DNA Methylation Alterations in the Pancreatic Juice of Patients with Suspected Pancreatic Disease

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Abstract

Molecular markers of pancreatic neoplasia could aid in the evaluation of visible pancreatic lesions and indicate neoplasia invisible to imaging. We evaluated methylation-specific PCR (MSP) assays that detect aberrantly methylated DNA for their use as markers of pancreatic neoplasia. Methylation analysis was done on pancreatic juice collected endoscopically or surgically from 155 individuals with suspected pancreatic disease: 56 patients had pancreatic ductal adenocarcinoma, 17 had intraductal papillary mucinous neoplasms, 26 had symptomatic chronic pancreatitis, 12 controls lacked evidence of pancreatic disease, and 44 were asymptomatic individuals at increased risk of developing familial pancreatic cancer undergoing screening for pancreatic neoplasia. Pancreatic juice DNA was analyzed for promoter methylation using conventional MSP assays for 17 genes. For six genes, pancreatic juice methylation was quantified using real-time quantitative MSP (QMSP; Cyclin D2, FOXEI, NPTX2, ppENK, p16, and TFF1). Quantifying pancreatic juice methylation using QMSP with a cutoff of >1% methylated DNA could better predict pancreatic cancer than detecting methylation using conventional MSP. In the endoscopic group, 9 of 11 patients with pancreatic cancer, but none of 64 individuals without neoplasia had ≥1% methylation for two or more of the best five QMSP assays (82% sensitivity and 100% specificity; \( P < 0.0001 \)). The prevalence of pancreatic juice methylation in patients with chronic pancreatitis was less than in patients with pancreatic cancer but higher than in controls and similar to high-risk individuals. The detection and quantification of aberrantly methylated DNA in pancreatic juice is a promising approach to the diagnosis of pancreatic cancer. (Cancer Res 2006; 66(2): 1208-17)

Introduction

Pancreatic ductal adenocarcinoma is the fourth leading cause of cancer death in the United States and has the lowest survival rate for any solid cancer (~2%; refs. 1, 2). This poor survival occurs in part because only ~10% to 15% of patients are diagnosed while they have small, surgically resectable cancers. Patients who undergo surgical resection have a 5-year survival of 15% to 40% (3). Unfortunately, only ~15% of this group have early-stage cancers (T1 or T2 tumors without lymph node metastases), and pancreatic cancer survival is better for patients with the smallest tumors (4–6).

The poor survival among patients with pancreatic cancer is particularly of concern to patients with inherited susceptibility to the disease (7, 8). For example, studies have found germ line BRCA2 gene mutations in ~5% to 15% of familial pancreatic cancer kindreds (9–11), and familial cancer syndromes due to mutations in the p16, hMLH, and rarely \( FancC \) (12, 13) genes can also cause familial pancreatic cancer. Patients with the Peutz-Jeghers syndrome have a high lifetime risk of developing pancreatic cancer (14). Although the genetic basis of most inherited pancreatic cancer is not known, an individual’s family history has been a useful indicator of their risk of developing pancreatic cancer (15, 16). Individuals that have several first-degree relatives with pancreatic cancer have a 6- to 32-fold increased risk for developing the disease (16). Patients with long-standing chronic pancreatitis, particularly those with hereditary pancreatitis, also have an increased risk of developing pancreatic cancer (17–19).

Clinical screening protocols for asymptomatic individuals with an increased risk of developing pancreatic cancer have detected preinvasive pancreatic neoplasms in some individuals that are curable with surgery (20, 21). These screening protocols use endoscopic ultrasound, computed tomography (CT) scanning, and genetic counseling. Individuals with a suspected pancreatic neoplasm undergo resection of the neoplasm (pancreatoduodenectomy or tail pancreatectomy, ref. 20) or total pancreatectomy to prevent new neoplasms from developing in the remnant pancreas (21, 22).

When a lesion is found in the pancreas, it can be difficult to determine if it is neoplastic; hence, many investigators have sought to find more accurate markers of pancreatic neoplasia. Such markers could help differentiate pancreatic cancers from chronic pancreatitis and to identify preinvasive pancreatic neoplasms, such as intraductal papillary mucinous neoplasms (IPMN) and pancreatic intraepithelial neoplasia (PanIN). PanINs are small neoplasms, ≤5 mm, more often found in the head of the gland, and are thought to be the commonest precursor to invasive pancreatic ductal adenocarcinoma (23). IPMNs are larger neoplasms measuring >5 mm that can give rise to pancreatic adenocarcinomas (23). Importantly, IPMNs can usually be cured with surgical resection if there is not an associated infiltrating pancreatic adenocarcinoma (24, 25).

With better diagnostic imaging, IPMNs are being diagnosed more frequently (25), whereas the vast majority of PanINs are too small to be detected using currently available imaging tests. Potentially, the detection of markers of advanced PanIN could be used to predict future pancreatic cancer risk among individuals undergoing pancreas screening.

