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CpG island methylation profile of pancreatic intraepithelial neoplasia

Norihiro Sato, Noriyoshi Fukushima, Ralph H Hruban and Michael Goggins

Departments of Pathology, Oncology and Medicine, The Sol Goldman Pancreatic Cancer Research Center, The Johns Hopkins Medical Institutions, Baltimore, MD, USA

Infiltrating adenocarcinoma of the pancreas is thought to develop through well-defined precursor lesions called pancreatic intraductal neoplasia (PanIN). Despite the exponential growth in our understanding of genetic events that characterize the progression of PanINs to invasive carcinoma, little is known about the role of epigenetic alterations in these precursor lesions. To define the timing and prevalence of methylation abnormalities during early pancreatic carcinogenesis, we investigated the CpG island methylation profile in the various grades of PanINs. Using methylation-specific PCR, we analyzed DNA samples from 65 PanIN lesions for methylation status of eight genes recently identified by microarray approach as aberrantly hypermethylated in invasive pancreatic cancer. Aberrant methylation at any of the eight genes was identified in 68% of all the PanIN lesions examined, and, notably, aberrant methylation was identified in more than 70% of the earliest lesions (PanIN-1A). The average number of methylated loci was 1.1 in PanIN-1A, 0.8 in PanIN-1B, 1.1 in PanIN-2, and 2.9 in PanIN-3 lesions ($P=0.01$ for PanIN -3 *vs* earlier PanINs). Among the genes analyzed, *NPTX2* demonstrated an increase in methylation prevalence from PanIN-1 to PanIN-2 ($P=0.0008$), and from PanIN-2 to PanIN-3 for *SARP2* ($P=0.001$), *Reprimo* ($P=0.01$), and *LHX1* ($P=0.03$). These results suggest that aberrant CpG island hypermethylation begins in early stages of PanINs, and its prevalence progressively increases during neoplastic progression.
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Accumulating evidence supports the hypothesis that infiltrating adenocarcinoma of the pancreas develops from noninvasive precursor lesions in the small ducts and ductules, called pancreatic intraductal neoplasia (PanIN).[1,2] Characterization of molecular basis for these precursor lesions may refine our understanding of pancreatic ductal carcinogenesis and also provide important insight into early pancreatic cancer detection strategies and novel targets for chemoprevention.[3] Many of the genetic abnormalities observed in invasive pancreatic cancer have also been observed in PanIN lesions. The reported genetic alterations in PanINs include activating point mutations in the *KRAS2* oncogene[4] and inactivation of *p16/CDKN2A*,[5] *TP53*,[6] *SMAD4/DPC4*,[6,7] and *BRCA2*.[2,8,9] Most of these genetic alterations have been detected in the histologically more advanced PanIN lesions (PanIN-2 and PanIN-3), and the initiating events in neoplastic progression within the pancreatic ducts remains unknown. In addition, telomere shortening is a common genetic abnormality observed in all stages of PanINs including the vast majority of earliest lesions (PanIN-1A).[10]

PanIN lesions may be particularly important in patients with a strong family history of pancreatic cancer.[11–13] Pancreata in patients with a strong family history of pancreatic cancer are remarkable for the presence of multifocal PanIN lesions, and these PanIN lesions are characteristically associated with a distinctive form of lobular parenchymal atrophy and are closely associated with the subtle EUS abnormalities seen in this group.[11] Identifying molecular markers of PanIN could be particularly valuable for these individuals.[12,13]

Aberrantly methylated genes is a particularly promising category of molecular markers of neoplasia. Aberrant CpG island hypermethylation is associated with the inactivation of critical tumor-suppressor genes in human cancers.[14] We have reported previously that many invasive pancreatic cancers harbor aberrant methylation of multiple genes.[15–42] Aberrant methylation of several genes has also been observed in intraductal papillary

