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# Identification and Characterization of Differentially Methylated CpG Islands in Pancreatic Carcinoma[1]

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## ABSTRACT

**To identify CpG islands differentially methylated in pancreatic adenocarcinoma, we used methylated CpG island amplification (MCA) coupled with representational difference analysis. Of 42 CpG islands identified by MCA/representational difference analysis, 7 CpG islands [methylated in carcinoma of the pancreas (MICP)] were differentially methylated in a panel of eight pancreatic cancer cell lines compared with normal pancreas. In a larger panel of 75 pancreatic adenocarcinomas, these 7 MICPs (*ppENK*, *Cyclin G*, ZBP, MICP25, 27, 36, and 38) were methylated in 93, 3, 9, 15, 48, 19, and 41% of cancers, respectively, by methylation-specific PCR but not in any of 15 normal pancreata. In pancreatic cancer cell lines, methylation of *ppENK*, a gene with known growth suppressive properties, was associated with transcriptional silencing that was reversible with 5-aza-2′-deoxycytidine treatment. Relationships between the methylation patterns of pancreatic adenocarcinomas and their clinicopathological features were also determined. Larger pancreatic cancers and those from older patients ($P = 0.017$) harbored more methylated loci than smaller tumors and those from younger patients ($P = 0.017$). *ppENK*, MICP25, and 27 were variably methylated in normal gastric, duodenal, and colonic mucosae.**

**These data indicate that aberrant methylation of *ppENK* and its transcriptional repression is a common event in pancreatic carcinogenesis.**

## INTRODUCTION

About one-half of all of the human genes have 5′ CpG islands, and these islands are usually associated with the 5′ regulatory regions of genes (1). The 5′ CpG islands of most nonimprinted genes are thought to remain unmethylated in normal cells but may become methylated during aging or tumorigenesis. Through interactions between methyl CpG binding proteins, histones, and histone deacetylases, 5′ CpG island methylation can contribute to changes in chromatin that cause transcriptional silencing (2). Promoter methylation is implicated in the transcriptional silencing of tumor suppressor and mismatch repair genes (*e.g.*, *p16*, *Rb*, *VHL*, *hMLH1*) in many cancers.

Pancreatic cancer is the fourth leading cause of cancer death in men and in women, and each year ~28,000 Americans die of the disease (3). Frequent genetic changes such as mutational activation of the K-*ras* oncogene and inactivation of the *p16*, *DPC4*, *p53*, *MKK4*, *STK11*, *TGFBR2*, *TGFBR1*, and ALK-4 tumor suppressor genes have been described in pancreatic cancer (4–6). Although we have identified previously genes aberrantly methylated in pancreatic cancers (7), there almost certainly are others. Costello *et al.* (8) have estimated that ~400 genes are aberrantly methylated in cancers and have found evidence for tumor-specific pattern of methylation. A better knowledge of the pattern of DNA methylation abnormalities in cancer may improve our understanding of the role of DNA methylation in tumorigenesis. In addition, the identification of differentially methylated CpG islands in cancer may lead to the discovery of novel genes with tumor suppressor properties. Finally, identified genes or loci could be used as cancer-specific markers for the early detection of cancer (9). In this study we used MCA[3] coupled with RDA to recover CpG islands differentially methylated in pancreatic adenocarcinoma (10). We chose MCA/RDA because the subtractive and kinetic enrichment of differentially methylated sequence by RDA (Fig. 1*A*) has the potential to clone out sequences methylated only in cancer (8, 11).

## MATERIALS AND METHODS

**Cell Lines and Samples.** Two pancreatic adenocarcinoma cell lines, PL3 (a CIMP+ cell line) and PL8 (a CIMP− cell line), were established and kindly provided by Dr. Elizabeth Jaffee at our institution. Additional pancreatic carcinoma cell lines examined included CAPAN1, CAPAN2, Panc1, Hs766T, MiaPaca2 (all from American Type Culture Collection, Rockville, MD) and Colo357 (from European Collection of Animal Cell Cultures, Salisbury, United Kingdom). Seventeen pancreatic cancer xenografts were selected at random from a total of 90 xenografts, which were established from the primary carcinomas as described previously (7). Forty-seven primary pancreatic adenocarcinomas, 15 normal pancreata, 5 pancreata from patients with chronic pancreatitis, and a panel of normal tissues were obtained from the resected surgical specimens at The Johns Hopkins Medical Institutions, Baltimore, MD. Frozen tissues or paraffin-embedded tissues were microdissected to obtain >40% neoplastic cellularity in the primary pancreatic adenocarcinomas, and 3 of the 15 frozen normal pancreatic tissues were also microdissected to enrich the normal ductal epithelium. DNA was extracted from microdissected primary pancreatic adenocarcinomas and normal tissues as well as from lymphocytes of four cancer-free individuals using standard methods.

