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Genome-Wide CpG Island Profiling of Intraductal Papillary Mucinous Neoplasms of the Pancreas

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Abstract

Purpose—Intraductal papillary mucinous neoplasms (IPMNs) are precursors to infiltrating pancreatic ductal adenocarcinomas. Widespread epigenetic alterations are characteristic of many cancers, yet few studies have systematically analyzed epigenetic alterations of neoplastic precursors. Our goal was to perform genome-wide CpG island methylation profiling to identify aberrantly methylated loci in IPMNs.

Experimental Design—We compared the CpG island methylation profiles of 6 IPMNs to normal primary pancreatic duct samples using methylation CpG island amplification (MCA) and Agilent CpG island microarray (MCAM) analysis. When selected 13 genes identified as differentially methylated by MCAM for methylation-specific PCR (MSP) analysis in an independent set of IPMNs and normal pancreas samples and performed expression analysis of selected genes.

Results—We identified 2,259 loci as differentially methylated in at least one of 6 IPMNs including 245 genes hypermethylated in IPMNs with high-grade dysplasia compared to normal pancreatic duct samples. Eleven of 13 genes evaluated by MSP were more commonly methylated in 61 IPMNs than in 43 normal pancreas samples. Several genes, (*BNIP3*, *PTCHD2*, *SOX17*, *NXP1*, *EBF3*), were significantly more likely to be methylated in IPMNs with high-grade than with lower-grade dysplasia. One gene, *Sox17*, demonstrated loss of protein expression by immunohistochemistry in 22% (19 of 88) of IPMNs. The most specific marker, *BNIP3*, was not methylated in any IPMNs with low-grade dysplasia or in normal pancreas samples.

Conclusions—IPMNs undergo extensive aberrant CpG island hypermethylation. The detection of genes selectively methylated in high-grade IPMNs such as *BNIP3* may have utility in the clinical evaluation of IPMNs.

Keywords

Intraductal papillary mucinous neoplasm; Pancreas; pancreatic cancer; DNA Methylation; CpG Island

INTRODUCTION

Pancreatic cancer is the 4th leading cause of cancer death in the United States. In 2011, it is estimated that 44,030 Americans will be diagnosed and 37,660 patients will die of pancreatic cancer (1). The 5-year survival for patients with all stages of infiltrating pancreatic ductal adenocarcinomas is only ~6% and is only ~23% for patients whose disease is detected early enough to undergo surgical resection with curative intent (1). Detecting early-stage pancreatic cancer and its precursors by screening high risk individuals is currently considered the best way to improve patient survival (2, 3). Intraductal papillary mucinous neoplasms (IPMNs) arise in the pancreatic ductal system and are distinct mucin-producing cystic precursor lesions of pancreatic ductal adenocarcinomas. IPMNs are large enough (usually more than 1 cm) to be detected by clinical imaging tests (4), unlike pancreatic intraepithelial neoplasias (PanINs), which are lesions of <5 mm (5). With the increasing use of abdominal imaging, IPMNs are commonly detected as incidental lesions(6).

Many of the molecular alterations of pancreatic ductal adenocarcinomas (reviewed in (7)) have been identified in IPMNs (8-10). Thus, the prevalence of *KRAS2* mutations increases in parallel with degree of dysplasia of IPMNs, and have been detected in ~80% and *TP53* mutations in ~50% of IPMNs (11) with high-grade dysplasia. In addition, somatic mutations of the *STK11/LKB1* (12) and *PIK3CA* genes (13) have been identified in ~10% of IPMNs. A low prevalence of chromosomal losses have also been identified in IPMNs (14). Compared with PanINs and pancreatic ductal adenocarcinomas, IPMNs rarely show inactivation of *DPC4/SMAD4* even in high-grade lesions (15, 16).

While genome-wide analyses of aberrant DNA methylation of pancreatic ductal adenocarcinomas have been described (17-21), no such DNA methylation analyses have been reported for IPMNs. Candidate gene analysis of genes methylated in pancreatic and other cancers has been performed in IPMNs revealing that several genes including *CDKN1C/p57KIP2*, *CDKN2A/p16*, *PENK*, *SOCS-1*, *RELN*, *TFPI2*, *SPARC*, and others undergo aberrant methylation often associated with gene silencing (18, 20, 22-25).

Genome-wide DNA methylation analysis of IPMNs has the potential not only to identify genes targeted in IPMNs but also provide a better understanding of the timing of aberrant methylation events during pancreatic tumorigenesis, thereby helping to elucidate the DNA methylation events important for tumor development. In this study, we utilized Methylated CpG island Amplification coupled with genome-wide CpG island microarrays (MCAM) followed by validation by methylation specific PCR (MSP) to identify differentially methylated genes in IPMNs.

METHODS

An overview of the experimental approach that we used in this study is summarized on Figure 1.

Cell Lines

Fourteen human pancreatic cancer cell lines, including A32-1, A38-5, BxPC3, Capan1, Capan2, CFPAC1, FamPanc, Miapaca2, Panc-1, Panc1.28, Panc2.5, PL4, PL8, and Su8686, were also used. An immortalized cell line derived from normal human pancreatic ductal epithelium (HPDE) was generously provided by Dr. Ming-Sound Tsao (University of Toronto, Canada).

Patients and Tissues

Tissues were obtained for methylation analysis and immunohistochemistry and from patients with IPMNs as well as from patients with pancreatic ductal adenocarcinoma and from disease controls. A summary of the demographics of the patients and their tissues used in this study is provided in Table 1 and Supplemental Table 1. Fresh-frozen sections from 61 IPMNs and 10 invasive ductal adenocarcinomas with an associated IPMN were collected from 71 patients who underwent surgical resection of their neoplasm at the Johns Hopkins Medical Institutions from 1999 to 2008. These patient samples were selected based on the availability of stored tissues with sufficient numbers of neoplastic cells for DNA methylation analysis. Fresh-frozen normal pancreatic tissues were obtained from 35 patients with ductal neoplasms (5 with IPMNs and 30 with an associated invasive pancreatic ductal adenocarcinomas), and 8 patients with benign pancreatic neuroendocrine neoplasms and used as control samples. Hematoxylin and eosin staining of frozen sections was performed to assess the degree of dysplasia using criteria described elsewhere (26-29). The IPMNs were classified by their grade of dysplasia using the new WHO classification into either low-, intermediate-, or high-grade dysplasia (30). IPMNs were also classified by their histologic subtype and localization of each IPMN as described elsewhere (27).

Formalin-fixed paraffin-embedded IPMN tissues were additionally retrieved from 88 patients, 75 of these cases were used to create tissue microarrays for immunohistochemical labeling. In addition, tissue microarrays of formalin-fixed paraffin embedded pancreatic ductal adenocarcinoma tissues from 111 patients who underwent surgical resection of the pancreas at our institution were also analyzed by immunohistochemistry. All specimens were collected and analyzed with the approval of the Johns Hopkins Committee for Clinical Investigation.