Correspondence: Dr M Goggins, MD, Departments of Pathology, Medicine, and Oncology, The Johns Hopkins Medical Institutions, Cancer Research II Building, 1550 Orleans Street, Baltimore, MD 21231, USA.
E-mail: mgoggins@jhmi.edu
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mucinous neoplasms of the pancreas,[43] and in a subset of PanIN lesions.[44–46] For example, we previously reported that *ppENK* is aberrantly methylated in 16% of 102 PanINs and that the prevalence of methylation increases with PanIN grade.[44] These findings suggest an important role of CpG island hypermethylation in early pancreatic carcinogenesis. We have also found that methylation of genes commonly methylated in pancreatic ductal adenocarcinomas is readily detectable in pancreatic juice samples of patients with invasive pancreatic cancer and can help distinguish patients with benign *vs* malignant pancreatic disease.[26,28,32] Sensitive and specific markers of PanIN would be a further advance as they could help identify curable preinvasive neoplasia among individuals at increased risk of developing pancreatic cancer who are undergoing screening. However, the prevalence of aberrant methylation of many genes in PanINs is not known.

To further characterize the timing and prevalence of aberrant DNA methylation during pancreatic ductal carcinogenesis, we investigated the CpG island methylation profile in various grades of PanINs. Using methylation-specific PCR, we analyzed DNA samples from a total of 65 PanIN lesions for methylation status of 8 genes that we identified and characterized as aberrantly hypermethylated in invasive pancreatic cancer.

Materials and methods

Tissues samples

Formalin-fixed paraffin-embedded blocks of resected pancreata from patients with various pancreatic disorders were collected from the archives of the Johns Hopkins Hospital and selected for microdissection of PanIN lesions. This study was approved by the Johns Hopkins Institutional Review Board. PanIN lesions were classified into PanIN-1A, PanIN-1B, PanIN-2, and PanIN-3 by two authors (NF and RHH), according to the previously described criteria.[47] Microdissection was performed as previously described.[44] Briefly, after deparaffinization and staining of slides with hematoxylin and eosin, pancreatic tissue surrounding the PanIN lesion was first removed by blade and needle. The isolated PanIN lesion was then collected by scratching the slide after placing a drop of TK buffer (200 μg/ml of proteinase K and 0.5% Tween 20) directly onto the microdissected tissue. The neoplastic cellularity of the microdissected PanINs has been estimated over 80%. Generally, 2000–4000 cells were dissected per PanIN. Genomic DNA was extracted from the microdissected tissues by incubating in 50 μl of TK buffer at 56°C overnight.

Methylation-Specific Polymerase Chain Reaction

DNA samples were treated with sodium bisulfite (Sigma Chemical Co., St Louis, MO, USA) for 16 h at 50°C. After purification with the Wizard DNA cleanup system (Promega, Madison, WI, USA), 1 μl of the bisulfite-treated DNA was amplified using primers specific for either methylated or unmethylated DNA. DNA was isolated and modified from an estimated average of 3000 cells or 10 ng per PanIN, corresponding to an input DNA of 200 pg per methylation-specific polymerase chain reaction (MSP) reaction. PCR conditions were as follows: 95°C for 5 min; then 40 cycles of 95°C for 20 s, 60–62°C for 20 s, and 72°C for 30 sec; and a final extension of 4 min at 72°C (primer sequences are available upon request). A volume of 5 μl of each PCR product was loaded onto 3% agarose gels and visualized by ethidium bromide staining.

Statistical Analysis

Statistical analysis was performed using χ^2-test or Mann–Whitney *U* non-parametric test. Differences were considered significant at $P<0.05$.

Results

Identification of Genes Aberrantly Methylated in Invasive Pancreatic Cancer

As previously reported, we used oligonucleotide microarrays to screen for genes that are induced after treatment of pancreatic cancer cells with the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine (5Aza-dC). Using this approach, we have identified a total of 475 genes that were markedly (>5-fold) induced after 5Aza-dC treatment in pancreatic cancer cell lines but not in a non-neoplastic pancreatic epithelial cell line.[26] Among this large panel of genes identified, eight genes were selected for the present analyses because these genes showed complete unmethylation in a panel of normal pancreatic ductal epithelia selectively microdissected using laser-capture microdissection. These eight genes were cadherin 3 (*CDH3*), candidate mediator of the p53-dependent G2 arrest (*reprimo*), claudin 5 (*CLDN5*), LIM homeobox protein 1 (*LHX1*), neuronal pentraxin II (*NPTX2*), secreted apoptosis related protein 2 (*SARP2*), secreted protein acidic and rich in cysteine (*SPARC*), and suppression of tumorigenicity 14 (*ST14*).