**MCA/RDA.** MCA/RDA was performed as described by Toyota *et al.* (10) with some modifications that may have improved the efficiency of the MCA/RDA technique.[4] Briefly, 5 μg of DNA was digested with *Sma*I and *Xma*I (New England Biolabs). The restriction fragments were ligated to RMCA adapter and amplified by PCR in 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl2, 50 mM KCl, 0.5 M betaine, 2% DMSO, 200 μM each deoxynucleotide triphosphate, 100 pmol of RMCA 24mer primer and 15 units of Taq polymerase (Life Technologies, Inc.) in a final reaction volume of 100 μl. The reaction mixture was incubated at 72°C for 5 min and at 95°C for 3 min, and then was subject 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. We included betaine in the PCR reaction and amplified the methylated templates under a higher annealing temperature (77°C). The combination of betaine and DMSO can uniformly amplify a mixture of DNA with different GC content (12). These modifications might have enhanced the amplification of distinct MICPs instead of Alu repetitive sequences that accounted for 60% of the recovered clones using the original protocol (10). The MCA amplicon from either the pancreatic cancer cell line PL3 or PL8 was

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[3] The abbreviations used are: MCA, methylated CpG island amplification; MSP, methylation-specific PCR; RDA, representational difference analysis; 5Aza-dC, 5-aza-2′-deoxycytidine; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase gene; RT-PCR, reverse transcription-PCR; MICP, methylated in carcinoma of the pancreas; PanIN, pancreatic intraepithelial neoplasia; RLGS, restriction landmark genome scanning; CIMP, CpG island methylator phenotype.

[4] Internet address: http://mdanderson.org/leukemia/methylation.

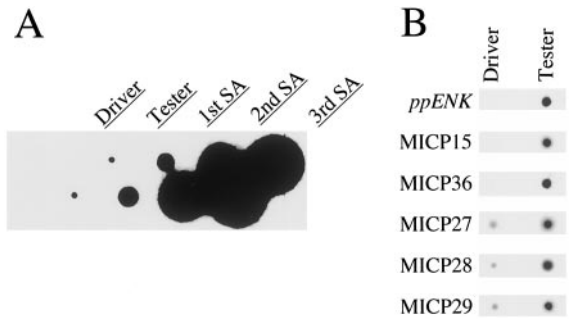


Fig. 1. Representative results of MICPs isolated by MCA/RDA. Dot blot analysis. *A*, an example of kinetic enrichment of methylated sequences by RDA. MCA products from the driver and the tester (PL8) and the PCR products from first (*1st SA*), second (*2nd SA*), and third (*3rd SA*) competitive hybridization/selective amplification were blotted onto the membrane and hybridized with a labeled *Cyclin G* probe. *B*, dot blot analysis using MICPs isolated from MCA/RDA as probes. First three MICPs were hybridized only to the tester (either PL3 or PL8), whereas next three MICPs were weakly hybridized to the driver as well as the tester.

used as the tester for RDA, and a MCA amplicon generated from a mixture of DNA from the normal pancreata of six different patients was used as the driver. RDA was performed on these MCA amplicons using different adapters, JMCA and NMCA. Sequences of adapters used for MCA/RDA are available at our website.[5] After the third round of competitive hybridization and selective amplification, the RDA difference products of second and third round amplifications were cloned into pBluescript II plasmid vector (Stratagene).

