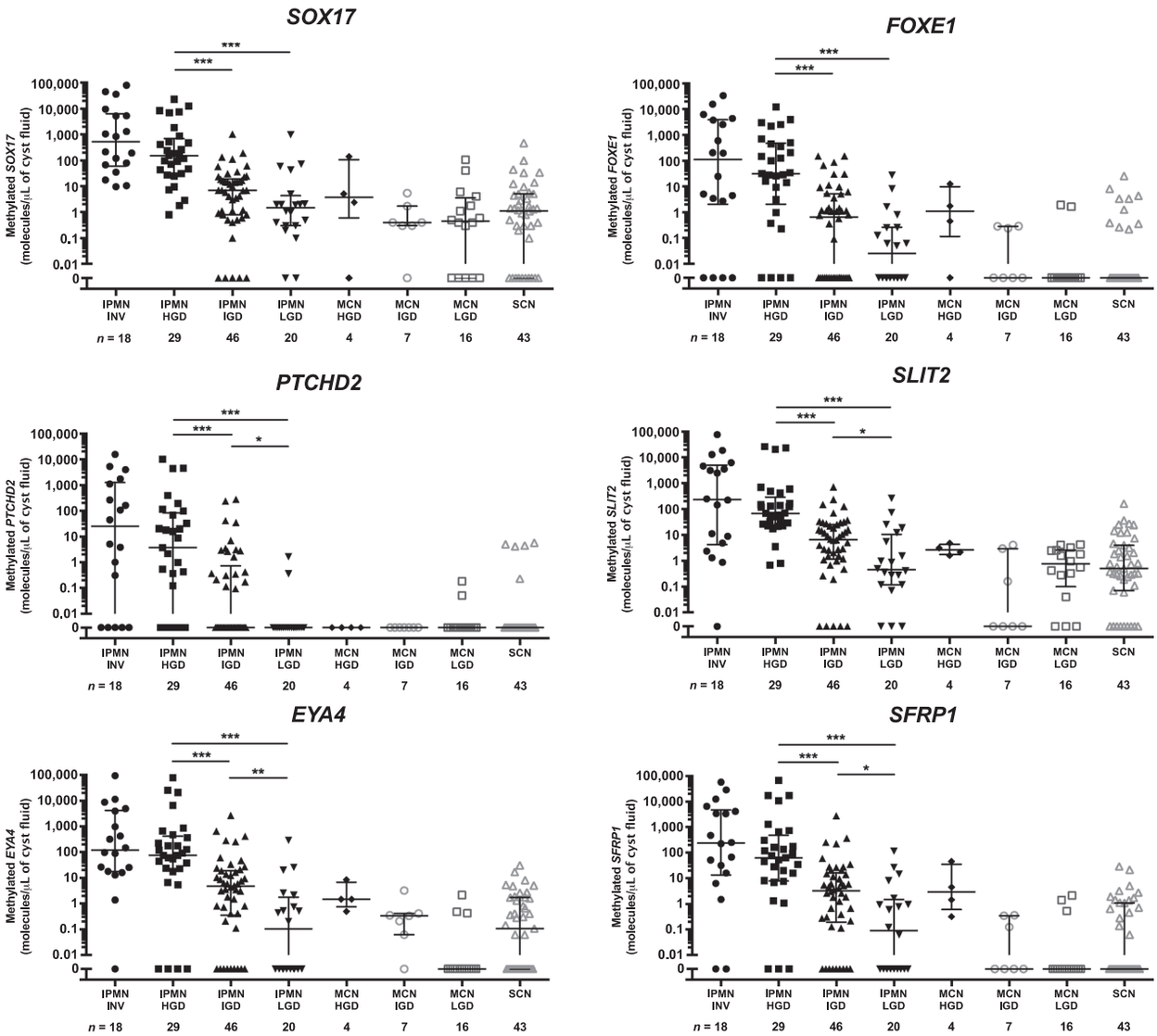

Figure 2.