Disclosure: Drs. Goggins and Sato have entered into licensing agreements with Oncomethylome Sciences and Epigenomics, who wish to develop into commercial products several of the methylated genes used in this study. Requests for reprints: Michael Goggins, The Sol Goldman Pancreatic Cancer Research Center, Johns Hopkins Medical Institutions, 632 Ross Building, 720 Rutland Avenue, Baltimore, MD 21205-2196. Phone: 410-955-3511; Fax: 410-614-0671; E-mail: mgoggins@jhmi.edu.

DOI: 10.1158/0008-5472.CAN-05-2664
Many genetic and epigenetic alterations occur during pancreatic tumorigenesis (26), but few such alterations are useful diagnostic markers. Genetic alterations include oncogene (K-ras and BRAF) and suppressor gene mutations (p16, p53, SMAD4, BRCA2, STK11, hMLH1, hCD4C, MMK4, and FancC). Mitochondrial mutations and microsatellite instability also occur in pancreatic cancers (27) as do many gene expression changes (28–30). Of the genetic alterations, mutant K-ras is most readily detectable in secondary sources such as pancreatic juice (27). K-ras mutations are not specific for invasive pancreatic cancer; they are found in the pancreatic juice and in the stool of patients with chronic pancreatitis, individuals who smoke, and in PanNs.

An alternative and promising strategy for diagnosing pancreatic neoplasia is the detection of aberrant DNA methylation. Promoter CpG island methylation, a common mechanism for silencing genes during tumorigenesis (31), is readily detected using methylation-specific PCR (MSP) analysis. We have previously identified a panel of genes that are aberrantly methylated and silenced in human pancreatic cancer tissues and are rarely methylated in nonneoplastic pancreas, including ppENK, SPARC, TFF2, FOXE1, NFX2, TSLC1, p16, p14, p57, and Cyclin D2 (32–39). For several of these genes, we have found that patients harboring methylated genes in their primary pancreatic cancers also have methylation detectable by MSP in their pancreatic juice that has been collected during their pancreatic surgery (36–38).

In this study, we evaluated the diagnostic use of detecting methylation in endoscopically collected pancreatic juice of patients undergoing investigation for pancreatic disease. Our study population included patients with exocrine pancreatic neoplasms, chronic pancreatitis, controls without evidence of pancreatic disease, and asymptomatic individuals undergoing pancreatic screening because of a family history of pancreatic cancer. We used conventional MSP for 17 genes, most of which undergo methylation during pancreatic cancer development. For six of these genes, we also quantified methylation using real-time MSP, and for these studies, we also analyzed perioperative pancreatic juice samples from patients undergoing pancreatic surgery. 

Materials and Methods

Patients and samples. Pancreatic juice was collected from within the pancreatic duct at the time of endoscopy or intraoperatively from 155 individuals as part of clinical research protocols approved by the Johns Hopkins Joint Committee on Clinical Investigation. Pancreatic juice samples were collected from five groups of patients with the following diagnoses after cannulation, 0.2 g/kg of human synthetic secretin (donated by Repligen, Inc., Waltham, MA) was infused i.v., and pancreatic juice was aspirated through the ERCP catheter for 5 minutes while the catheter was still in the pancreatic duct. The diagnosis and grading of chronic pancreatitis was made from ERCP findings using the Cambridge criteria (20, 21), when applicable. An ERCP score of 3 was considered consistent with moderate pancreatitis; a score of 1 or 2 with mild pancreatitis and 0 with normal. The pancreas was separated from ERCP findings using the Cambridge criteria (20, 21), when applicable. Eighty-two of the 155 patients had pancreatic cancer, pancreatitis, or both; 11 had primary pancreatic cancer (32, 38, 41).

In 98% (38), Cyclin D2 is reported to be methylated in 71% (38), p16 in 73% (38), p53 in 93% (38), SPARC in 19%, and PP2A in 64%. Mitochondrial mutations and microsatellite instability also occur in pancreatic cancers (27) as do many gene expression changes (28–30). Of the genetic alterations, mutant K-ras is most readily detectable in secondary sources such as pancreatic juice (27). K-ras mutations are not specific for invasive pancreatic cancer; they are found in the pancreatic juice and in the stool of patients with chronic pancreatitis, individuals who smoke, and in PanNs.

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Quantitative methylation analysis by real-time MSP. DNA templates were amplified by fluorescence-based quantitative real-time MSP (QMSP; Methylation; ref. 44). Briefly, primers and probes were designed to amplify specifically bisulfite converted promoter DNA of NPTX2. Cyclin D2, FOXE1, TFPI2, pepEnk, p16, HPP1, NTPX2, FOXE1, CLDNS, Cyclin D2, and WNT7A; and 20 pg for SPARC and reprimo.