**DNA Sequencing of Clones and Dot Blot Hybridization.** The clones recovered from each cell line after MCA/RDA were amplified with T3 and T7 primers and then sequenced using KS primer as recommended by the manufacturer (Sequitherm Excel; Epicentre Technologies). To determine the methylation status of MCA/RDA MICPs in pancreatic cancer and normal pancreas, we first screened MICPs by hybridizing them to a dot blot of MCA products of pancreatic cancers and normal pancreata. Plasmid DNA containing each independent clone was prepared and digested with *Sma*I. DNA fragments were recovered from agarose gel and used as a probe for dot blot hybridization. Aliquots (1 $\mu$l) of the mixture of 10XSSC and MCA products from the driver and from the tester (PL3 and PL8) both before and after each of the three rounds of RDA competitive hybridization/selective amplification were blotted onto nylon membranes in duplicate. Similarly, MCA products from six pancreatic cell lines (CAPAN1, CAPAN2, Panc1, Hs766T, MiaPaca2, and Colo357) and from eight other normal pancreata were also blotted onto the membranes. The membranes were hybridized with $^{32}$P-labeled probes overnight, washed, and exposed to a Kodak X-ray film.

**Bisulfite Modification, Bisulfite-modified Genomic Sequencing, and MSP.** The bisulfite treatment was carried out for 16 h at 50°C using 1 $\mu$g of genomic DNA, as reported previously (7). Genomic sequencing was performed on bisulfite-treated DNA to examine the methylation status of 10–20 CpG dinucleotides located in and/or around *Sma*I sites of each clone in 22 pancreatic tissues (8 cancer cell lines, 6 primary adenocarcinomas, and 8 normal pancreata; Ref. 7). Genomic sequencing of the coding sequence of *cyclin G* was also performed in PL8. We interpreted the level of methylation of each clone by quantifying the level of methylation of each CpG site by comparing the intensity of unconverted cytosine with that of cytosine plus thymidine. Generally, in pancreatic cancer cell lines, the level of methylation observed at each CpG dinucleotide was consistent throughout the CpG island. Therefore, we graded the average level of methylation of each sequence into 4 grades: 0–10%, 11–30%, 31–70%, and 71–100%.

MSP was performed as described previously (13) and to acquire optimal specificity, each primer pair contained four to six CpG sites, and high specific annealing temperatures were used. The primers and the specific annealing temperatures for each clone are available at our website.[6] If validated MSP primers sets specific for methylated and unmethylated templates reveal that there is only amplification of methylated templates, we conclude that the sample is 100% methylated. Methylated and unmethylated templates were identified by bisulfite-modified sequencing. In describing MSP results performed on CpG islands that were normally unmethylated in non-neoplastic pancreas, we termed a pancreas cancer sample as "methylated" if MSP yielded any methylated templates.

**RT-PCR and 5Aza-dC Treatment.** Five pancreatic cancer cell lines (PL3, PL8, CAPAN2, Panc1, and MiaPaca2) and four normal pancreata were used for RT-PCR analysis. The cell lines were treated with demethylating agent 5Aza-dC (Sigma Chemical Co.) at a final concentration of 1 $\mu$M for 5 days. Total RNA was prepared using TRIzol (Life Technologies, Inc.), reverse-transcribed and amplified. As a control for cDNA integrity, *GAPDH* was also amplified. Primer sequences for RT-PCR are available at our website.[7]

**Statistics.** The primary outcome variable in this study was the observed number of 7 MICP loci found to be methylated in 64 pancreatic cancers. Wilcoxon's rank-sum test compared the observed number of methylated loci by tumor differentiation (poorly *versus* well or moderately differentiated), lymph node status (0 or 1 *versus* >1 node positive), and prior CIMP classification (CIMP positive *versus* CIMP negative). Simple linear regression assessed the relationship between the observed number of methylated loci and these covariates: age, age squared, and tumor diameter (in cm). Multivariate linear regression assessed the simultaneous contribution of the clinicopathological and demographic variables to the observed number of methylated loci. All of the tests were two-sided. A $P$ of $< 0.05$ signified statistical significance.