IPMNs were carefully manually microdissected from frozen sections to collect neoplastic cells. Normal pancreatic ductal epithelial cells were selectively microdissected from frozen sections adjacent to IPMN, pancreatic ductal adenocarcinoma, or well-differentiated pancreatic neuroendocrine tumors using the PALM micro laser system (Carl Zeiss microimaging Inc., North America, Thornwood, NY).

DNA Extraction

Genomic DNA was extracted from the microdissected samples with QIAamp DNA Micro Kit (Qiagen, Inc., Valencia, CA) following the manufacturer's protocol. Extracted DNA was quantified by either Quantifiler (Applied Biosystems, Foster City, CA) or PicoGreen Assay (Invitrogen, Carlsbad, CA).

Methylated CpG island Amplification (MCA)

MCA was performed on DNA from 6 IPMNs (1 IPMN with low-grade, 3 intermediate-grade, and 2 with high-grade dysplasia) and DNA from matched normal pancreatic samples as previously described with minor modifications (31). In brief, 500 nanograms of DNA was digested with *SmaI* and *XmaI* (New England Biolabs, Inc., Ipswich, MA). Unmethylated *SmaI* sites are eliminated by *SmaI* digestion, which leaves a blunt end fragment. In contrast, CCCGGG sites with methylated CpG are not cut by *SmaI*. These methylated *SmaI* sites can then be digested with *XmaI*, which is the non-methylation-sensitive *SmaI* isoschizmer, leaving a CCGG overhanging sticky end. Adaptors are ligated to these sticky ends, and PCR is performed to amplify the methylated sequences. Restriction fragments were ligated to an RMCA adaptor and amplified by PCR in a 100 μ l volume containing 200 pmol of RMCA 24-mer primer, 600 mM Tris-SO₄, 2mM MgSO₄, 160 mM (NH₄)₂SO₄, 200 μ M each dNTP, 2% v/v DMSO, 0.5 M betaine and 2 U of Platinum Taq Hifidelity polymerase (Invitrogen). The reaction mixture was incubated at 72C° for 5 min and at 95 C° for 3 min, and then

subjected to 25 cycles of 1 min at 95 °C and 3 min at 77 °C followed by a final extension of 10 min at 77 °C.

Agilent 244K human promoter and CpG island microarrays

Agilent's 244K Human promoter chip-on-chip microarray contains 195 K CpG island probes and 50 K non-CpG island probes and interrogates 27,800 CpG islands covering 21MB with an average of 8 probes per island. Array hybridization was performed by the Sidney Kimmel Cancer Center Microarray Core Facility at Johns Hopkins. Briefly, 2 µg of MCA amplicon was labeled with either Cy3-dUTP or Cy5-dUTP (Perkin Elmer, Waltham, MA) by using BioPrime DNA Labeling System (Invitrogen). These dye-labeled amplicons were then mixed and co-hybridized to each of the 244K human promoter Chip on-chip microarrays. After the hybridization, the microarray slides were washed, dried and scanned using an Agilent G2505B scanner.

Data were extracted with Agilent Feature Extraction 9.1 software. Methylation-specific sites were called with Agilent Genomic Workbench Standard Edition 5.0.14 software for methylation (CH₃) analysis, which calculates the normalized log₂-signal ratios (NLR) and combined Z-scores for each probe. The cut-off of 4-fold differential methylation [normalized log₂ signal ratio (NLR) >2 or <-2] which was validated previously (31), was used in order to identify differentially methylated probes in IPMN DNA relative to normal pancreatic duct DNA.

Methylated gene patterns were analyzed for evidence of associated Gene network and pathway analysis using Ingenuity Pathways Analysis (Ingenuity systems, Redwood City, CA; <http://www.ingenuity.com>) software.

Bisulfite Treatment of DNA

Microdissected DNA was treated by sodium bisulfite (Sigma, St. Louis, MO) for 3 hours at 50°C as previously described (32). The bisulfite DNA was purified with Wizard DNA clean up system (Promega, Madison, WI).

Bisulfite sequencing

The methylation status of the 5' CpG islands of *PCDH17* and *SOX17* was determined by bisulfite sequencing as described previously (31). Thirty nanograms (ng) of bisulfite-treated DNA in 3 µl was amplified by PCR with RDA buffer. PCR conditions were as follows: (a) 95°C for 5 min; (b) 45 cycles of 95°C for 20 s, 62°C for 20 s and 72°C for 30 s; and (c) a final extension of 4 min at 72°C. Amplicons were purified with the QIAquick PCR purification kit (Qiagen, Germantown, MD). Sequence analysis was carried out at the Synthesis and Sequencing Facility at Johns Hopkins University using a 3730xl DNA Analyzer (Applied Biosystems). PCR primer sequences for *PCDH17* and *SOX17* genes are summarized on Supplemental Table 2.

Methylation Specific PCR (MSP)

MSP was performed as previously described using 10 ng of input DNA for each MSP reaction (25). Primer sequences for the 11 genes identified as differentially methylated are provided in Supplemental Table 2.

5-aza-2'-deoxycytidine Treatment

Cell lines were treated with 1 µmol/L of 5-aza-2'-deoxycytidine (5-aza-dC; Sigma) for 4 days as we described previously (17).

RNA Isolation and Real Time-PCR

Total RNA was extracted using the mirVana miRNA Isolation Kit (Ambion) and reverse transcribed using Superscript III Reverse Transcriptase and oligodT (Invitrogen) for quantitative reverse transcriptase-PCR (RT-PCR) following the manufacturer's protocol. cDNAs for SOX17 and EBF3 were quantified with SYBR Green PCR Master Mix (Applied Biosystems). PCR was performed with a StepOnePlus Real time PCR system (Applied Biosystems). GAPDH was used as a reference for SYBR Green. Relative expression was determined with the $\Delta\Delta C_t$ method. The primer sequences and PCR conditions are summarized on Supplemental Table 2.

Immunohistochemistry

Immunohistochemistry was performed as previously described on 88 IPMNs, including 75 IPMNs on tissue microarrays and 13 IPMNs on conventional slides, and 111 pancreatic ductal adenocarcinomas on tissue microarrays. The antigen retrieval step was carried out in a steam pressure cooker containing preheated high pH antigen retrieval buffer (DakoCytomation, Glostrup, Denmark) at 95°C for 20 min. To minimize non-specific antibody binding, sections were incubated with protein blocker (DakoCytomation) for 10 min, and stained using the LSAB Detection method (DAKO, Carpinteria, CA). Primary anti-Sox17 (GT15094, anti-goat antibody, Neuromics Antibodies, Edina, MN, 1:400 dilution) monoclonal antibodies was applied manually to the sections and incubated for 30 min at room temperature. The remaining steps were performed using an automated immunostainer (DakoCytomation). Immunolabeled sections were lightly counterstained in with hematoxylin, dehydrated in ethanol, and cleared in xylene. Immunohistochemical labeling of sox17 protein was scored using a previously described histologic score quantifying both the area and intensity of labeling (33). Labeling Intensity was designated as 0–2 for absent, weak, and strong, and the area of labeling was designated as 0–4 for <5%, 5–25%, 26–50%, 51–75, and >76%, respectively (33). Sox17 expression was classified as reduced when <50% of tumor cells were labeled.