Quantification of methylated target molecules per μL of cyst fluid samples among 183 merged samples from the discovery and validation sets. The longer horizontal bar represents the median value and shorter ones represent values of the 75th and 25th percentiles, respectively. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

with an AUC for predicting invasive cancer/high-grade dysplasia versus lower grades of dysplasia or no dysplasia (AUC 0.888; sensitivity, 78.4%; specificity, 85.6%; accuracy 83.6%, cutoff $\geq$ 25 methylated target molecules/μL cyst fluid (Table 2). Similar results were found when we limited the comparison to the 113 cases of IPMN, (methylated *SOX17*; diagnostic sensitivity, 83.0%; specificity, 81.8%; AUC, 0.883; Table 2). Methylated *EYA4* (cutoff: 12 methylated target molecules/μL of cyst fluid) and *SFRP1* (cutoff: 6 methylated molecules/μL of cyst fluid) also had high diagnostic accuracy in the whole set, 83.1% and 82.0%, respectively (Table 2), but a little lower in the IPMN set, 77.9% and 75.2%, respectively. Similar methylated DNA levels were observed for most markers in the 27 cases with MCN but with only four MCN cases with high-grade dysplasia statistical power was limited (Fig. 2).

The number of methylated target molecules detected in a cyst is a function of cyst fluid DNA concentrations, which are somewhat higher in cysts with invasive cancer or high-grade dysplasia (Supplementary Fig. S10). We therefore examined marker performance if the number of target molecules detected was divided by cyst fluid DNA concentrations, or bisulfite-modified DNA concentrations (as measured by amplified bisulfite-modified *ACTB* molecules). As shown in Supplementary Fig. S11, there was very little difference in marker performance when marker levels were normalized by cyst fluid volume, total cyst fluid DNA, or amplified bisulfite-modified cyst fluid DNA.

We next examined how well diagnostic accuracy could be improved when methylated DNA markers were combined. For all the biomarkers, higher levels of methylated DNA were more

Table 2. Diagnostic performance for predicting of malignancy in pancreatic cyst fluid

Marker gene	AUC	Cutoff[a]	Sensitivity (%, 95% CI)	Specificity (%, 95% CI)	Accuracy (%)	LR+	LR−
All cases ($n = 183$)							
SOX17	0.888	≥25	78.4 (64.7–88.7)	85.6 (78.4–91.1)	83.6	5.5	0.3
FOXE1	0.828	≥2	74.5 (60.4–85.7)	84.1 (76.7–89.9)	81.4	4.7	0.3
PTCHD2	0.768	≥1	62.8 (48.1–75.9)	87.1 (80.2–92.3)	80.3	4.9	0.4
SLIT2	0.845	≥17	72.6 (58.3–84.1)	82.6 (75.0–88.6)	79.8	4.2	0.3
EYA4	0.846	>12	76.5 (62.5–87.2)	85.6 (78.4–91.1)	83.1	5.3	0.3
SFRP1	0.853	>6	78.4 (64.7–88.7)	83.3 (75.9–89.3)	82.0	4.7	0.3
IPMN cases ($n = 113$)							
SOX17	0.883	≥25	83.0 (69.2–92.4)	81.8 (70.4–90.2)	82.3	4.6	0.2
FOXE1	0.798	≥ 2	76.6 (62.0–87.7)	77.3 (65.3–86.7)	77.0	3.4	0.3
PTCHD2	0.766	≥ 1	68.1 (52.9–80.9)	80.3 (68.7–89.1)	75.2	3.5	0.4
SLIT2	0.816	≥ 17	78.7 (64.3–89.3)	72.7 (60.4–83.0)	75.2	2.9	0.3
EYA4	0.813	> 12	83.0 (69.2–92.4)	74.2 (62.0–84.2)	77.9	3.2	0.2
SFRP1	0.816	> 6	83.0 (69.2–92.4)	69.7 (57.2–80.4)	75.2	2.7	0.2

Abbreviations: AUC, area under the curve; CI, confidence interval; LR+, positive likelihood ratio; LR−, negative likelihood ratio.
[a]The values of methylated target molecules in pancreatic cyst fluid (molecules/μL of cyst fluid).