## RESULTS

**Identification of Differentially Methylated Sequences.** The strategy of MCA/RDA has been reported previously (10). Ninety-six randomly selected clones recovered from each cell line were subjected to DNA sequencing, and 66 clones were revealed to be independent. Only 3 clones contained Alu-repetitive sequences. The subsequent probing of labeled MICPs to MCA products of tester and driver by dot blot hybridization revealed that 42 of 66 MICPs (MICP1–42) were differentially methylated in the tester compared with the driver (Fig. 1). These 42 MICPs were also variably methylated in the other 6 pancreatic cancer cell lines examined (data not shown).[8] All of the 42 MICPs had a GC content of >50%, and 40 (95%) had a sequence uniqueness sustaining the criteria of CpG island (14). The DNA homology search of each clone with the BLAST program (National Center for Biotechnology Information) demonstrated that 38 of the 42 (90%) MICPs had significant homologies to known human sequences including 11 MICPs matched to human gene sequences and 10 MICPs matched to human ESTs. Five MICPs were also matched or contained a part of CpG islands isolated previously (10, 15), and 12 MICPs had significant homology to high-throughput genome sequences in the three international nucleotide sequence databases: DDBJ (DNA Databank of Japan), European Molecular Biology Laboratory, and GenBank. The remaining 4 had no significant homology to known sequences. Interestingly, 3 MICPs (MICP1, 14, and 23) matched to CpG islands originally recovered from colorectal cancer cell line using the same technique (named MINT 23, 20, and 32, respectively; Ref. 10).

**Characterization of the Methylation Status of Cloned CpG Islands by Bisulfite Sequencing.** For 30 of the 42 MICPs, methylation was detected in 2 or more of 8 normal pancreata by dot blot analysis, suggesting that these MICPs could be frequently methylated in normal pancreas. These CpG islands including those of 8 known genes [CSX, FLJ00083, *GAD1*, ICAM5, HLH (helix loop helix DNA binding protein), MCT3, *PAX5*, and SMO (smoothened gene)] were isolated by MCA/RDA, because relatively fewer DNA templates were methylated in normal pancreas. Therefore, only the remaining 12 MICPs (and MICP3 = FLJ0083) were additionally analyzed by bisulfite sequencing. For 7 of the MICPs (*Cyclin G*, *ppENK*, ZBP,

[5] Internet address: http://pathology2.jhu.edu/pancreas/prim0425.htm.
[6] Internet address: http://pathology2.jhu.edu/pancreas/prim0425.htm.
[7] Internet address: http://pathology2.jhu.edu/pancreas/prim0425.htm.
[8] Internet address: www.pathology.jhu.edu/pancreas/gogginslab for a table describing these 42 differentially methylated MICPs.

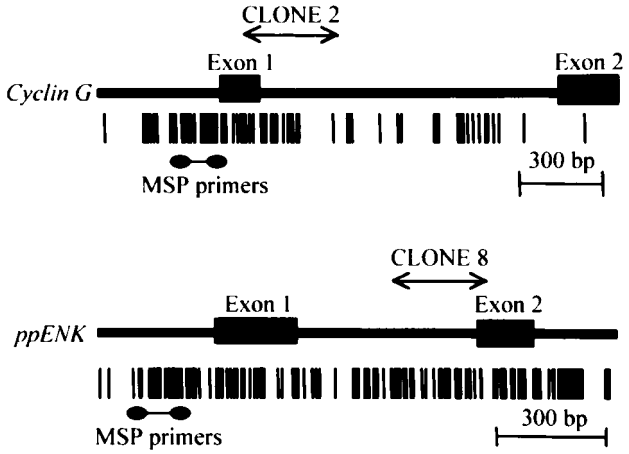


Fig. 2. CpG plot across the 5′ CpG islands of *Cyclin G* and *ppENK* showing the relation between isolated clones and their corresponding genes. The positions of MSP primers are also indicated. ■, exons.

MICP25, 27, 36, and 38), methylation was restricted to pancreatic cancers. A map of the CpG island of *ppENK* and *cyclin G* is shown in Fig. 2. In the case of 6 MICPs methylation bisulfite sequencing revealed methylation in DNA from pancreata samples as well as cancer DNA. A summary of the level of methylation of these 13 MICPs is shown in Fig. 3. DNA from pancreatic cancer cell lines and from pancreatic cancer xenografts does not have DNA from contaminating stroma; in these samples we were able to identify whether aberrant methylation in the carcinoma was complete or partial. Bisulfite sequencing revealed that the level of methylation of *ppENK*, *MICP27*, and MICP38 was often ~100% (see Fig. 3).