Statistical Analysis

The mean number of methylated genes in subtypes of IPMN were compared by Student T-test and Mann-Whitney U-test. The proportion of genes methylated in each group was compared by ANOVA and post-hoc Duncan's tests. Associations between categorical variables were examined with Pearson's chi-square and Fisher's exact tests. Linear regression analysis was performed to examine the relationship between patients' age and the number of methylated genes. Statistical analyses were performed with SPSS version 17 (IBM SPSS, Somers, NY, USA). A P-value < 0.05 was considered statistically significant.

RESULTS

Characteristics of Cases

The 6 patients whose IPMNs were analyzed by MCA microarrays included 3 males and 3 females, their mean age was 65.8±11.9 years (range 48 to 79 years). One patient had an IPMN with low-grade dysplasia (6T), 3 had IPMNs with intermediate-grade dysplasia (2T, 55T, 60T), and 2 had IPMNs with high-grade dysplasia (4T, 11T). The pooled normal pancreatic duct samples were obtained from 3 of these patients (2T, 4T, and 6T).

Identification of differentially methylated loci between IPMN and normal pancreatic ducts

Six MCA experiments were performed using the Agilent 244K CpG island microarrays with each MCA experiment comparing an IPMN to pooled laser-captured microdissected normal pancreatic duct samples. A≥4-fold differential methylation [normalized log₂ signal ratio

(NLR) >2 or <-2] in the IPMNs relative to the normal pancreas was used in order to identify differentially methylated probes. This cut-off was validated using bisulfite sequencing (31). Based on this criterion, the number of probes and genes differentially methylated in each paired IPMN/normal pancreatic duct sample was 1338 probes/ 745 genes (6T, low-grade dysplasia); 893 probes/ 515 genes (2T, intermediate-grade dysplasia); 1204 probes/ 726 genes (55T, intermediate-grade); 1158 probes/ 688 genes (60T, intermediate-grade dysplasia) 1295 probes/ 710 genes (4T, high-grade dysplasia); 8449 probes, 5091 genes (11T, high-grade dysplasia). Overall, there were 10,323 uniquely hypermethylated probes in at least one of the 6 IPMN/normal pancreatic duct pairs representing 2,195 loci and 2,244 genes (Supplemental Table 3).

To identify more commonly hypermethylated genes, we determined the probes and genes hypermethylated in 2 or more of the 6 IPMNs evaluated by MCA microarray analysis. The resulting list included 2,406 hypermethylated probes representing 692 loci and 708 genes (Supplemental Table 4).

Understanding the molecular events associated with high-grade IPMN neoplasia is particularly important not only because of their potential biological importance but also because IPMNs with high-grade dysplasia (high-grade IPMNs) warrant surgical resection and markers of high-grade IPMNs could have diagnostic utility. We therefore identified probes differentially methylated in the 2 high-grade IPMNs compared to normal pancreatic duct samples. This comparison yielded 840 probes, corresponding to 245 genes (Supplemental Table 5). Of these 245 hypermethylated genes, 53 genes were only hypermethylated in both of the high-grade IPMNs (Supplemental Table 6).

Many on this list of 245 hypermethylated genes have been identified as aberrantly methylated in ductal adenocarcinomas of the pancreas, including *ADCYAP1*, *ALK*, *CCNA1*, *EYA4*, *GALR1*, *LHX1*, *LHX2*, *LIN28*, *MYOD1*, *MDF1*, *NPY*, *NRN1*, *NRXN1*, *PAX1*, *PENK*, *PITX2*, *TFPI2*, *SALL3*, *SLIT2*, *SOX17*, *TLX3*, and *ZNF415* (17, 18, 20, 31, 34). We also confirmed our previous findings of aberrant methylation of the genes *TFPI2*, *SARP2/SFRP1*, *PENK*, and *CLDN5* in a subset of IPMNs (24, 25). The genes, *DLX1*, *EVX2*, and *TBX18*, have been reported to be abnormally hypermethylated in other cancers (Supplemental Table 7).

Some important genes were methylated in only one of the IPMNs including *CDKN2A/p16* (Supplemental Table 2). Several genes methylated in <10% of pancreatic cancers such as *CDH1/e-cadherin* and *hMLH1* (23, 24) were not identified as methylated in any of the IPMNs.

Differentially Hypermethylated Genes in IPMNs vs. Pancreatic Cancers

We compared the number of differentially methylated genes in IPMNs to those of pancreatic cancers obtained using the same MCAM strategy utilized to profile IPMNs (35). The median number of probes methylated in the IPMNs was 1249.5 and for pancreatic cancer it was 7713 ($p=0.018$, Mann-Whitney U test). The median number of hypermethylated genes in IPMNs relative to normal pancreatic duct (506 ± 1804.0 genes) was significantly lower than in 9 pancreatic cancers relative to normal pancreatic duct (2285.7 ± 608.2 genes) ($P=0.04$).

Validation of hypermethylated genes by MCA microarray analysis

To confirm the accuracy of our MCA microarray data, we utilized MSP to evaluate the methylation status of 13 genes in an independent set of 61 IPMNs and 43 normal pancreas samples. Genes that were selected had multiple probes identified as differentially methylated by MCAM. These genes were analyzed in an independent set of IPMNs and normal

pancreas samples. This analysis was limited to 13 genes because the amount of available DNA from our tumor samples available for these assays after bisulfite-modification was limited. Indeed, three of the genes were tested in a smaller set of IPMNs and normal pancreas samples (*BNIP3*, *PTCHD2*, *LIN28*) because of limited sample availability. Most of these genes, including *BNIP3*, *PTCHD2*, *LIN28*, *NXP1*, *SOX17*, *EBF3*, *PAX6*, *FOXF2*, *PCDH17*, and *SALL3*, were identified as methylated by MCAM analysis in the high-grade IPMNs. Representative images of results of MSP are depicted in Supplemental Figure 1. The demographic and clinicopathological information of the 65 patients whose IPMNs were studied is summarized on Table 1 and Supplemental Table 1. Two of 6 IPMN samples used for MCA microarray were included in the validation, one an IPMN with low-grade dysplasia, and the other an IPMN with intermediate dysplasia and the results of MSP and MCAM were 100% concordant in these 2 samples.

Overall, the genes tested for methylation by MSP analysis confirmed the results of the MCAM array analysis, showing that the majority of genes tested were commonly methylated in the IPMNs, compared to much lower prevalence of methylation in normal pancreas (<10% of samples for most genes evaluated). Two of the 13 genes tested (*IRX2* and *HoxA5*) were not identified as differentially methylated in IPMNs relative to normal pancreatic duct samples by MSP (methylated DNA was as prevalent in normal as in IPMN samples).