specific but less sensitive at distinguishing cyst fluids from cases with high-grade dysplasia ± an invasive cancer from cases with lower grades of dysplasia. Therefore, we used higher (more specific) levels of methylated DNA as cutoffs to evaluate marker combinations. Several two-gene marker combinations were modestly superior to *SOX17* alone (the best single marker) including those combining *SOX17* with *FOXE1* or *EYA4* (accuracy 86.3% or 85.8%, respectively; Table 3). The accuracy of three-gene combinations was not significantly better than any two-gene combination. The highest diagnostic accuracy among the combined marker panels in this series was achieved using four genes (which was only slightly higher than the best two gene combinations), *FOXE1* (cutoff: 125.0 methylated molecules/μL of cyst fluid), *SLIT2* (130.0), *EYA4* (23.7), and *SFRP1* (31.9), which had an 88.0% accuracy (84.3% sensitivity, 89.4% specificity; Table 3).

Because aging influences the propensity for DNA methylation, we examined the correlation between patient age and cyst fluid methylated DNA levels. There was no correlation between patient age and the number of methylated target molecules among patients with IPMN (results shown for cases with intermediate-grade dysplasia; Supplementary Fig. S12A). There was also no correlation between methylated markers and age among patients with SCN apart from *EYA4* (Supplementary Fig. S12B). To determine if ethnicity was an important factor in our results, we compared methylation results between the two institutions (Johns Hopkins Hospital and Asan Medical Center) but did not find any evidence for differences in marker behavior between the two centers (Supplementary Fig. S13).

To evaluate the diagnostic accuracy of the cyst fluid markers in relation to clinical criteria, we classified patient cysts by their preoperative imaging features into those with "high-risk stigmata,""worrisome features," and "no concerning features" as defined by the 2012 International Consensus Guidelines (7). Among the 183 cases, 27 (71%) of 38 with "high-risk stigmata" and 23 (20%) of 116 with "worrisome features" had invasive

Table 3. Diagnostic performance of combining pancreatic cyst fluid markers

Combination (cutoff[a])	Sensitivity (%)	Specificity (%)	Accuracy (%)	LR+	LR−
Two-gene combination					
SOX17 (59.0), *FOXE1* (12.0)	78.4	89.4	86.3	7.39	0.24
SOX17 (60.0), *PTCHD2* (3.7)	76.5	89.4	85.8	7.21	0.26
SOX17 (24.7), *SLIT2* (66.0)	80.4	84.1	83.1	5.05	0.23
SOX17 (140.0), *EYA4* (23.7)	78.4	88.6	85.8	6.90	0.24
SOX17 (140.0), *SFRP1* (20.0)	74.5	87.9	84.2	6.15	0.29
FOXE1 (12.0), *PTCHD2* (0.97)	72.5	85.6	82.0	5.04	0.32
FOXE1 (12.0), *SLIT2* (49.0)	74.5	90.2	85.8	7.57	0.28
FOXE1 (12.0), *EYA4* (12.5)	80.4	84.8	83.6	5.31	0.23
FOXE1 (12.0), *SFRP1* (31.0)	74.5	89.4	85.2	7.03	0.29
PTCHD2 (3.4), SLIT2 (16.8)	74.5	81.1	79.2	3.93	0.31
PTCHD2 (8.8), *EYA4* (12.5)	78.4	84.8	83.1	5.18	0.25
PTCHD2 (0.53), *SFRP1* (20.0)	78.4	82.6	81.4	4.50	0.26
SLIT2 (130.0), *EYA4* (25.2)	72.5	90.2	85.2	7.37	0.30
SLIT2 (73.0), *SFRP1* (16.0)	76.5	88.6	85.2	6.73	0.27
EYA4 (26.0), *SFRP1* (16.0)	78.4	86.4	84.2	5.75	0.25
Three-gene combination					
SLIT2 (22.9), *EYA4* (22.9), *SFRP1* (31.9)	84.3	82.6	83.1	4.84	0.19
SOX17 (78.0), *EYA4* (22.9), *SFRP1* (31.9)	88.2	85.6	86.3	6.13	0.14
Four-gene combination					
SOX17 (78.0), *FOXE1* (203.0), *EYA4* (25.2), *SFRP1* (31.9)	86.3	86.4	86.3	6.33	0.16
FOXE1 (125.0), *SLIT2* (130.0), *EYA4* (23.7), *SFRP1* (31.9)	84.3	89.4	88.0	7.95	0.18
Five-gene combination					
SOX17 (78.0), *FOXE1* (203.0), *SLIT2* (66.0), *EYA4* (23.7), *SFRP1* (31.9)	88.2	86.4	86.9	6.47	0.14

Abbreviations: LR+, positive likelihood ratio; LR−, negative likelihood ratio.
[a]The values of methylated target molecules in pancreatic cyst fluid (molecules/μL of cyst fluid).