**MSP Analysis of *Cyclin G*, *ppENK*, ZBP, MICP25, 27, 36, and 38.** To identify low level of methylation of MICPs in pancreatic tissues, we next designed MSP primers for the 7 CpG islands differentially methylated in pancreatic cancers by bisulfite sequencing (*Cyclin G*, *ppENK*, ZBP, MICP25, 27, 36, and 38). We examined the methylation status of these CpG islands in 75 pancreatic adenocarcinomas (including 11 pancreatic cancer cell lines, 17 pancreatic cancer xenografts, and 47 primary pancreatic adenocarcinomas), 5 DNA samples from pancreata with chronic pancreatitis, as well as 15 normal pancreata including three specimens enriched in normal ductal epithelium. None of these 7 MICPs harbored methylation in normal pancreas by MSP (Fig. 4; Table 1). Because methylation of these 7 MICPs was not found in non-neoplastic pancreata, we defined any amplification of methylated templates as indicating "methylation." Such aberrant methylation of the 7 was detected in 9–100% of the pancreatic cancer cell lines analyzed and in 2–92% of the 64 (Pxs and

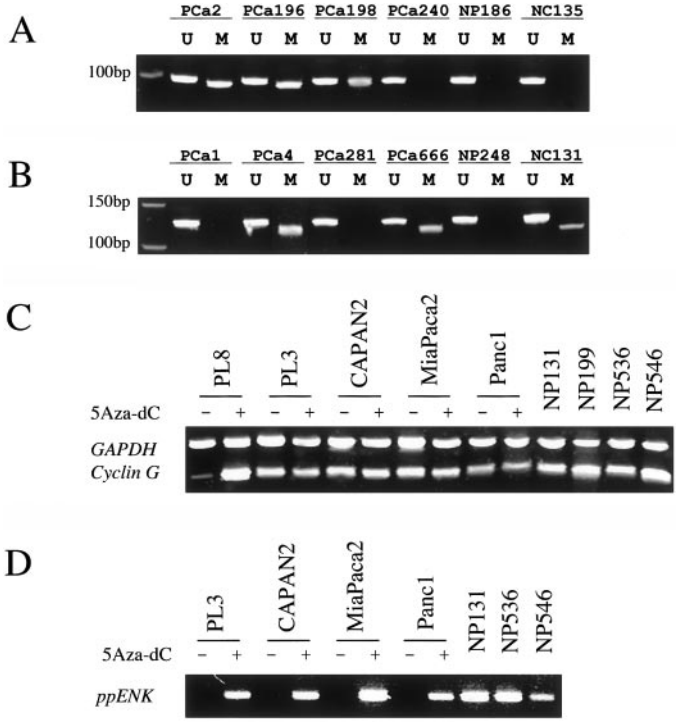


Fig. 4. MSP analyses of MICP36 (*A*) and MICP25 (*B*) in primary pancreatic adenocarcinomas and normal tissues. The PCR products in the *Lanes U* and *M* indicate the presence of unmethylated and methylated templates, respectively. *PCa*, primary pancreatic carcinoma; *NP*, normal pancreas; *NC*, normal colonic mucosa. Expression of *Cyclin G* (*C*) and *ppENK* (*D*) in pancreatic cancer cell lines and normal pancreata by RT-PCR analysis. *Cyclin G* and *ppENK* were coamplified with *GAPDH* to ensure the RNA integrity. *Cyclin G* was expressed at low level in the methylated cell line PL8 compared with unmethylated cell lines (PL3, CAPAN2, MiaPaca2, and Panc1) and four normal pancreata. 5Aza-dC treatment increased Cyclin G expression in PL8. All methylated cell lines examined (PL3, CAPAN2, MiaPaca2, and Panc1) lacked expression of *ppENK*, whereas all three normal pancreata expressed *ppENK*. Treatment of four cell lines with 5Aza-dC restored the *ppENK* expression. The expected sizes of the PCR products are 306 bp for *GAPDH*, 207 bp for *Cyclin G*, and 179 bp for *ppENK*.