By MSP analysis, significantly more genes were methylated in IPMNs than in normal pancreata and higher grade IPMNs had more methylated genes than lower-grade IPMNs. Thus, **48.4±21.9%** of genes were methylated in IPMNs with low-grade dysplasia, **52.2±24.2%** in IPMNs with intermediate-grade dysplasia, **73.9±20.9%** in IPMNs with high-grade dysplasia, and 85.1±18.5% in IPMNs with an associated invasive adenocarcinoma ($P<0.0001$, ANOVA; Supplemental Figure 2). More genes were methylated in IPMNs with high-grade dysplasia or with associated invasive adenocarcinoma than IPMNs with low-grade dysplasia or with intermediate-grade dysplasia ($P<0.05$, post-hoc Duncan test). And more genes were methylated in IPMNs with low-grade dysplasia or IPMNs with intermediate-grade dysplasia than in normal pancreata ($P<0.05$, post-hoc Duncan test). These results are summarized in Table 2 and Supplemental Figure 2. These results are consistent with our previous investigations of IPMN methylation investigating candidate genes that had been identified as aberrantly methylated in pancreatic ductal adenocarcinomas (25, 36). In this previous study, we also found an overall trend for a higher prevalence of aberrant methylation in higher grade IPMNs, but this was limited to some genes (e.g. *TFPI-2*, *TSLC1*, *SARP2*, *UCHL1*, *CLDN5*), whereas for other genes, aberrant methylation is common even low-grade IPMNs (*SPARC*, *RELN*).

Since IPMNs can be subclassified by their anatomic localization and their histological subtype, we compared DNA methylation profiles within these subgroups. The percentage of genes methylated in main-duct IPMNs (**71.3±23.8%**) was significantly higher than those in branch-duct type (**44.4±20.0%**, $P<0.0001$). Main-duct IPMNs are more likely to be associated with high-grade lesions and invasive adenocarcinomas, and once histologic grade is taken into account, the relationship between the number and pattern of methylated genes with IPMN location was no longer significant. IPMNs also can be subclassified by their histological phenotypes into pancreatobiliary, intestinal and gastric subtypes. We found that the percentage of genes methylated in pancreatobiliary type IPMNs (**72.8±22.6%**) was significantly higher than gastric type IPMN (**51.2±22.5%**, $P=0.001$, Student-T test), but not after adjusting for grade of IPMN. Similarly, there was a borderline difference in the percentage of genes methylated in gastric vs. intestinal-type IPMNs (64.9±26.5)($P=0.08$), but this difference was also not significant after adjusting for neoplastic grade.

The number of genes methylated in normal pancreatic tissues from patients with ductal neoplasms was marginally but not significantly higher than in those from patients with well-differentiated pancreatic endocrine neoplasms (13.0 ± 13.9 vs. $4.7 \pm 9.3\%$, $p=0.08$, Mann-Whitney U-test) which could be related to patient age as the mean age of patients with ductal neoplasia (65 ± 12 years) was significantly higher than those with well-differentiated pancreatic endocrine neoplasms (53 ± 13 years, $P=0.02$, Mann-Whitney U-test). Among patients with IPMNs, we found a significant correlation between patient age and the number of methylated genes in their IPMN ($P < 0.001$, $R=0.34$, Linear regression analysis). There was no evidence that this was related to IPMN grade as there was no significant difference in patient age by IPMN grade in our study population. These results are similar to our previous analysis of methylation in pancreatic samples (25) where with a different panel of genes we observed a small but significantly higher number of methylated genes in the normal pancreata of patients with ductal neoplasms compared to patients with neuroendocrine tumors (25).

We next compared the progression of individual gene methylation by neoplastic grade. Aberrant hypermethylation of *BNIP3* was more common in IPMNs with high-grade dysplasia (57%, 8/14 cases) than those with intermediate-(20%, 3/15 cases, $P=0.06$), or low-grade dysplasia (0%, 0/8 cases, $P=0.02$) and more common than in normal pancreas samples (0%, 0/18 cases, $P < 0.001$). Similarly, *PTCHD2* methylation was also significantly more common in IPMNs with high-grade dysplasia (50%, 7/14 cases) than those with low-grade dysplasia (0%, 0/8 cases, $P=0.02$) or normal pancreas (6%, 1/18 cases, $P=0.001$). *PTCHD2* methylation is also detected in a smaller percentage of IPMNs with intermediate-grade dysplasia (27%, 3/11 cases). There was also evidence of increasing methylation of *EBF3* ($P=0.01$; chi-square test), *SOX17* ($P=0.01$), and *NXP1* ($P=0.001$) by neoplastic grade, but not for any of the other genes analyzed, *PAX6* ($P=0.16$), *SALL1* ($P=0.68$), *FOXF2* ($P=0.13$), *SALL3* ($P=0.14$) or *PCDH17* ($P=0.13$). The presence of methylation in at least one of the two markers most specifically associated with high-grade dysplasia (*BNIP3* and *PTCHD2*) had a sensitivity of 71% (10/14).

SOX17 Expression Analysis

To evaluate the expression of one of the aberrantly methylated genes in IPMNs, we selected *SOX17*, a gene with an available antibody to its protein product. (There were no commercially antibodies available that had been shown to work for immunohistochemistry to the protein products of other genes we analyzed by MSP). We next examined the expression of the protein product of one of the genes commonly methylated in IPMNs by immunohistochemistry. Among 88 resected primary IPMNs (see Figure 2 for representative images), Sox17 protein expression was significantly lower in IPMNs than in associated normal pancreatic duct (histologic score 3.0 ± 2.2 vs. 7.8 ± 0.7 , Student *T*-test, $p < 0.001$). Sox17 expression was absent in 27% (4/15) of low-grade IPMNs, 21% (6/29) of intermediate-grade IPMNs, and 20% (9/44) of high-grade IPMNs. Reduced expression of *sox17* was observed in 60% (9/15) of low-grade IPMNs, 69% (20/29) of intermediate-grade IPMNs, and 75% (33/44) of high-grade IPMNs. Loss of Sox17 expression in IPMNs was not associated with clinicopathologic factors, such as histologic subtype, grade and tumor location. We also observed complete loss of Sox17 in a similar proportion of pancreatic ductal adenocarcinomas (29%, 32/111).

We also found evidence for epigenetic regulation of Sox17 expression in pancreatic cancer cells. *SOX17* RNA was upregulated in several pancreatic cancer cell lines, including A32-1, Capan1, FamPanc, and Panc2.5 after 5-aza-dC treatment (Supplemental Figure 3). We also performed bisulfite sequencing of *SOX17* in pancreatic cancer cell lines and found methylation of most of the CpGs in the 5' region of *SOX17* in 6 pancreatic cancer cell lines tested, and it was predominantly unmethylated in 6 normal pancreatic samples, and the non-

neoplastic cell line, HPDE (Supplemental Figure 4). Similarly, 5' CpGs of *PCDH17* were predominantly methylated in 11 pancreatic cancer samples, and predominantly unmethylated in 5 of 8 normal pancreatic tissue samples (Supplemental Figure 5).