Methylation Analysis of Multiple Genes in PanINs

DNA samples from a total of 65 PanIN lesions (17 PanIN-1A, 21 PanIN-1B, 15 PanIN-2, and 12 PanIN-3) were analyzed in the present study. These PanIN lesions were derived from 20 pancreata resected for pancreatic ductal adenocarcinoma (11 patients, mean age of 62), chronic pancreatitis (4 patients, mean age of 58), and other neoplasms (5 patients, mean age of 67), including ampullary cancer, common bile duct cancer, well-differentiated

pancreatic endocrine neoplasm, and mucinous cystic neoplasm. There was no significant difference in age between these three disease groups.

To detect methylation abnormalities in these microdissected PanIN samples, we utilized MSP. Using this sensitive assay, we were able to detect *ppENK* amplification from an initial 50 μl of DNA samples containing ~200–400 cells dissected from archival tissues.[44] Representative MSP results of *SARP2* gene are shown in Figure 1. In most PanINs containing methylated alleles, unmethylated alleles were also detected in the same samples, reflecting contamination by normal cells (such as stromal cells and inflammatory cells), partial methylation of the CpG island, hemimethylation involving one allele of the gene, or clonal heterogeneity of neoplastic cells.

The overall prevalence of CpG island methylation at each gene locus was 30% for *Reprimo*, 28% for *SPARC*, 23% for *SAPR2*, 20% for *NPTX2*, 14% for *LHX1*, 13% for *CLDN5*, 13% for *CDH3*, and 0% for *ST14*. We next compared the methylation patterns of each gene among different grades of PanIN (Figure 2). For several genes such as *CDH3* and *SPARC*, the methylation frequencies were similar throughout all the PanIN grades. On the other hand, the prevalence of methylation at *NPTX2* significantly increased from PanIN-1 to PanIN-2 (6 *vs* 46%, $P=0.0008$). Furthermore, the methylation frequency significantly increased from PanIN-2 to PanIN-3 for *SARP2* (20 *vs* 83%, $P=0.001$), *reprimo* (20 *vs* 67%, $P=0.01$), and *LHX1* (7 *vs* 42%, $P=0.03$). There was no correlation between methylation frequency for each gene and clinicopathological features including age, gender, and underlying disease (not shown).

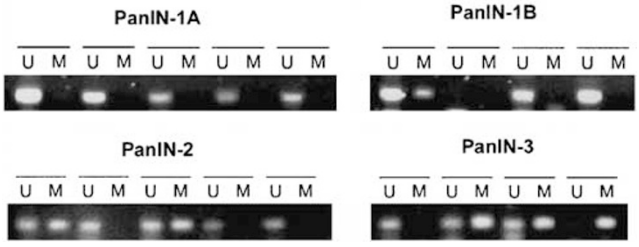

Figure 1 Methylation-specific PCR analysis of *SARP2* in various grades of PanINs. The PCR products in lanes U and M indicate the presence of unmethylated and methylated templates, respectively.