Quantitative methylation analysis by real-time MSP. DNA templates were amplified by fluorescence-based quantitative real-time MSP (QMP; Methylation; ref. 44). Briefly, primers and probes were designed to amplify specifically bisulfite converted promoter DNA of NPTX2. Cyclin D2, FOXE1, TFPI2, pepEnk, p16, and β-actin (used as the internal reference gene to quantify the amount of modified DNA in a sample; http://pathology2.jh.edu/pancreas/pancreaticjuice.html). The primers used for QMSP were the same as was used for conventional MSP apart from Cyclin D2 (primers amplified nucleotides 442-562 of HSU47284). QMSP was done with 3 µL of bisulfited DNA in 20 µL PCR reactions in 384-well clear optical reaction plates using the 7900 L PCR reactions in 384-well clear optical reaction plates using the 7900 HT Sequence Detection System (Applied Biosystems) and included water blanks, positive and negative controls. To quantify the amount of methylated DNA in a DNA sample, a standard curve was generated containing serially diluted bisulfite-modified DNA known to be completely methylated (e.g., Colo357 DNA for p16; ref. 33) and AsPC1 DNA (38) for the other genes. The ratio of the level of methylated DNA to the level of modified DNA from the β-actin quantification yielded the percentage of methylated DNA in a sample. QMSP intra-assay and inter-assay variation were determined. The intra-assay variation for quantifying a standard DNA was determined by performing the same QMSP assay six times on a DNA standard. The intra-assay variation using 60 and 6 ng of input DNA of the FOXE1 QMSP was 3.8% and 12.8%, respectively. Similarly, for NPTX2 it was 7.5% and 10.7%, respectively. The inter-assay variation of the QMSP assays ranged from 8.2% to 19.5% using 60 ng of input DNA to 9.2% to 16.9% using 6 ng of input DNA. An estimate of the lower limit of sensitivity of each QMSP was determined by mixing 12 ng of unmethylated DNA with input amounts of completely methylated DNA (10%, 5%, 1%, 0.5%, 0.1%, and 0%). These DNA mixtures were then bisulfite modified and assayed. Using 12 ng of input DNA, the QMSP assays could detect methylation if it was present at a concentration of ≥1% for FOXE1 and 0.1% for the NPTX2 assay.

**Statistical analysis.** Analyses were stratified according to the mode of collection of pancreatic juice into endoscopic pancreatic juice and surgical pancreatic juice. In the endoscopic group, methylation profiles of patients with a normal pancreas were compared with patients with a pancreatic cancer, chronic pancreatitis, IPMN, or those in the high-risk group. Among the high-risk group, DNA methylation status was compared between those with endoscopic evidence of moderate pancreatic disease (i.e., ERCP findings consistent with moderate chronic pancreatitis) and those with mild or no pancreatic disease. Among the surgical group, patients with pancreatitis were compared with those with pancreatic cancer or with an IPMN. Additionally, methylation profiles were also compared between those with lower-grade IPMN (i.e., IPMN-adenoma or a borderline IPMN) with those with high-grade IPMN (IPMNs with a grade of carcinoma in situ or IPMNs with an associated infiltrating adenocarcinoma). The sensitivity and specificity were calculated for each of the markers and marker panels. Separate comparisons were made for the conventional MSP results and quantitative MSP results. The average number of genes methylated in pancreatic juice in each group was compared using Student’s t test and Wilcoxon rank sum test. The relationship of methylation with age was determined using Pearson’s correlation coefficient. χ² tests and Fisher’s exact tests were used to determine differences in the proportion of methylated genes or the probability of having one or more genes methylated. A two-tailed P < 0.05 was used to assess statistical significance. Statistical analysis was done using the Excel statistics software (Microsoft, Redmond, WA), STATA version 8.2 software.