| | Age | *Cyclin G* | *MCT3* | *PAX5* | *ppENK* | MICP20 | MICP21 | MICP25 | MICP27 | MICP36 | MICP38 | zbp | MICP3 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| NP3 | 71 | ○ | ◔ | ○ | ○ | ○ | ◕ | ○ | ○ | ○ | ○ | ○ | ◔ |
| NP281 | 36 | ○ | ◔ | ◔ | ○ | ◔ | ◕ | · | ○ | ○ | ○ | ○ | ◕ |
| NP397 | 52 | ○ | ◕ | ◔ | ○ | ◔ | ◕ | ○ | ○ | ○ | ○ | ○ | ◕ |
| NP536 | 59 | ○ | ◔ | ◔ | ○ | ◔ | ◕ | ○ | ○ | ○ | ○ | ○ | ◕ |
| NP546 | 66 | ○ | ◕ | ◔ | · | ◔ | ◕ | ○ | ○ | ○ | ○ | ○ | ◕ |
| NP666 | 34 | ○ | ◔ | ◔ | ○ | ○ | ◕ | ○ | ○ | ○ | ○ | ○ | ◕ |
| NP676 | 84 | ○ | ◕ | ◔ | ○ | ◔ | ◕ | ○ | ○ | ○ | ○ | ○ | ◕ |
| NP914 | 73 | ○ | ▒ | ◔ | ○ | ◔ | ◕ | ○ | ○ | ○ | ○ | ○ | ◕ |
| PCa196 | 84 | ○ | ● | ◕ | ● | ○ | ◔ | ○ | ● | ◔ | ● | ○ | ● |
| PCa198 | 76 | ○ | ◕ | ◔ | ◔ | ◕ | ◕ | ○ | ◔ | ○ | ◕ | ○ | ◕ |
| PCa240 | 50 | ○ | ◕ | ◔ | ○ | ◕ | ◕ | ○ | ○ | ○ | ◔ | ○ | ◕ |
| PCa248 | 67 | ○ | ◕ | ◕ | ◕ | ◔ | ◕ | ○ | ○ | ○ | ○ | ○ | ◕ |
| PCa281 | 36 | ○ | ◕ | ○ | ● | ○ | ◕ | ○ | ○ | ○ | ○ | ○ | ◕ |
| PCa666 | 34 | ○ | ● | ◕ | ● | ◕ | ◕ | ○ | ◔ | ◕ | ● | ○ | ◕ |
| PL3 | | ○ | ● | ○ | ● | ◕ | ○ | ● | ● | ◕ | ◕ | ○ | ● |
| PL8 | | ◕ | · | ◕ | · | ● | ● | ○ | ● | ◔ | ● | ◔ | ● |
| MiaPaca2 | | ○ | ● | ● | ● | ● | ● | ◕ | ● | ○ | ● | ◕ | ● |
| Colo357 | | ○ | ● | ● | ● | ● | ○ | ◕ | ● | ◕ | ● | ◔ | ● |
| CAPAN2 | | ○ | ● | ● | ● | ● | ● | ○ | ◔ | ○ | ◕ | ○ | ● |
| CAPAN1 | | ○ | ● | ● | ● | ● | ● | ○ | ● | ○ | ◕ | ○ | ● |
| Panc1 | | ○ | ● | ◕ | ● | ◔ | ● | ○ | ○ | ○ | ◕ | ○ | ● |
| Hs766T | | ○ | ◕ | ◕ | ● | ○ | ○ | ○ | ○ | ○ | ◕ | ○ | ● |

Fig. 3. A summary of the average level of methylation of selected MCA/RDA MICPs by bisulfite-modified genomic sequencing. The specimen number and the age of the patients are in the *left column*. *Empty oval*, 0–10% methylation; *white oval with black dots*, 11–30% methylation; *black oval with white dots*, 31–70% methylation; *black oval*, 71–100% methylation; ·, not determined. *NP*, normal pancreas; *PCa*, primary pancreatic adenocarcinoma.

Table 1 *The results of MSP*

| | *n* | *Cyclin G* | *ppENK* | *ZBP* | MICP25 | MICP27 | MICP36 | MICP38 |
|---|---|---|---|---|---|---|---|---|
| Pancreatic cancer cell lines | 11 | 9%[a] | 100% | 27% | 27% | 73% | 45% | 91% |
| Pancreatic cancer xenografts | 17 | 0% | 94% | 18% | 6% | 59% | 18% | 24% |
| Primary pancreatic adenocarcinomas | 47 | 2% | 91% | 2% | 15% | 38% | 13% | 36% |
| All pancreatic adenocarcinomas | 75 | 3% | 93% | 9% | 15% | 48% | 19% | 41% |
| Chronic pancreatitis | 5 | 20% | 40% | 0% | 20% | 0% | 0% | 0% |
| Normal gastric mucosae | 6 | ND[b] | ND | 50% | 17% | 0% | 0% | ND |
| Normal duodenal mucosae | 8 | ND | 100%[d] | 50% | 75% | 0% | 0% | ND |
| Normal colonic mucosae | 6 | ND | 100%[e] | 33% | 100% | 0% | 0% | ND |
| Normal lymphocytes | 4 | 0% | 0% | 0% | 0% | 0% | 0% | ND |
| Normal pancreata | 15 | 0% | 0% | 0% | 0% | 0% | 0% | 0% |