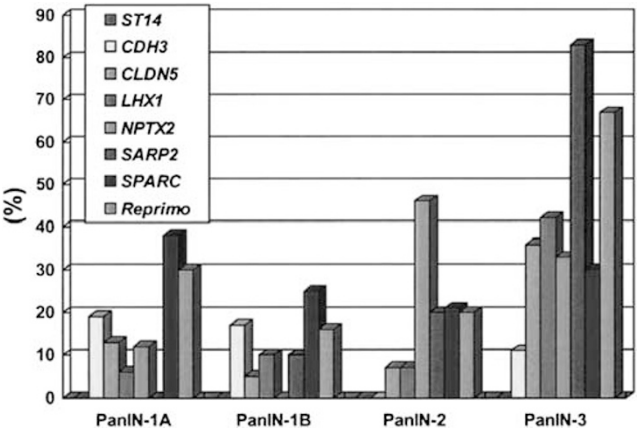

Figure 2 Frequency of aberrant CpG island methylation at eight genes in various grades of PanINs. The methylation frequency significantly increased from PanIN-1 to PanIN-2 for *NPTX2* (6 *vs* 46%, $P=0.0008$) and from PanIN-2 to PanIN-3 for *SARP2* (20 *vs* 83%, $P=0.001$), *reprimo* (20 *vs* 67%, $P=0.01$), and *LHX1* (7 *vs* 42%, $P=0.03$).

The methylation profile of 10 genes (including *ppENK* and *p16* as previously reported[44]) for each PanIN lesion is summarized in Figure 3. Aberrant methylation of at least one of the eight genes examined was found in 44 (68%) of the 65 PanIN lesions, including 12 (71%) of the PanIN-1A, 12 (57%) of the PanIN-1B, 9 (60%) of the PanIN-2, and 11 (92%) of the PanIN-3 lesions. The number of methylated loci for each PanIN lesion ranged from 0 to 6, and the average number of methylated loci was 1.1 in PanIN-1A, 0.8 in PanIN-1B, 1.1 in PanIN-2, and 2.9 in PanIN-3, showing an increase from PanIN-2 to PanIN-3 and PanIN-1 to PanIN-3, but not from PanIN1 to PanIN-2 ($P=0.01$, Mann–Whitney *U*-test).

Of note, 6 (9%) of 65 PanINs (1 PanIN-1A, 1 PanIN-2, and 4 PanIN-3) harbored aberrant methylation involving 4 or more of the 8 genes, suggesting a hypermethylator phenotype. These six lesions were derived from four resected pancreata from patients with an invasive cancer (three patients with pancreatic ductal adenocarcinoma and one patient with common bile duct carcinoma). Interestingly, 5 (83%) of the 6 PanIN lesions with this phenotype showed concordant methylation at *ppENK*, whereas only 4 (7%) of the remaining 59 lesions showed methylation at this gene ($P<0.0001$; Figure 3).

Discussion

In order to determine the timing and prevalence of CpG island hypermethylation during early pancreatic ductal carcinogenesis, we analyzed 65 PanIN lesions for methylation status of eight genes recently identified as aberrantly methylated in invasive pancreatic cancer but not in normal pancreatic ductal epithelium. We found aberrant methylation at multiple CpG islands in all grades of PanIN and a progressive increase in the overall methylation frequency from low-grade to high-grade PanINs. These results provide evidence that aberrant CpG island methylation is involved in early pancreatic ductal carcinogenesis and further support our previous findings of aberrant methylation of *p16*, *ppENK*, and *TSLC1* in a subset of PanINs.

In the present study, we were able to detect aberrant methylation even in the lowest grade PanIN lesions (PanIN-1A). Remarkably, aberrant