Table 4. Diagnostic performance of pancreatic cyst fluid methylated DNA biomarkers among cysts classified by their clinical features

Marker gene	Cutoff[a]	Sensitivity (%, 95% CI)	Specificity (%, 95% CI)	Accuracy (%)	LR+
Worrisome features ($n = 116$)					
SOX17	≥25	78.3 (56.3–92.5)	85.7 (77.2–92.0)	84.5	5.48
FOXE1	≥2	65.2 (42.7–83.6)	84.7 (76.0–91.2)	80.2	4.26
PTCHD2	≥1	52.2 (30.6–73.2)	89.8 (82.0–95.0)	81.9	5.11
SLIT2	≥17	73.9 (51.6–89.8)	83.7 (74.8–90.4)	81.0	4.53
EYA4	>12	60.9 (38.5–80.3)	86.7 (78.4–92.7)	81.0	4.59
SFRP1	>6	73.9 (51.6–89.8)	85.7 (77.2–92.0)	82.8	5.17
High-risk stigmata ($n = 38$)					
SOX17	≥25	77.8 (57.7–91.4)	63.6 (30.8–89.1)	73.7	2.14
FOXE1	≥2	81.5 (61.9–93.7)	54.6 (23.4–83.4)	73.7	1.79
PTCHD2	≥1	74.1 (53.7–88.9)	72.7 (39.0–94.0)	73.7	2.72
SLIT2	≥17	70.4 (49.8–86.3)	72.7 (39.0–94.0)	71.1	2.58
EYA4	>12	88.9 (70.8–97.7)	54.6 (23.4–83.4)	79.0	1.96
SFRP1	>6	81.5 (61.9–93.7)	45.5 (16.8–76.6)	71.1	1.49

Abbreviations: AUC, area under the curve; CI, confidence interval; LR+, positive likelihood ratio.
[a]The values of methylated target molecules in pancreatic cyst fluid (molecules/μL of cyst fluid).

cancer or high-grade dysplasia in their resection specimen (Supplementary Fig. S14). *SOX17* and *SFRP1* had somewhat higher ability to discriminate the high-grade dysplasia/invasive cancer from cystic lesions of lower neoplastic grade (as measured by AUC values) among cases classified as having "worrisome features" than those classified as having "high-risk stigmata" (Table 4).

We investigated if the detection of a positive cyst fluid methylation test was an independent predictor of grade of dysplasia when results were adjusted for other clinical factors (age, gender, cyst fluid appearance, cyst fluid DNA quantity, cyst size, cyst location, MPD dilatation, MPD communication, and mural nodule) used to evaluate patients (7, 8). Multivariate analysis revealed that cyst fluid methylation remained an independent predictor of the grade of dysplasia high-grade dysplasia/invasive cancer versus lower grades of dysplasia (Supplementary Table S3). A positive *SOX17* methylation test had the highest adjusted odds ratio for predicting grade of dysplasia (9.76, 95% confidence interval [CI], 3.74–27.67), followed by *SFRP1* (adjusted odds ratio, 8.75; 95% CI, 3.37–24.42; Supplementary Table S3).

We also examined if methylated DNA markers combined with endoscopic ultrasound guided fine needle aspiration (EUS-FNA) cytology could improve the diagnostic accuracy for predicting cancer or high-grade dysplasia over cytology alone. Among cases that had FNAs sent for cytology, positive cytology (cancer cells) was 47.8% sensitive and 100% specific for the presence of high-grade dysplasia or invasive cancer. Cyst fluid methylated *SOX17* DNA combined with cytology improved the diagnostic accuracy for prediction of neoplastic grade over cytology alone (87.2% vs. 74.5%; Supplementary Table S4).