**Results**

DNA methylation analysis of pancreatic juice using conventional MSP. There were significantly more genes methylated in the pancreatic juice samples of patients with pancreatic cancer than...
in juice from patients without pancreatic cancer (Fig. 1; Table 2). Overall, the mean percentage of genes in the 17-gene panel that were methylated in the juice of patients with pancreatic cancer (17 genes × 11 juice samples = 187 methylated genes) was 63 ± 16.2% (118 methylated genes of 187 total) compared with 11.8 ± 13.3% (24 of 205) for the normal pancreas controls (P < 0.0001). The percentage of genes methylated in patients with pancreatic cancer was also higher than in patients with chronic pancreatitis (24.6 ± 16.8%), who in turn had a higher percentage of methylated genes than the normal pancreas controls. Two of the 17 genes did not provide significant discrimination for pancreatic cancer (FHIT and reprimo); two other genes were almost never methylated in any juice sample consistent with results in primary pancreatic cancers (CDH3 and DAPK). Using the most discriminating 13 genes in the panel, an average of 71.3 ± 29.4% (9.3 ± 3.8 genes) of the genes were methylated in the pancreatic cancer group (PC) compared
with $26.7 \pm 20.1\%$ (3.3 ± 2.6 genes per individual) in the chronic pancreatitis (CP) group and $10.9 \pm 12.5\%$ (1.4 ± 1.6 genes per case) in the normal pancreas (N) group (PC versus N; PC versus CP; PC versus CP + N; all $P < 0.001$). The percentage of methylated genes was also higher in the PC group compared with the high-risk group (27.4 ± 16.4%; $P < 0.0001$) and in the CP group compared with the normal pancreas group (CP versus N; $P = 0.019$). Receiver operator characteristics analysis indicated that using a cutoff of seven, or eight methylated genes was equally superior for discriminating cases and controls. Thus, 8 of 11 (73%) patients with pancreatic cancer had methylation of ≥7 of the 13 genes in the panel in their endoscopic pancreatic juice. In contrast, only three patients (11.5%) in the pancreatitis group and none in the normal pancreas groups had methylation of seven or more of the genes in the panel (3 of 26 in the combined pancreatitis/normal pancreas group; $P < 1 \times 10^{-5}$). Three of 38 individuals (7.9%) in the high-risk group (without a detectable neoplasm) had more than seven methylated genes in their pancreatic juice (see below). The methylation of some genes showed more discrimination for pancreatic cancer than others: NPTX2 (sensitivity, 90.9%; specificity, 77.8%), Cyclin D2 (sensitivity, 81.8%; and specificity, 92.6%), SARPI2 (sensitivity, 81.8%; specificity, 77.8%), FOXE1 (sensitivity, 72.7%; specificity, 77.8%), SPARC (sensitivity, 90.9%; specificity, 70.4%), and ppEnk and TFF2 (both: sensitivity, 72.7%; and specificity, 81.5%).

**DNA methylation analysis of pancreatic juice using QMSP.** Because the conventional MSP assays detect but do not quantify methylation levels in pancreatic juice, we designed real-time quantitative methylation assays using promising markers as indicated by the conventional MSP assays. We used the same study population as was used for the conventional MSP assays, with the exception of two samples that did not amplify DNA with the β-actin QMSP assay (one normal control and one from the familial pancreatic cancer screening group. In addition, we also included 73 patients undergoing pancreatic surgery in the QMSP analyses. (This group was not included in the conventional MSP panel above as we have been previously evaluated several genes in this panel using surgical pancreatic juice samples refs. 36–38). There was almost no detectable methylation of these genes by QMSP in frozen normal pancreatic parenchyma from nine individuals who had undergone pancreatic surgery (Table 3). Because methylation is rarely detected in normal pancreas, we suspected that the pancreatic juice methylation detected in controls derived from a small percentage of DNA molecules. Pancreatic juice samples from patients with pancreatic cancer usually had methylation of ≥1% of total DNA, and this level of methylation was rarely detected in controls. One percent or more methylation was thus chosen as the cutoff for calling a QMSP result “positive for methylation.” By this criterion, several genes were accurate discriminators of pancreatic cancer by QMSP.

### Table 2. Percentage of methylated genes detected by conventional MSP in endoscopic pancreatic juice

<table>
<thead>
<tr>
<th>Patient group</th>
<th>% Methylated genes* (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>$10.9 \pm 12.5^{b}$</td>
</tr>
<tr>
<td>Chronic pancreatitis</td>
<td>$26.8 \pm 19.3^{c}$</td>
</tr>
<tr>
<td>High-risk group</td>
<td>$27.4 \pm 16.4^{d}$</td>
</tr>
<tr>
<td>High-risk group with neoplasms</td>
<td>$36.2 \pm 15^{e}$</td>
</tr>
<tr>
<td>Pancreatic adenocarcinoma</td>
<td>$71.2 \pm 23.7^{a}$</td>
</tr>
</tbody>
</table>

**NOTE:** Different superscripts indicate $P < 0.001$ by Student's $t$ test (a versus b, a versus c, and a versus d).

*13 genes analyzed.