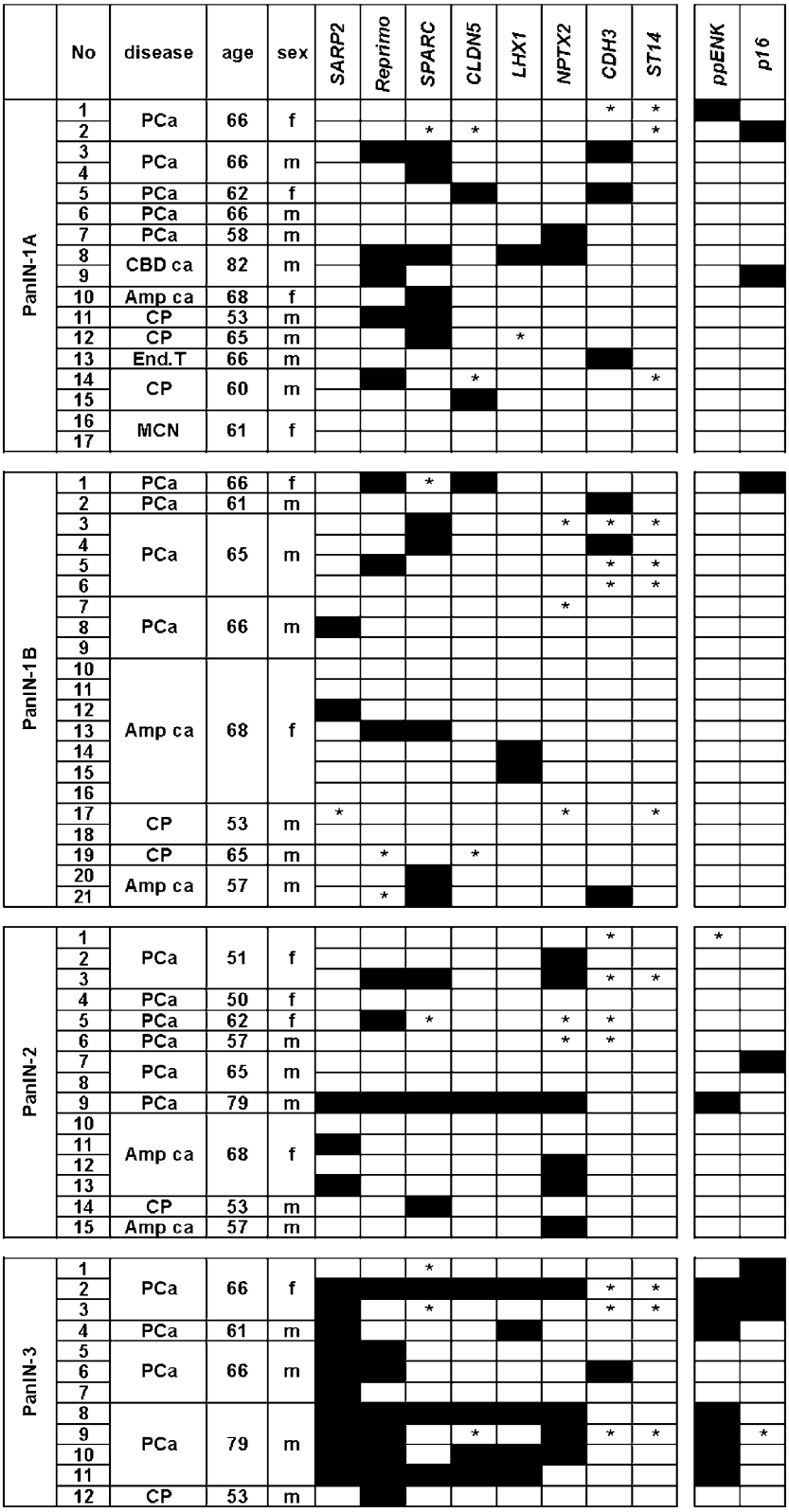

Figure 3 Methylation profiles of 10 genes (8 genes analyzed in this study and 2 genes (*ppENK* and *p16*) in a previous study) in PanINs determined by MSP. PCa, pancreatic adenocarcinoma; CP, chronic pancreatitis; CBD ca, common bile duct cancer; Amp ca, ampullary cancer; End.T, endocrine tumor; MCN, mucinous cystic tumor. Filled boxes, methylated alleles; open boxes, unmethylated alleles; *, not determined.

methylation at any of the eight genes tested was identified in more than 70% of PanIN-1A lesions. Consistent with our present results, other investigators have reported aberrant methylation of several genes in the earliest neoplastic lesions (or even in non-neoplastic lesions associated with malignancy) of several other tumor types. For example, aberrant methylation of several CpG islands (including *hMLH1* and *HPP1*) has been detected in aberrant crypt foci and hyperplastic polyps of the colon.[48,49] Aberrant methylation of *E-cadherin*, *hMLH1*, and *p16* has been frequently detected in non-neoplastic gastric epithelia in patients with gastric cancer.[50] Similarly, hypermethylation of *14-3-3 sigma* has been reported in atypical hyperplasias and apparently normal breast epithelium adjacent to breast cancer.[51] These previous reports and our present results raise the possibility that aberrant CpG island methylation is one of the earliest events during neoplastic progression of human cancers.