We also determined to what extent the methylated DNA markers predicted cyst type. Because the methylated DNA markers were good predictors of grade of dysplasia and most of the cases with high-grade dysplasia/cancer were IPMNs, it is not surprising that in our series, positive methylated DNA results were more common in cases with IPMN than with MCN or SCN (Supplementary Fig. S15). To account for this, we compared cases with the same neoplastic grade (Supplementary Table S5) and found for several genes (*SOX17*, *PTCHD2*, *SLIT2*, and *EYA*); cyst fluids from IPMNs with high-grade dysplasia had significantly higher methylated DNA levels than MCN cyst fluids with high-grade dysplasia.

We also determined if there was any association between cyst fluid methylated DNA and cyst fluid mutations. We stratified cyst fluids according to the most commonly mutated genes (*KRAS* and *GNAS*). Sixty-six cyst fluids, (64.0%) harbored mutant *KRAS* DNA and 59 cyst fluids, (57.3%) harbored mutant *GNAS* DNA (Supplementary Fig. S16A). However, there was no association between cyst fluid mutation status and the number of methylated genes (Supplementary Fig. S16B). We calculated a methylation score for each cyst fluid (defined as the average number of methylated genes above the cutoff for predicting grade of dysplasia). Methylation scores were significantly higher in cysts with HGD/invasive cancer (Supplementary Fig. S16C), but there was no association between the cyst fluid methylation score and the presence or absence of mutated *KRAS* and/or *GNAS* (Supplementary Fig. S16D). Similarly, there was no evidence that cyst fluid *KRAS* or *GNAS* mutation status affected the diagnostic performance of individual methylated genes for predicting a cyst's grade of dysplasia (Supplementary Fig. S17).

Discussion

In the present study, we found quantifying levels of cyst fluid methylated DNA using digital-droplet PCR technology (dd-QMSP) could reliably predict whether cysts had high-grade dysplasia/ invasive cancer or a lower grade of dysplasia. Combining methylated DNA markers with cytology also yielded higher diagnostic accuracy for predicting the grade of dysplasia than cytology alone. For patients with suspected IPMNs, reliable prediction of the grade of dysplasia is usually the most important question clinicians need to answer when evaluating patients. Many patients are followed who have pancreatic cysts with worrisome features that could be followed rather than undergo resection. A molecular evaluation of their pancreatic cyst could help predict the malignant potential of the cyst before evidence of malignancy is detected by imaging. Most pancreatic cysts have low malignant potential and do not require cyst fluid analysis for patient management, but many pancreatic cysts pose management dilemmas and better characterization of these cysts with accurate and reliable cyst fluid markers should help clinicians reduce diagnostic uncertainty (24, 26, 27, 33). Our best aberrantly methylated marker panel had similar diagnostic accuracy (88.0%) to our recently reported telomerase activity measurement (88.2%;

ref. 33), and to a next-generation sequencing-based mutated gene/chromosomal marker panel (24), for predicting the histologic grade of dysplasia of IPMNs (HGD/invasive cancer vs. lower grades of dysplasia). Further investigation is needed to determine the optimal combination of these cyst fluid markers for predicting the grade of dysplasia and type of pancreatic cyst. While cyst fluid mutations (especially *KRAS*, *GNAS*, *VHL*, *TP53*, *SMAD4*, *RNF43* and *CDKN2A*) can accurately classify a pancreatic cyst, additional markers may be needed to predict the grade of dysplasia of a pancreatic cyst. It should be noted that our marker panel was developed from methylated genes identified as differentially methylated in high-grade IPMNs. In keeping with this, we found that markers such as methylated *SOX17* and *EYA4*, originally identified as elevated in IPMNs with high-grade dysplasia, were not elevated in MCN cyst fluids with high-grade dysplasia. In keeping with this, methylated *SOX17* achieved similarly high diagnostic accuracy as a marker of grade of dysplasia in both the IPMN group and the whole study population.