### Table 3. Methylation by QMSP in pancreatic juice

<table>
<thead>
<tr>
<th>Patient group</th>
<th>% Genes methylated (mean ± SD)</th>
<th>% Patients with methylation*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≥1 gene</td>
<td>≥2 genes</td>
</tr>
<tr>
<td>Pancreatic juice</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endoscopically collected pancreatic juice</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal control ($n = 11$)</td>
<td>$3.6 \pm 8.1^{i}$</td>
<td>$16.7^{i}$</td>
</tr>
<tr>
<td>Chronic pancreatitis ($n = 15$)</td>
<td>$2.5 \pm 6.8^{i}$</td>
<td>$12.5^{i}$</td>
</tr>
<tr>
<td>High-risk group ($n = 43$)</td>
<td>$6.2 \pm 12.3^{i}$</td>
<td>$25.6^{i}$</td>
</tr>
<tr>
<td>Mild or no pancreatic abnormalities ($n = 22$)</td>
<td>$0.9 \pm 9^{i}$</td>
<td>$4.4^{i}$</td>
</tr>
<tr>
<td>Moderate pancreatic abnormalities ($n = 15$)</td>
<td>$9.3 \pm 5.8^{i}$</td>
<td>$46.6^{i}$</td>
</tr>
<tr>
<td>High-risk group with neoplasms ($n = 6$)</td>
<td>$13.3%$</td>
<td>$33%$</td>
</tr>
<tr>
<td>Pancreatic adenocarcinoma ($n = 11$)</td>
<td>$47.2 \pm 24.1$ (reference)</td>
<td>$91$ (reference)</td>
</tr>
<tr>
<td>Surgically collected pancreatic juice</td>
<td>$5.5 \pm 9.3^{i}$</td>
<td>$27.3^{i}$</td>
</tr>
<tr>
<td>Chronic pancreatitis ($n = 11$)</td>
<td>$9 \pm 21.8^{i}$</td>
<td>$35.3^{i}$</td>
</tr>
<tr>
<td>IPMN ($n = 17$)</td>
<td>$27.0 \pm 22.2$ (reference)</td>
<td>$80$ (reference)</td>
</tr>
<tr>
<td>Pancreatic adenocarcinoma ($n = 45$)</td>
<td>$2.2 \pm 6.7$</td>
<td>$11.1$</td>
</tr>
<tr>
<td>Normal pancreatic parenchyma ($n = 9$)</td>
<td></td>
<td>$0$</td>
</tr>
</tbody>
</table>

**NOTE:** For reference, see text.

*Five genes (cyclin D2, TFF2, ppENK, NPTX2, and FOXE1).

$i P < 0.001$.

* $P < 0.01$.

$P < 0.05$.
example, the sensitivity of FOXE1 methylation for pancreatic carcinoma in endoscopic pancreatic juice was 82% and the specificity was 98.5%. The p16 QMSP assays was less useful. p16 methylation was not detected at the 1% level in any juice samples from a patient with pancreatic cancer (Fig. 2).

Using the five most discriminating QMSP assays, the mean percentage of genes that were methylated (≥1% concentration of methylated DNA) in the juice of patients with pancreatic cancer (47.2 ± 24.1%, 26 of 55 genes) was significantly higher than in the normal pancreas controls (3.6 ± 8.1%, 2 of 60; P < 0.001), as well as in the chronic pancreatitis (2.5 ± 6.8%, 2 of 80; P < 0.001), and in the high-risk groups (6.2 ± 12.3%, 12 of 195; P < 0.001; Table 3). Nine of 11 patients with pancreatic cancer but in none of the 64 individuals without detectable pancreatic neoplasia that underwent endoscopic juice collection had ≥1% methylation of more than one gene in their endoscopic juice (82% sensitivity and 100% specificity for pancreatic cancer; P < 0.001). The use of other cutoffs of methylated DNA concentrations to decide if a test was positive

Figure 2. Analysis of endoscopic and surgical pancreatic juice using QMSP. Abbreviations: C, Caucasian; AA, African American; O, others; PJS, Peutz-Jeghers syndrome; C.w., consistent with. **, EUS/ERCP findings at the time of pancreatic juice collection. PI EIPMNHR04 also had a separate focus of microinvasive adenocarcinoma adjacent to PanIN. ^^, baseline screening normal, follow-up screening identified a pancreatic neoplasm. EHR98 was subsequently found to have a pancreatic adenocarcinoma. EHR40 had a follow-up endoscopic ultrasound consistent with IPMN, patient considering surgery.
or negative provided less optimal discrimination between disease groups. For example, the use of a 2% concentration of methylated DNA for one or more genes in the panel reduced the sensitivity of the test panel to 82% and 63%, respectively, for identifying patients with pancreatic cancer. The 2% concentration cutoff did increase specificity as no patient with a normal pancreas had more 2% methylation of any gene, but it did not change the prevalence of positives detected in patients with pancreatitis. Reducing the diagnostic cutoff to 0.5% methylated DNA did not change the sensitivity for detecting patients with pancreatic cancer but increased the number of false positives with pancreatitis to 20%.