Discussion

In this study we find that the pancreatic precursor neoplasms known as IPMNs are characterized by widespread aberrant hypermethylation affecting several hundred CpG islands. Extensive aberrant hypermethylation has been demonstrated in pancreatic and other invasive cancers, but the timing of such aberrant methylation during tumor development has not been well established. In our study we find that even low-grade IPMNs harbor aberrant methylation of hundreds of CpG islands, with evidence of increased levels of methylation with increasing neoplastic grade. Consistent with this trend is the finding that significantly more genes are methylated in pancreatic ductal adenocarcinomas than in IPMNs.

Among the genes tested, both *BNIP3* and *PTCHD2* had promising performance characteristics as diagnostic markers of IPMNs with high-grade dysplasia. Aberrant methylation of *BNIP3* has been described in pancreatic ductal adenocarcinomas, but not in IPMNs (37). *PTCHD2/DISP3* is a trans-membrane protein and shares a PTCH/DISP homologous domain and aberrant methylation of *PTCHD2/DISP3* has not been described in any solid cancer.

Our MCAM analysis identified other genes that were selectively methylated in high-grade IPMNs including the gene *TFPI-2* which we have previously described as much more commonly methylated in high-grade IPMNs and infiltrating adenocarcinomas than in low- or intermediate-grade of IPMNs (25). Since most IPMNs are detected incidentally when they are small neoplasms of low malignant potential, they are managed by surveillance and only undergo resection when there is a concern for high-grade neoplasia or malignancy. Since imaging tests cannot differentiate low-grade from high-grade neoplastic lesions, markers that can reliably identify the grade of neoplasia in IPMNs could help determine the optimal timing of resection for patients undergoing surveillance (38). Genes with methylation profiles highly specific for high-grade dysplasia in IPMNs could be useful as markers for detection in fine needle aspirates of IPMN samples (3, 38-40).

Of the 2244 genes identified as aberrantly methylated in any IPMN, many have been identified as aberrantly methylated in pancreatic cancers, including genes bound by polycomb and those involving pathways and gene families commonly targeted in pancreatic ductal adenocarcinomas. For example, of the 331 aberrantly methylated genes involved in transcription regulation, many were members of the homeobox superfamily. Indeed, 41 of the 245 aberrantly methylated genes in high-grade IPMNs were homeobox genes. Homeobox genes encode transcription factors and play a key role in development by activating or repressing down-stream target genes involved in many cellular processes, including adhesion, apoptosis, body patterning, differentiation, migration, proliferation, polarity and proliferation and are commonly observed in cancers (41).

Several members of the Wnt signaling pathway were also found to be hypermethylated in IPMNs by MCAM analysis including multiple genes previously shown to be epigenetically silenced both in invasive pancreatic and other types of cancer such as *FZD10*, *SFRP1*, *APC2*, *SOX11* and *SOX17*. *SOX17* is expressed in the developing pancreas and has an important role in endoderm development (42). Sox17 represses the Wnt/ β -catenin signaling pathway (43) and its silencing has been reported in colorectal and stomach cancers (43, 44). We and others have previously described aberrant hypermethylation of *SOX17* in pancreatic cancers (31, 34). In the present study, we find that aberrant hypermethylation of *SOX17* and

silencing of its protein product is evident early during pancreatic neoplastic development and the prevalence of methylation increases with IPMN grade, and this was associated with silencing of *SOX17* expression in ~1/4 of IPMNs and pancreatic ductal adenocarcinomas.

Another gene we found to be commonly methylated in IPMNs was the putative tumor suppressor gene *EBF3/COE3* which encodes a transcription factor involved in regulating differentiation and neurogenesis (45). Although aberrant methylation of *EBF3* has not been reported in pancreatic neoplasms previously, such methylation has been reported in several cancers, including glioblastoma and head and neck squamous cell carcinomas (45, 46). Furthermore, *EBF3* mutation has been described (47). Methylation of *EBF3* by MSP was detected in only 1 of 40 normal pancreas tissues.

Many genes involved in cell adhesion were also hypermethylated in IPMNs, including members of the cadherin superfamily *CDH2*, *CDH4*, *PCDH7*, *PCDH8*, *PCDH10*, *PCDH17*, and *PCDHB7*. Loss of the protein product of *CDH2* (n-cadherin) expression has been reported in infiltrating pancreatic ductal adenocarcinomas (48). We and others have previously reported occasional silencing, mutation and focal loss of expression of *CDH1/E-cadherin* in pancreatic cancers (49, 50). Interestingly, occasional somatic mutations of cadherins and protocadherins have been identified in pancreatic cancers (47).

In summary, genome-wide CpG island methylation profiling of IPMNs reveals that IPMNs harbor aberrant methylation of hundreds of CpG islands relative to normal pancreatic duct and that aberrant CpG island methylation increases with IPMN grade. The specificity of methylation of genes such as *BNIP3* for high-grade dysplasia highlights the potential diagnostic applications of methylated genes in the evaluation of IPMNs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Statement of Translational Relevance

Pancreatic cancer is the fourth leading cause of cancer death in the United States. Intraductal papillary mucinous neoplasms (IPMNs) are an important precursor to pancreatic adenocarcinoma. They are commonly detected as asymptomatic lesions during abdominal scanning and pancreatic screening of individuals at high risk of developing pancreatic cancer. Most IPMNs are first detected as small lesions of low malignant potential necessitating regular imaging until their IPMN has features suggesting progression to adenocarcinoma. Molecular marker analysis of IPMNs to identify high-grade dysplasia and invasive cancer would permit optimal timing for surgical resection of IPMNs. In addition, a better understanding of molecular mechanisms that arise in pancreatic cancer precursors would improve our understanding of pancreatic cancer biology. In this study we describe the first genome-wide analysis of IPMNs and identify hundreds of aberrantly hypermethylated genes in IPMNs and identify several genes such as *BNIP3* whose hypermethylation is highly specific for high-grade IPMNs.

Methylated CpG island amplification (MCA) array analysis of IPMN

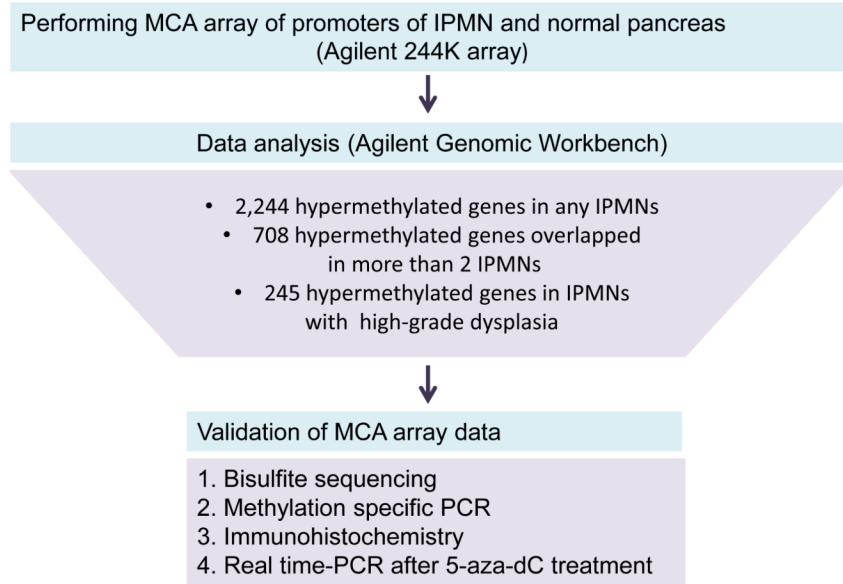


Figure 1. An overview of the experimental approach used to evaluate differential DNA methylation in intraductal papillary mucinous neoplasms