In the present study, all enrolled patients underwent surgical resection and had defined histology. While having the "gold standard" of histology is a strength of the study, the cyst fluids were obtained by fine-needle aspiration at the surgical pathology bench. One limitation of our study is the lack of available EUS-guided samples so that analysis of paired fluids collected from preoperative EUS-guided aspirates and surgical resection specimens could be performed to further evaluate the diagnostic utility of the cyst fluid markers reported. Prospective studies are needed that evaluate the clinical utility of using pancreatic cyst fluid markers along with pancreatic imaging and clinical criteria to help decide which patients need surgery, surveillance or neither. Such studies could also evaluate the additive diagnostic value of comparing the results of serially collected cyst fluid samples from same individual.

In conclusion, quantification of aberrant methylated DNA in pancreatic cyst fluid samples has potential as a predictive biomarker for the presence of high-grade dysplasia/invasive carcinoma within a cyst, and one with high sensitivity and specificity. Further study with a large cohort, including matched endoscopically and surgically collected cyst fluid samples is warranted to investigate the utility of using methylated DNA biomarkers in the evaluation and risk stratification of patients undergoing pancreatic screening and surveillance.

Disclosure of Potential Conflicts of Interest

A. Lennon is a consultant/advisory board member for Novo Nordisc and Olympus. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: T. Hata, M.J. Weiss, C.L. Wolfgang, A.M. Lennon, R.H. Hruban, M.G. Goggins
Development of methodology: T. Hata, K. Tamura, M.G. Goggins
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Hata, M. Dal Molin, S.-M. Hong, M. Suenaga, J. Yu, H. Sedogawa, A.M. Lennon, R.H. Hruban, M.G. Goggins
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T. Hata, K. Tamura, J. Yu, C.L. Wolfgang, M.G. Goggins
Writing, review, and/or revision of the manuscript: T. Hata, M. Dal Molin, J. Yu, M.J. Weiss, C.L. Wolfgang, A.M. Lennon, R.H. Hruban, M.G. Goggins
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Dal Molin, J. Yu, M.G. Goggins
Study supervision: M.G. Goggins
Other (next-generation sequencing–related experiment support): K. Tamura

Grant Support

This work was supported by Susan Wojcicki and Dennis Troper, NIH grants (CA62924, R01CA176828, and U01 CA210170), and the Rolfe Pancreatic Cancer Foundation. M. Goggins is the Sol Goldman Professor of Pancreatic Cancer Research.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received September 6, 2016; revised December 22, 2016; accepted January 17, 2017; published OnlineFirst February 1, 2017.