We examined methylation patterns using QMSP in surgically obtained pancreatic juice. As with the endoscopic juice analyses, differences in methylation in surgically collected pancreatic juice were clearly evident between patient groups. Methylation was more often detected in the endoscopic than in the surgical juice samples, perhaps because endoscopic juice samples collected after the pancreatic ductal system has been lavaged are more likely to contain pancreatic cancer DNA than surgically obtained juice samples (Fig. 2). None of the patients with chronic pancreatitis had methylation of more than one gene in their surgical pancreatic juice by QMSP (0 of 11) compared with 6% (1 of 17) of patients with an IPMN and 40% (18 of 45) of patients with pancreatic adenocarcinoma (chronic pancreatitis versus pancreatic carcinoma; \( P = 0.0001 \)). The amount of methylation detected by QMSP tended to increase with the grade of IPMN; more methylated genes detected in pancreatic juice from IPMNs with carcinoma in situ or with an associated infiltrating ductal or colloid adenocarcinoma, than among IPMNs of adenoma or borderline grade (\( P = 0.051 \)). The NPTX2 and FOXE1 genes were most often methylated in the surgical pancreatic juice of patients with pancreatic ductal adenocarcinoma (42.2% and 40%, respectively), less frequently in samples of IPMN without cancer (11.8% and 5.6%, respectively) but rarely in patients with chronic pancreatitis (9.1% and 0%, respectively). In contrast, the prevalence of \( p16 \) methylation (>1%) was similar in patients of chronic pancreatitis (18.2%), IPMN (23.5%), and in pancreatic cancer (11%) figures similar to those found for the patients that underwent the endoscopic juice analyses (Table 3; Fig. 2).

**Pancreatic juice methylation among high-risk individuals undergoing screening.** Using the 13-gene conventional MSP panel, the high-risk group had more methylation in their pancreatic juice (27.4 ± 16.4%) than normal controls (\( P < 0.0001 \)) but similar to patients with chronic pancreatitis (27.2 ± 19%; Table 2).

Among the 44 high-risk individuals that underwent ERCP and pancreatic juice collection as part of their screening protocol, 15 had pancreatograms that met the Cambridge criteria for moderate pancreatitis, 20 had abnormalities consistent with mild pancreatitis, six had an IPMN, and three were normal. The women in the study were slightly older than the men (51.1 ± 5.9 versus 47.9 ± 6.2 years; \( P = 0.079 \)). Conventional MSP assays did not identify significant differences in the prevalence of methylation between the moderate and mild pancreatitis high-risk groups.

Four of the high-risk kindred had Peutz-Jeghers syndrome. Their mean number of genes methylated by conventional MSP was similar to that found in the familial pancreatic carcinoma kindreds (33.8% versus 27.4%).

To date, six of the high-risk relatives who underwent screening and pancreatic juice collection had pathologically confirmed neoplasms: five had IPMNs, one patient with an IPMN also had an area of microinvasive adenocarcinoma, and one patient had a metastatic pancreatic adenocarcinoma diagnosed by needle biopsy that was detected months after follow-up endoscopic ultrasound indicated a probable neoplasm in the pancreas. Two of these six individuals had their IPMN detected at baseline; in the remaining four individuals, the IPMN or pancreatic cancer was identified during follow-up screening ~1 year after their pancreatic juice collection. The number of aberrantly methylated genes detected by the 13-gene conventional MSP panel in these six individuals (4.7 ± 2.1 genes methylated, or 36.6 ± 15% of the gene panel methylated) was higher than that found in those with normal pancreata (\( P < 0.05 \)) but similar to that found in the remaining 37 individuals in the high-risk group that did not have an IPMN (27.4 ± 16.4% genes methylated). ERCP changes suggestive of mild or moderate pancreatitis were seen in five of the six individuals in the high-risk group with an IPMN, although these changes can also result from the IPMN or its mucin production. Pathologic examination of the surgical resections from five members of this group revealed that three also had PanIN and one patient did not have evidence of pancreatitis, suggesting that the ERCP changes that mimic chronic pancreatitis may have resulted from PanIN. One other patient in the high-risk group had endoscopic ultrasound and ERCP changes indicative of an IPMN with evidence of IPMN on fine needle aspiration cytology of the lesion but has thus far preferred to undergo continued surveillance rather than surgical resection (see Figs. 1 and 2). Four additional patients from the CAPS2 study (EHR8, EHR26, EHR29, and EHR38) who had pure pancreatic juice collections had small (≤1 cm) focal endoscopic ultrasound abnormalities (such as focal duct dilation, nodules, or cysts) that were not considered strongly suspicious for neoplasia are undergoing continuing surveillance.

**Concordance of conventional MSP and QMSP.** We examined the concordance between conventional MSP and QMSP results done on the same sample for the seven genes that were assayed (FOXE1, NPTX2, peENK, TPPI2, Cyclin D2, and \( p16 \)) in the endoscopically collected pancreatic juice. Because a pancreatic juice sample was considered negative by QMSP if the level of methylation was <1%, we defined discordance as results where the QMSP result for the gene was positive and the conventional MSP result was negative or vice versa. Four of the five cases of discordance between conventional MSP and QMSP occurred in individuals with IPMNs. This was consistent with moderate pancreatitis being slightly older than those that had only mild pancreatitis or had a normal pancreas by imaging (51.5 ± 5.9 versus 47.9 ± 6.2 years; \( P = 0.079 \)). Conventional MSP assays did not identify significant differences in the prevalence of methylation between the moderate and mild pancreatitis high-risk groups.