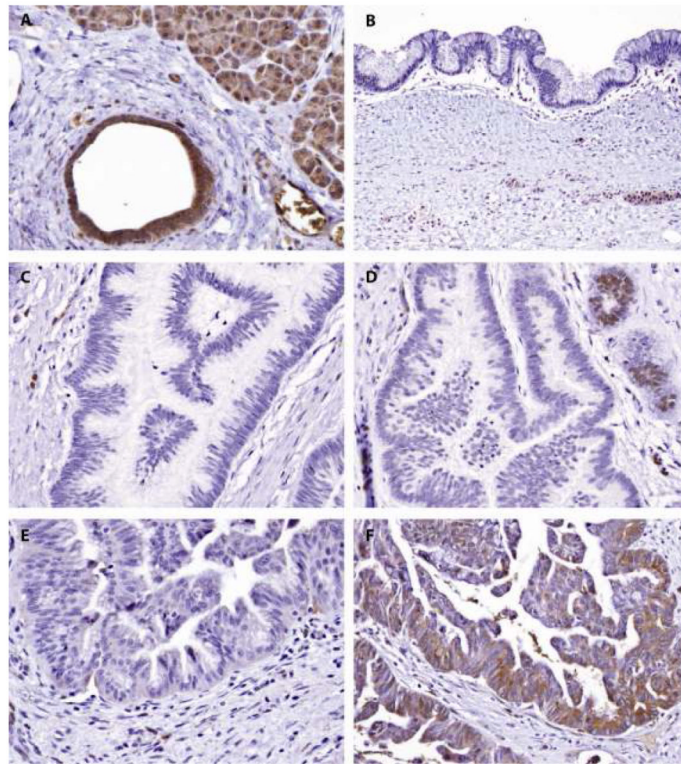


Figure 2.

Immunohistochemical analysis of Sox17 protein expression of IPMNs (A) Sox17 labeling in normal pancreatic ductal epithelial cells and acinar cells (x20). Lack of tumoral sox17 expression in (B) an IPMN with low-grade dysplasia (x10), (C-D) an IPMN with intermediate-grade dysplasia (x20), and (E) an IPMN with high-grade dysplasia (x20). (F) An IPMN with high-grade dysplasia expressing sox17. Stromal fibroblasts expressed sox17.

Table 1
Clinicopathologic characteristics of patients with IPMNs

Parameter	Number (percentage)
Age (mean±Standard deviation)	68.5±10.5
Sex	
Male	34 (52%)
Female	31 (48%)
Age (mean, year)	68.5+10.4
Ethnicity	
Caucasian	59 (90%)
African American	3 (5%)
Other	3 (5%)
Association with adenocarcinoma	
Yes	10 (15%)
No	55 (85%)
Degree of dysplasia	
IPMN, low-grade dysplasia	14 (22%)
IPMN, intermediate dysplasia	19 (29%)
IPMN, high-grade dysplasia	32 (49%)
Tumor location	
Main duct	43 (66%)
Branch duct	17 (26%)
Unknown	5 (8%)
Histologic subtype	
Gastric	23 (35%)
Intestinal	13 (20%)
Pancreatobiliary	29 (45%)

Table 2

Summary of methylation profiles of each grade of IPMNs, and IPMNs with associated invasive adenocarcinomas arising in IPMNs, and peritumoral normal pancreatic tissues determined by MSP.

Patients No IPMN with low-grade dysplasia	Age	Sex	Ethnicity	IPMN ductal subtype	Histologic subtype	SOX17	PAX6	SALL1	SALL3	FOXF2	NXP1	EBF3	PCDH17	LIN28A	PTCHD2	BNIP3	# of methylated genes	% of methylated genes
01L	82	M	C	Main	Gastric	U	U	M	M	M	U	U	NT	NT	NT	NT	3/7	43%
12L	76	F	C	Main	Gastric	M	M	M	M	NT	U	U	NT	NT	NT	NT	6/8	75%
17L	77	F	C	Main	Gastric	M	M	M	M	NT	NT	NT	NT	NT	NT	NT	4/4	100%
32L	69	M	C	Main	Gastric	M	U	M	M	M	U	U	M	NT	NT	NT	5/8	63%
34L	47	M	C	Branch	Gastric	U	U	M	M	U	U	U	M	M	U	U	4/11	36%
37L	72	F	C	Branch	Gastric	U	U	M	M	M	U	U	M	M	U	U	5/11	45%
38L	66	F	C	Main	Gastric	U	U	M	M	M	U	M	M	NT	NT	NT	5/8	63%
39L	63	M	C	Unknown	Gastric	U	M	M	U	U	M	U	M	U	U	U	4/11	36%
40L	57	M	C	Branch	Gastric	U	U	M	M	U	U	M	M	M	U	U	5/11	45%
43L	72	F	C	Branch	Gastric	U	M	M	M	M	M	M	U	M	U	U	7/11	64%
46L	72	F	C	Branch	Gastric	U	U	U	M	M	U	U	U	NT	NT	NT	2/8	25%
47L	56	F	C	Branch	Gastric	U	U	M	M	U	U	U	U	M	U	U	3/11	27%
57L	53	F	C	Branch	Gastric	M	U	M	M	U	U	U	U	U	U	U	3/11	27%
63L	59	M	C	Branch	Gastric	U	M	U	M	U	U	U	U	M	U	U	3/11	27%
IPMN with intermediate dysplasia						4/14	5/14	12/14	13/14	6/12	2/13	3/13	6/11	6/8	0/8	0/8	0/8	48%
						29%	33%	86%	93%	50%	15%	23%	55%	75%	0%	0%	0%	
14I	69	M	C	Main	Pancreatobiliary	U	U	M	M	M	M	M	NT	NT	NT	NT	5/7	71%
18I	60	F	AA	Main	Pancreatobiliary	M	M	M	M	U	U	U	U	NT	NT	NT	4/8	50%
22I	74	F	C	Main	Pancreatobiliary	M	M	M	M	U	U	M	M	M	NT	U	7/10	70%
27I	73	M	C	Main	Pancreatobiliary	M	M	M	M	M	M	M	M	M	NT	M	10/10	100%
33I	79	M	C	Main	Intestinal	U	M	U	U	U	U	NT	NT	NT	NT	NT	1/6	17%
36I	82	M	C	Unknown	Gastric	M	M	U	U	M	M	NT	NT	NT	NT	NT	4/6	67%
41I	55	F	C	Branch	Gastric	U	U	M	M	M	U	M	M	NT	NT	NT	5/8	63%
44I	78	F	C	Unknown	Pancreatobiliary	U	U	M	M	M	M	M	U	M	NT	U	6/10	60%
49I	81	F	C	Branch	Gastric	M	U	M	M	M	M	U	U	M	U	U	6/11	55%