References

1. Stark A, Donahue TR, Reber HA, Hines OJ. Pancreatic cyst disease: a review. JAMA 2016;315:1882–93.
2. Lennon AM, Wolfgang C. Cystic neoplasms of the pancreas. J Gastrointest Surg 2013;17:645–53.
3. Farrell JJ. Prevalence, Diagnosis and management of pancreatic cystic neoplasms: current status and future directions. Gut Liver 2015;9: 571–89.
4. Crippa S, Salvia R, Warshaw AL, Dominguez I, Bassi C, Falconi M, et al. Mucinous cystic neoplasm of the pancreas is not an aggressive entity: lessons from 163 resected patients. Ann Surg 2008;247:571–9.
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. Law JK, Ahmed A, Singh VK, Akshintala VS, Olson MT, Raman SP, et al. A systematic review of solid-pseudopapillary neoplasms: are these rare lesions? Pancreas 2014;43:331–7.
7. 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.
8. Masica DL, Dal Molin M, Wolfgang CL, Tomita T, Ostovaneh MR, Blackford A, et al. A novel approach for selecting combination clinical markers of pathology applied to a large retrospective cohort of surgically resected pancreatic cysts. J Am Med Inform Assoc 2017;24:145–52.
9. Brune K, Abe T, Canto M, O'Malley L, Klein AP, Maitra A, et al. Multifocal neoplastic precursor lesions associated with lobular atrophy of the pancreas in patients having a strong family history of pancreatic cancer. Am J Surg Pathol 2006;30:1067–76.
10. Shi C, Klein AP, Goggins M, Maitra A, Canto M, Ali S, et al. Increased prevalence of precursor lesions in familial pancreatic cancer patients. Clin Cancer Res 2009;15:7737–43.
11. Yu J, Sadakari Y, Shindo K, Suenaga M, Brant A, Almario JA, et al. Digital next-generation sequencing identifies low-abundance mutations in pancreatic juice samples collected from the duodenum of patients with pancreatic cancer and intraductal papillary mucinous neoplasms. Gut 2016. DOI: 10.1136/gutjnl-2015-311166.
12. 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: pii: gutjnl-2015-310162. doi: 10.1136/gutjnl-2015-310162. [Epub ahead of print].
13. 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.
14. 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:epub 2016/02/26.
15. Yamada S, Fujii T, Murotani K, Kanda M, Sugimoto H, Nakayama G, et al. Comparison of the international consensus guidelines for predicting malignancy in intraductal papillary mucinous neoplasms. Surgery 2016; 159:878–84.
16. Mukewar S, de Pretis N, Aryal-Khanal A, Ahmed N, Sah R, Enders F, et al. Fukuoka criteria accurately predict risk for adverse outcomes during follow-up of pancreatic cysts presumed to be intraductal papillary mucinous neoplasms. Gut 2016; pii: gutjnl-2016-311615. doi: 10.1136/gutjnl-2016-311615. [Epub ahead of print].
17. 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.
18. 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.
19. Cizginer S, Turner BG, Bilge AR, Karaca C, Pitman MB, Brugge WR. Cyst fluid carcinoembryonic antigen is an accurate diagnostic marker of pancreatic mucinous cysts. Pancreas 2011;40:1024–8.
20. 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.
21. 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.
22. Furukawa T, Kuboki Y, Tanji E, Yoshida S, Hatori T, Yamamoto M, et al. Whole-exome sequencing uncovers frequent GNAS mutations in intraductal papillary mucinous neoplasms of the pancreas. Sci Rep 2011;1:161.
23. Singhi AD, Nikiforova MN, Fasanella KE, McGrath KM, Pai RK, Ohori NP, et al. Preoperative GNAS and KRAS testing in the diagnosis of pancreatic mucinous cysts. Clin Cancer Res 2014;20:4381–9.
24. Springer S, Wang Y, Dal Molin M, Masica DL, Jiao Y, Kinde I, et al. A combination of molecular markers and clinical features improve the classification of pancreatic cysts. Gastroenterology 2015;149:1501–10.
25. Khalid A, Zahid M, Finkelstein SD, LeBlanc JK, Kaushik N, Ahmad N, et al. Pancreatic cyst fluid DNA analysis in evaluating pancreatic cysts: a report of the PANDA study. Gastrointest Endosc 2009;69:1095–102.
26. Jones M, Zheng Z, Wang J, Dudley J, Albanese E, Kadayifci A, et al. Impact of next-generation sequencing on the clinical diagnosis of pancreatic cysts. Gastrointest Endosc 2016;83:140–8.
27. Singhi AD, Zeh HJ, Brand RE, Nikiforova MN, Chennat JS, Fasanella KE, et al. American Gastroenterological Association guidelines are inaccurate in detecting pancreatic cysts with advanced neoplasia: a clinicopathologic study of 225 patients with supporting molecular data. Gastrointest Endosc 2016;83:1107–17.e2.