A more detailed summary is described in Canto et al., submitted for publication.
Correlation between age and methylation in pancreatic juice. The differences in methylation observed between patient groups did not differ when we took age into account. In multivariate models, age was not a significant predictor of the percentage of gene methylated (data not shown). We and others have found that promoter methylation of many genes increases with age in nonneoplastic tissues (32, 41, 45, 46). In the normal pancreas controls, we did find that methylation in pancreatic juice increased with patient age by conventional MSP ($R = 0.65$, $P = 0.021$). In contrast, there was no correlation between the number of methylated genes and patient age among patients in the chronic pancreatitis ($R = 0.12$), in the high-risk group ($R = 0.12$), or among the patients with pancreatic cancer ($R = 0.07$). There was also no significant relationship between age and methylation as measured by QMSP among any of the diagnostic groups.

Discussion

In this study, we show the diagnostic utility of quantifying aberrantly methylated DNA concentrations in pancreatic juice. Using a QMSP panel of five genes, 82% of patients with pancreatic adenocarcinoma had at least 1% methylation of two or more genes in their endoscopically obtained pancreatic juice, whereas not one of 64 individuals in the nonneoplastic groups had this threshold of methylation. These results compare favorably to other pancreatic juice markers, such as telomerase activity, which have been used to differentiate benign from malignant pancreatic diseases (47). These results indicate that methylation analysis of pancreatic juice can differentiate pancreatic cancer from other pancreatic lesions. QMSP could also reliably discriminate pancreatic cancer from chronic pancreatitis in the operative pancreatic juice groups, although the total number of methylated genes detected in operative pancreatic juice samples was somewhat less than that found in endoscopic juice, perhaps reflecting the small amounts of pancreatic juice that can be aspirated during surgery without secretin stimulation.

As has been previously observed by others, the conventional MSP assays detected methylation in pancreatic juice that was below levels detectable by QMSP. As with QMSP, conventional MSP results also showed significant discrimination for pancreatic cancer over disease controls. Eight of 11 patients with pancreatic cancer had detectable methylation of more than half of the genes tested in their endoscopic pancreatic juice, compared with only 3 of 15 patients in the pancreatitis group and in none of those with a normal pancreas ($P < 1 \times 10^{-6}$). Overall, QMSP assays could better discriminate between disease groups.

Analysis of resected IPMNs for several genes indicates that aberrant DNA methylation is less prevalent in IPMNs than in pancreatic cancers and is more prevalent in high-grade than in low-grade IPMNs (37). Consistent with this pattern, less pancreatic juice methylation was detected by conventional MSP in patients with low-grade IPMNs versus IPMNs with carcinoma. Thus, the prevalence of pancreatic juice methylation in patients with IPMN may reflect their small size and low grade, and our marker panel was chosen for their prevalence in pancreatic cancers not in IPMNs. Our current panel could not distinguish patients with an IPMN (without an associated invasive pancreatic cancer) from those with chronic pancreatitis. Excess DNA methylation is seen in chronic inflammatory settings, such as during ulcerative colitis, gastritis, and cholecystitis (32, 41, 46, 48, 49). It is also possible that IPMNs shed less DNA than carcinomas. It is possible that using fine needle aspirates of IPMNs as a sample source would provide more neoplastic DNA for DNA methylation assays and facilitate the diagnosis of IPMNs when cytology is not diagnostic. Further studies are needed to identify markers that are sensitive for identifying low-grade IPMNs and that can distinguish such lesions from inflammatory lesions of the pancreas.

The biological basis for the diagnostic accuracy of our marker panel for pancreatic cancer derived from the choice of methylated genes, the assays used for their detection, and the greater concentration of methylated copies of these genes in the pancreatic juice of patients with pancreatic cancer compared with those without cancer. Refining the marker panel with other aberrantly methylated genes may improve methylation-based cancer diagnosis. In addition, further analyses of promoter regions to identify where methylation most closely predicts neoplasia can inform the designs of QMSP assays to create optimal MSP markers (50). Our MSP assays generally targeted regions just upstream to the transcriptional start site of each gene. Most studies have found that cancer-specific methylation is usually located in this region (50). The propensity for low-level methylation to also occur with aging or in nonneoplastic settings highlights the need to test the specificity of MSP assays in large appropriately age-matched control populations. Noninvasive PanINs arise increasingly with age, and these PanINs may be responsible for some of the low-level methylation we found in the pancreatic juice of controls without evidence of pancreatic disease (51). Just as some genes are rarely mutated in early PanINs (e.g., p53 and SMAD4; ref. 52), some genes have a propensity undergo methylation late in precancerous development (e.g., TP7-2; ref. 37).