We also observed that the average number of methylated loci significantly increased from PanIN-2 to PanIN-3, suggesting that methylation abnormalities may play a major role in the transition from low-grade (PanIN-1 and PanIN-2) to high-grade PanINs (PanIN-3). A similar stepwise progression of methylation abnormalities has been implicated in the progression of various cancer types, including bladder cancer,[52] gastric cancer,[53] and esophageal cancer.[54–58] Belinsky *et al*[59] examined the timing for *p16* methylation during sequential progression of squamous cell carcinoma of the lung and found that the methylation frequency increased during disease progression from basal cell hyperplasia (17%) to squamous metaplasia (24%) to carcinoma *in situ* (50%) lesions. We previously demonstrated that aberrant methylation of multiple loci in another precursor lesion in the pancreas, intraductal papillary mucinous neoplasms of the pancreas (IPMN), increases with histological grade of malignancy.[22] These findings suggest that progressive increase in methylation frequency may be involved in the sequential progression of various tumor types.

Many of the genes analyzed in this study have been found to be functionally important in a variety of cell functions such as cell cycle regulation (*reprimo*), cell proliferation and adhesion (*SPARC*), apoptosis (*SARP2*), cell adhesion (*CDH3*), neuronal uptake or synapse formation (*NPTX2*), and tight junction barrier (*CLDN5*). For example, *reprimo* is a downstream mediator of p53-induced G2 cell cycle arrest, and its overexpression induces cell cycle arrest at the G2 phase, suggesting that it has tumor-suppressor properties.[60] SPARC is a matricellular glycoprotein involved in diverse biological processes[61] and is frequently silenced by aberrant methylation in pancreatic adenocarcinomas.[23] Several lines of evidence suggest a tumor-suppressor role of SPARC in certain tumor types.[62–65] We have recently demonstrated that stromal SPARC patterns independently predict outcome in patients with pancreatic ductal adenocarcinoma.[66] *SARP2* is an apoptosis-related gene that interacts the Wnt oncogenic signaling pathway.[67] Transfection of *SARP2* into breast cancer cells results in an increased sensitivity to different proapoptotic stimuli,[67] implying that epigenetic inactivation of *SARP2* can confer cellular resistance to apoptosis. Therefore, aberrant methylation at these genes in PanIN lesions may contribute to neoplastic progression of pancreatic cancer, although the functional consequences of these epigenetic abnormalities remain to be determined.

By analyzing the methylation status of multiple genes, we found that the patterns of methylation during the progression of PanINs were variable among genes. For example, *NPTX2* showed an increase in methylation prevalence from PanIN-1 to PanIN-2, whereas the prevalence of methylation at other genes such as *SARP2* increased from PanIN-2 to PanIN-3. By contrast, some genes (eg, *SPARC*) were aberrantly methylated at similar frequencies throughout all grades of PanINs. These results suggest that specific genes can be targeted for aberrant methylation at different stages of pancreatic neoplastic progression. The genes that are abnormally methylated in early low-grade PanIN lesions may be targets for chemoprevention, while those that are abnormally methylated in late high-grade lesions may be useful as markers of early detection.

In conclusion, by analyzing the CpG island hypermethylation profile at multiple genes in various grades of PanINs, we demonstrate a progressive increase in the prevalence of methylation with increasing histological grade of PanINs. Our present results provide further evidence for a role of epigenetic abnormalities in early pancreatic ductal carcinogenesis.

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Conflict of interest/disclosure

None.

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