28. Matthaei H, Wylie D, Lloyd MB, Dal Molin M, Kemppainen J, Mayo SC, et al. miRNA biomarkers in cyst fluid augment the diagnosis and management of pancreatic cysts. Clin Cancer Res 2012;18:4713–24.
29. Maker AV, Katabi N, Qin LX, Klimstra DS, Schattner M, Brennan MF, et al. Cyst fluid interleukin-1beta (IL1beta) levels predict the risk of carcinoma in intraductal papillary mucinous neoplasms of the pancreas. Clin Cancer Res 2011;17:1502–8.
30. Schmidt CM, Yip-Schneider MT, Ralstin MC, Wentz S, DeWitt J, Sherman S, et al. PGE(2) in pancreatic cyst fluid helps differentiate IPMN from MCN and predict IPMN dysplasia. J Gastrointest Surg 2008;12:243–9.
31. Zikos T, Pham K, Bowen R, Chen AM, Banerjee S, Friedland S, et al. Cyst fluid glucose is rapidly feasible and accurate in diagnosing mucinous pancreatic cysts. Am J Gastroenterol 2015;110:909–14.
32. Sadot E, Basturk O, Klimstra DS, Gonen M, Lokshin A, Do RK, et al. Tumor-associated neutrophils and malignant progression in intraductal papillary mucinous neoplasms: an opportunity for identification of high-risk disease. Ann Surg 2015;262:1102–7.
33. Hata T, Dal Molin M, Suenaga M, Yu J, Pittman M, Weiss MJ, et al. Cyst fluid telomerase activity predicts the histologic grade of cystic neoplasms of the pancreas. Clin Cancer Res 2016 May 26. pii: clincanres.0311.2016. [Epub ahead of print].
34. Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. Nat Rev Genet 2002;3:415–28.
35. 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;18:700–12PMC3271174.
36. Kisiel JB, Raimondo M, Taylor WR, Yab TC, Mahoney DW, Sun Z, et al. New DNA methylation markers for pancreatic cancer: discovery, tissue validation, and pilot testing in pancreatic juice. Clin Cancer Res 2015;21:4473–81.
37. Vincent A, Omura N, Hong SM, Jaffe A, Eshleman J, Goggins M. Genome-wide analysis of promoter methylation associated with gene expression profile in pancreatic adenocarcinoma. Clin Cancer Res 2011; 17:4341–54.
38. Omura N, Li CP, Li A, Hong SM, Walter K, Jimeno A, et al. Genome-wide profiling of methylated promoters in pancreatic adenocarcinoma. Cancer Biol Ther 2008;7:1146–56.
39. Sato N, Maitra A, Fukushima N, van Heek NT, Matsubayashi H, Iacobuzio-Donahue CA, Rosty C, Goggins M. Frequent hypomethylation of multiple genes overexpressed in pancreatic ductal adenocarcinoma. Cancer Res 2003;63:4158–66.
40. Sato N, Fukushima N, Maitra A, Matsubayashi H, Yeo CJ, Cameron JL, et al. Discovery of novel targets for aberrant methylation in pancreatic carcinoma using high-throughput microarrays. Cancer Res 2003;63: 3735–42.
41. Nones K, Waddell N, Song S, Patch AM, Miller D, Johns A, et al. Genome-wide DNA methylation patterns in pancreatic ductal adenocarcinoma reveal epigenetic deregulation of SLIT-ROBO, ITGA2 and MET signaling. Int J Cancer 2014;135:1110–8.
42. Hong SM, Kelly D, Griffith M, Omura N, Li A, Li CP, et al. Multiple genes are hypermethylated in intraductal papillary mucinous neoplasms of the pancreas. Mod Pathol 2008;21:1499–507.
43. Sato N, Ueki T, Fukushima N, Iacobuzio-Donahue CA, Yeo CJ, Cameron JL, et al. Aberrant methylation of CpG islands in intraductal papillary mucinous neoplasms of the pancreas. Gastroenterology 2002;123:365–72.
44. Sato N, Fukushima N, Chang R, Matsubayashi H, Goggins M. Differential and epigenetic gene expression profiling identifies frequent disruption of the RELN pathway in pancreatic cancers. Gastroenterology 2006;130:548–65.
45. Sato N, Parker AR, Fukushima N, Miyagi Y, Iacobuzio-Donahue CA, Eshleman JR, et al. Epigenetic inactivation of TFPI-2 as a common mechanism associated with growth and invasion of pancreatic ductal adenocarcinoma. Oncogene 2005;24:850–8.
46. 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.
47. Matsubayashi H, Canto M, Sato N, Klein A, Abe T, Yamashita K, et al. DNA methylation alterations in the pancreatic juice of patients with suspected pancreatic disease. Cancer Res 2006;66:1208–17.
48. Parsi MA, Li A, Li CP, Goggins M. DNA methylation alterations in endoscopic retrograde cholangiopancreatography brush samples of patients with suspected pancreaticobiliary disease. Clin Gastroenterol Hepatol 2008;6:1270–8.
49. Pea A, Yu J, Rezaee N, Luchini C, He J, Dal Molin M, et al. Targeted DNA sequencing reveals patterns of local progression in the pancreatic remnant following resection of intraductal papillary mucinous neoplasm (IPMN) of the pancreas. Ann Surg 2016 Jul 18. [Epub ahead of print].