Patient heterogeneity was evident in pancreatic juice methylation patterns, as has been found for tumor methylation patterns. An example of tumor heterogeneity with respect to alterations in methylation is seen with $p16$. $p16$ methylation is only found in pancreatic cancers that have not genetically inactivated $p16$ ($\sim 15-20\%$ of pancreatic cancers; ref. 42). Hence, the detection of $p16$ methylation in pancreatic juice was not a sensitive marker for identifying pancreatic cancer: it was found in the pancreas of some of the disease controls, and in some patients with IPMNs (53).

We also found that asymptomatic individuals from familial pancreatic cancer kindreds show abnormal levels of methylation compared with controls. Additional prospective studies involving these kindred are needed to determine if DNA methylation abnormalities increase in pancreatic juice as early-stage pancreatic neoplasms develop. This would permit their early diagnosis and management. Individuals from these kindred frequently show mild to moderate parenchymal and pancreatic duct abnormalities that resemble changes of chronic pancreatitis (20, 21) despite the absence of symptoms or clinical signs of chronic pancreatitis in most kindred. The QMSP analysis showed that high-risk patients’ pancreatic juice methylation profile correlated with the severity of their pancreatic abnormalities by ERCP. That is, 7 of 15 high-risk individuals with moderately abnormal pancreaticograms had a positive QMSP result in their juice compared with only 1 of 22 individuals with mildly abnormal or normal pancreaticograms ($P = 0.007$). Although ERCP is the gold standard for the diagnosis and grading of chronic pancreatitis, autopsy studies indicate that structural changes in the pancreatic duct that mimic chronic pancreatitis can occur in individuals without pancreatitis (54). Some asymptomatic high-risk individuals who have gone to surgery have had evidence of chronic pancreatitis histologically (20, 21).
but this is often detected in the setting of an associated obstructing neoplasm that could have caused secondary pancreatitis. Thus, it is not certain that the structural pancreatic changes seen in high-risk individuals by endoscopic ultrasound/ERCP in the absence of an obstructing neoplasm are due to pancreatitis. Pancreatic function testing of high-risk individuals with pancreatic structural abnormalities may shed light on this question (55). Similarly, the prevalence and extent of histologic pancreatitis in this population will eventually become clarified as more of these individuals undergo pancreatic resection. The resected pancreata of many of the high-risk individuals contain PanINs (20, 21), raising the possibility that the endoscopic ultrasound and ERCP abnormalities found in many of these individuals reflect the presence of PanIN rather than chronic pancreatitis (56, 57). One hypothesis is that microscopic PanIN can obstruct small ductules and cause secondary changes in the parenchyma resembling pancreatitis. The ability to reliably detect and quantify PanIN using molecular assays in high-risk individuals would help identify individuals needing more surveillance to detect advanced pancreatic neoplasia and could also open up the option of enrolling affected individuals in chemoprevention trials.

The mode of collection of pancreatic juice can influence methylation results. In previous studies, we have shown that low-level methylation of CpG islands is a common feature of duodenal mucosa; thus, methylation is commonly detected in the pancreatic juice of controls if it is collected from the duodenal lumen rather than from the pancreatic duct (36, 41). Thus, the optimal method of collecting pancreatic juice for pancreas methylation analysis is during direct cannulation of the pancreatic duct. Secretin stimulation for pancreatic juice collection has been used for pancreatic function testing for many years and is very safe (55), but direct cannulation of the pancreatic duct during ERCP carries with it a small but significant risk of pancreatitis. The high risk of pancreatic cancer among familial pancreatic cancer kindreds may warrant ERCP and pancreatic juice collection as part of a screening protocol if it provided useful information. However, it would be preferable if pancreatic juice could be collected without the need for an ERCP. One less invasive method of pancreatic juice collection would be using a catheter that capped the Ampulla of Vater to permit aspiration of secretin-stimulated pancreatic juice during endoscopy without pancreatic duct cannulation.

In conclusion, we find that quantitative methylation analysis of pancreatic juice can accurately distinguish patients with pancreatic cancer from those without pancreatic cancer. Future studies involving larger patient populations and additional aberrantly methylated genes will hopefully reveal the optimal strategy of using pancreatic juice methylation analysis for the early diagnosis and early detection of pancreatic cancer.

Acknowledgments

Received 8/1/2005; revised 9/9/2005; accepted 10/27/2005.

Grant support: National Cancer Institute Specialized Programs of Research Excellence in Gastrointestinal Cancer grants CA62924 and RO1CA90709, Michael Rolfe Foundation, and Sankyo Life Science Foundation (H. Matushabayashi).

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DNA Methylation Alterations in the Pancreatic Juice of Patients with Suspected Pancreatic Disease

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