Patients No IPMN with low-grade dysplasia	Age	Sex	Ethnicity	IPMN ductal subtype	Histologic subtype	SOX17	PAX6	SALL1	SALL3	FOXF2	NXP1	EBF3	PCDH17	LIN28A	PTCHD2	BNIP3	# of methylated genes	% of methylated genes
50I	72	F	C	Branch	Gastric	M	M	M	M	M	M	U	U	M	NT	U	7/10	70%
51I	61	F	C	Branch	Intestinal	M	M	M	M	M	M	U	M	NT	NT	NT	7/8	88%
55I	84	F	C	Main	Pancreatobiliary	U	M	M	M	FA	U	M	U	U	M	U	4/10	40%
58I	61	M	C	Branch	Intestinal	U	U	M	M	M	M	FA	U	U	U	U	4/10	40%
59I	80	M	C	Main	Pancreatobiliary	FA	FA	M	U	U	M	U	M	M	U	U	4/9	44%
60I	72	F	H	Branch	Intestinal	U	M	U	U	U	U	U	U	M	M	U	3/11	27%
62I	56	M	C	Branch	Gastric	U	U	U	U	U	U	U	M	U	U	U	1/11	9%
64I	73	M	C	Branch	Gastric	M	U	M	FA	U	U	M	M	NT	NT	NT	4/7	57%
65I	56	M	C	Main	Pancreatobiliary	U	U	FA	M	M	U	U	U	M	U	U	3/10	30%
67I	87	F	C	Main	Intestinal	U	U	M	U	FA	M	U	M	M	M	M	6/10	60%
69I	72	F	H	Main	Intestinal	NT	NT	NT	NT	NT	NT	NT	NT	M	U	M	2/3	67%
74I	66	M	C	Main	Intestinal	NT	NT	NT	NT	NT	NT	NT	NT	M	U	U	1/3	33%
73I	66	M	C	Main	Gastric	NT	NT	NT	NT	NT	NT	NT	NT	M	U	U	1/3	33%
IPMN with high-grade dysplasia																		
05H	81	M	C	Main	Pancreatobiliary	M	U	M	M	M	M	U	U	NT	NT	NT	5/8	63%
06H	35	M	C	Main	Intestinal	FA	U	U	M	M	U	NT	NT	NT	NT	NT	2/5	40%
07H	68	F	C	Main	Pancreatobiliary	M	M	U	M	M	M	M	M	NT	NT	NT	7/8	88%
09H	59	M	C	Unknown	Pancreatobiliary	NT	M	U	M	M	U	NT	U	NT	NT	NT	3/6	50%
13H	57	M	C	Unknown	Pancreatobiliary	U	M	M	M	M	M	U	U	NT	NT	NT	5/8	63%
15H	68	M	C	Main	Pancreatobiliary	M	M	M	M	M	NT	NT	NT	NT	NT	NT	4/4	100%
19H	74	F	C	Main	Gastric	M	M	M	M	M	M	NT	NT	NT	NT	NT	6/6	100%
20H	65	M	C	Main	Pancreatobiliary	M	M	M	M	M	M	M	M	M	U	M	8/8	100%
23H	80	M	C	Main	Intestinal	M	M	M	M	M	NT	NT	NT	NT	NT	NT	4/4	100%
25H	66	F	C	Main	Pancreatobiliary	FA	M	M	M	M	U	U	U	M	M	M	5/7	71%
26H	56	M	C	Main	Pancreatobiliary	M	U	M	M	M	M	M	M	U	U	M	7/8	88%
28H	76	M	C	Main	Pancreatobiliary	M	M	M	M	U	U	U	M	M	U	U	5/8	63%
31H	80	F	C	Main	Intestinal	M	U	M	M	M	M	M	M	M	M	M	7/8	88%

Patients No IPMN with low-grade dysplasia	Age	Sex	Ethnicity	IPMN ductal subtype	Histologic subtype	SOX17	PAX6	SALL1	SALL3	FOXF2	NXP1	EBF3	PCDH17	LIN28A	PTCHD2	BNIP3	# of methylated genes	% of methylated genes
42H	76	M	C	Main	Intestinal	U	M	M	M	M	M	M	M	NT	NT	NT	7/8	88%
45H	76	M	O	Main	Pancreatobiliary	M	U	M	U	M	NT	NT	M	NT	NT	NT	5/7	71%
48H	63	M	C	Main	Intestinal	M	M	M	M	M	U	U	M	M	M	M	6/8	75%
52H	74	F	C	Main	Pancreatobiliary	M	M	M	M	M	M	M	M	M	M	U	8/8	100%
53H	53	M	C	Main	Intestinal	M	M	M	M	FA	U	U	M	M	M	M	6/7	86%
54H	50	M	O	Main	Pancreatobiliary	M	U	U	M	U	M	M	U	M	U	M	4/8	50%
56H	66	M	C	Main	Intestinal	FA	M	M	M	FA	U	U	U	NT	NT	NT	4/6	67%
61H	68	M	C	Branch	Gastric	U	U	M	M	M	U	M	U	M	U	U	4/8	50%
66H	58	M	C	Main	Pancreatobiliary	FA	M	FA	M	U	U	M	U	M	U	U	3/6	50%
71H	35	M	C	Main	Intestinal	NT	NT	NT	NT	NT	NT	NT	NT	M	M	U	2/3	67%
72H	81	M	C	Main	Pancreatobiliary	NT	NT	NT	NT	NT	NT	NT	NT	M	U	U	1/3	33%
75H	60	M	C	Main	Pancreatobiliary	NT	NT	NT	NT	NT	NT	NT	NT	M	M	M	3/3	100%
						14/17	15/22	16/21	21/22	15/18	15/20	9/16	10/18	13/14	7/14	8/14	74%	
IPMN with associated invasive adenocarcinoma						82%	68%	76%	95%	83%	75%	56%	56%	93%	50%	57%		
03CA	79	F	C	Main	Pancreatobiliary	NT	NT	NT	NT	M	FA	NT	M	NT	NT	NT	2/2	100%
04CA	77	F	C	Main	Intestinal	NT	M	M	M	M	M	M	NT	NT	NT	NT	6/6	100%
07CA	68	F	C	Main	Pancreatobiliary	U	U	U	M	M	M	M	M	NT	NT	NT	5/8	63%
12CA	76	F	C	Main	Gastric	FA	FA	M	M	U	U	NT	NT	NT	NT	NT	2/4	50%
18CA	60	F	AA	Main	Pancreatobiliary	M	M	M	M	U	U	M	NT	NT	NT	NT	5/7	71%
22CA	74	F	C	Main	Pancreatobiliary	U	M	M	M	NT	NT	NT	M	NT	NT	NT	4/5	80%
24CA	83	F	AA	Main	Pancreatobiliary	M	M	M	M	M	M	M	M	NT	NT	NT	8/8	100%
25CA	66	F	C	Main	Pancreatobiliary	M	U	M	M	FA	M	M	M	NT	NT	NT	7/8	88%
27CA	73	M	C	Main	Pancreatobiliary	M	M	M	M	M	M	M	M	NT	NT	NT	8/8	100%
28CA	76	M	C	Main	Pancreatobiliary	M	M	M	M	M	M	M	M	NT	NT	NT	8/8	100%
Normal from endocrine neoplasm						5/8	6/8	9/10	9/10	6/9	7/9	8/8	7/7				85%	
						63%	75%	90%	90%	67%	78%	100%	100%					

Patients No IPMN with low-grade dysplasia	Age	Sex	Ethnicity	IPMN ductal subtype	Histologic subtype	SOX17	PAX6	SALL1	SALL3	FOXF2	NXPPI	EBF3	PCDH17	LIN28A	PTCHD2	BNIP3	# of methylated genes	% of methylated genes
NE01	46	M	C	NA	NA	U	U	U	U	U	U	M	U	NT	NT	NT	1/8	13%
NE02	63	F	C	NA	NA	U	U	U	U	U	U	U	U	NT	NT	NT	0/8	0%
NE03	42	F	C	NA	NA	U	U	U	U	U	U	U	U	NT	NT	NT	0/8	0%
NE04	39	F	C	NA	NA	U	U	U	U	NT	NT	NT	U	NT	NT	NT	0/5	0%
NE08	56	F	C	NA	NA	U	U	U	M	U	U	U	M	NT	NT	NT	2/8	25%
NE09	78	M	C	NA	NA	U	U	U	U	U	U	U	U	NT	NT	NT	0/8	0%
NE10	41	F	C	NA	NA	U	U	U	U	U	U	U	U	NT	NT	NT	0/8	0%
NE11	57	M	C	NA	NA	U	U	U	U	U	U	U	U	NT	NT	NT	0/8	0%
						0/8	0/8	0/8	1/8	0/7	0/7	1/7	1/8				13%	5%
Normal from IPMN or cancer																		
NLPDA01	62	M	AA	NA	NA	U	U	U	U	M	U	U	U	NT	NT	NT	1/8	13%
NLPDA02	61	M	C	NA	NA	U	U	U	U	U	U	U	U	M	U	U	1/11	9%
NLPDA03	78	F	C	NA	NA	U	U	U	M	U	NT	U	U	M	U	U	2/10	20%
NLPDA04	59	F	C	NA	NA	U	U	U	U	U	U	U	U	M	U	U	1/11	9%
NLPDA05	56	F	C	NA	NA	U	U	U	U	U	U	U	U	U	U	U	0/11	0%
NLPDA06	78	M	C	NA	NA	U	U	U	M	M	U	U	U	U	U	U	2/11	18%
NLPDA07	61	F	C	NA	NA	U	U	FA	M	U	FA	U	M	NT	NT	NT	2/6	33%
NLPDA08	76	M	C	NA	NA	U	U	U	M	U	U	U	U	U	M	U	2/11	18%
NLPDA09	51	M	C	NA	NA	M	M	M	M	U	U	U	M	NT	NT	NT	5/8	63%
NLPDA10	70	M	C	NA	NA	U	M	U	U	U	U	U	NT	NT	NT	NT	1/8	13%
NLPDA11	78	F	C	NA	NA	U	U	U	U	U	M	U	U	NT	NT	NT	1/8	13%
NLPDA14	59	M	C	NA	NA	U	U	U	U	U	U	U	U	NT	NT	NT	0/8	0%
NLPDA15	56	M	O	NA	NA	U	U	U	M	NT	U	NT	NT	NT	NT	NT	1/5	20%
NLPDA16	72	M	C	NA	NA	U	U	U	U	U	U	NT	NT	NT	NT	NT	0/6	0%
NLPDA17	72	M	C	NA	NA	M	U	M	U	U	U	U	U	M	U	U	3/11	27%
NLPDA18	76	M	C	NA	NA	M	M	U	M	U	U	U	U	NT	NT	NT	3/8	38%
NLPDA21	60	F	C	NA	NA	U	U	M	U	U	U	U	U	M	U	U	2/11	18%
NLPDA22	31	M	C	NA	NA	U	U	U	U	U	U	U	U	U	U	U	0/11	0%

Patients No IPMN with low-grade dysplasia	Age	Sex	Ethnicity	IPMN ductal subtype	Histologic subtype	SOX17	PAX6	SALL1	SALL3	FOXF2	NXPPI	EBF3	PCDH17	LIN28A	PITCHD2	BNIP3	# of methylated genes	% of methylated genes
NLPDA23	53	M	C	NA	NA	U	U	U	U	U	U	U	U	U	U	U	0/11	0%
NLPDA24	58	M	C	NA	NA	U	U	M	U	U	U	U	U	U	U	U	1/11	9%
NLPDA25	59	F	C	NA	NA	U	M	M	U	M	U	U	U	U	U	U	3/11	27%
NLPDA26	51	M	C	NA	NA	U	U	U	U	U	U	U	U	U	U	U	0/11	0%
NLPDA27	60	F	C	NA	NA	U	U	U	U	U	U	U	U	U	U	U	0/11	0%
NLPDA28	54	M	C	NA	NA	U	U	U	U	U	U	U	U	U	U	U	0/11	0%
NLPDA29	52	M	C	NA	NA	U	U	U	U	U	U	U	U	NT	NT	NT	0/8	0%
NLPDA31	66	M	C	NA	NA	U	M	U	M	M	U	U	U	U	U	U	3/11	27%
NLPDA32	62	M	C	NA	NA	U	U	U	U	U	U	U	U	U	U	U	0/11	0%
NLPDA33	84	F	C	NA	NA	U	U	U	U	U	U	U	U	NT	NT	NT	0/8	0%
NLPDA34	81	M	C	NA	NA	U	U	U	U	U	U	U	U	NT	NT	NT	0/8	0%
NLPDA35	66	M	C	NA	NA	U	U	U	U	U	M	U	U	NT	NT	NT	1/8	13%
NLPDA36	64	F	C	NA	NA	U	U	U	U	U	U	U	U	NT	NT	NT	0/8	0%
NLPDA37	70	F	C	NA	NA	U	U	U	M	M	U	U	U	NT	NT	NT	2/8	25%
NLPDA38	78	M	C	NA	NA	U	U	U	U	M	U	U	U	NT	NT	NT	1/8	13%
NLPDA39	74	F	C	NA	NA	U	U	U	M	U	U	U	U	NT	NT	NT	1/8	13%
NLPDA40	88	F	C	NA	NA	U	U	U	M	M	U	U	U	U	U	U	2/11	18%
	3/35					9%	5/35	5/34	11/35	7/34	2/33	0/33	2/32	5/18	1/18	0/18		13%
							14%	15%	31%	21%	6%	0%	6%	28%	6%	0%		

M, methylated; U, unmethylated; NT, not tested;

FA, failed to amplify; NA, not applicable.

M box indicates completely methylated

M box indicates partially methylated