[a] The percentage of methylation in each box indicates the percentage of specimens that harbor methylation by MSP at that locus.
[b] ND, not determined.
[c] Number examined.
[d] 4 samples examined.
[e] 3 samples examined.

Pcas) pancreatic adenocarcinomas and pancreatic cancer xenografts analyzed. Because the primary pancreatic carcinomas harbor neoplastic and non-neoplastic tissue, MSP typically identified the presence of both methylated and unmethylated templates. DNA from one chronic pancreatitis tissue containing PanIN (16) harbored methylated templates of *Cyclin G*, *ppENK*, and MICP25, and DNA from another chronic pancreatitis sample also had methylated templates of *ppENK*. A summary of the results of the methylation profiles of these 7 MICPs in each of the 75 pancreatic adenocarcinomas is shown in Table 2.

Aberrant methylation of these 7 CpG islands methylated in pancreatic carcinoma did not occur randomly. In the 64 cancers for which clinical and pathological information was available (11 of the 75 pancreatic cancers analyzed were cell lines), the number of loci simultaneously methylated in a pancreatic cancer increased significantly with patient age (0.28 methylations per decade of age; $P = 0.017$) and the size of their tumor (0.156 methylations per centimeter of tumor diameter; $P = 0.017$) but not lymph node status ($P = 0.37$) or tumor differentiation ($P = 0.11$) in the surgical resection specimen (see Fig. 5). Of the 75 pancreatic cancers analyzed, the methylation status of 29 cases had been characterized previously using a panel of candidate genes methylated in cancer (7). These 29 cancers included 7 CIMP+, 7 CIMP-intermediate, and 15 CIMP− cancers. In this subset of 29 cases, pancreatic carcinomas identified previously as CIMP+ harbored methylation of a greater number of the 7 MICPs than those cancers classified previously as CIMP− (3.4 *versus* 1.25 methylations; $P = 0.002$).

Because tissue-specific methylation differences can be found in normal tissues (11), we also determined if the MICPs we found aberrantly methylated in pancreatic cancer compared with normal pancreas are methylated in other normal tissues. By MSP, MICP36 and MICP38 were not methylated in any normal gastrointestinal tissues, whereas *ppENK*, MICP25, and MICP2 7 were partially methylated in DNA samples from normal gastric, duodenal, and colonic mucosae (Fig. 4*B*; Table 1). Amplification of methylated templates of these MICPs was always weaker in normal mucosae compared with the primary pancreatic adenocarcinomas (Fig. 4*B*), suggesting that there were fewer methylated DNA templates in these mucosae. None of these 7 CpG islands were methylated in peripheral blood lymphocytes from normal individuals. Of the CpG islands that harbored methylation in histologically normal tissues that were bisulfite sequenced, the number of methylated CpGs in the CpG island was similar in the normals compared with the cancers, but the relative percentage of methylated templates were often less than in the cancer samples as determined by comparing the density of C to T at each CpG.

**Expression of Cyclin G and ppENK in Pancreatic Cancer and Effect of 5-Azacytidine.** PpENK causes growth suppression (17, 18), and *Cyclin G* is a target for transcriptional activation by *p53* and *p73* (19, 20) and may augment apoptosis, although growth promoting properties for cyclin G have also been reported (21, 22). Therefore, we examined expression of *ppENK* and *Cyclin G* using RT-PCR in 4 and 5 pancreatic cell lines, respectively. Partial methylation (~50%) of the 5′ CpG island of *Cyclin G* in PL8 (Fig. 4*C*) was associated with decreased expression of *Cyclin G* by RT-PCR. The 5′ CpG island of

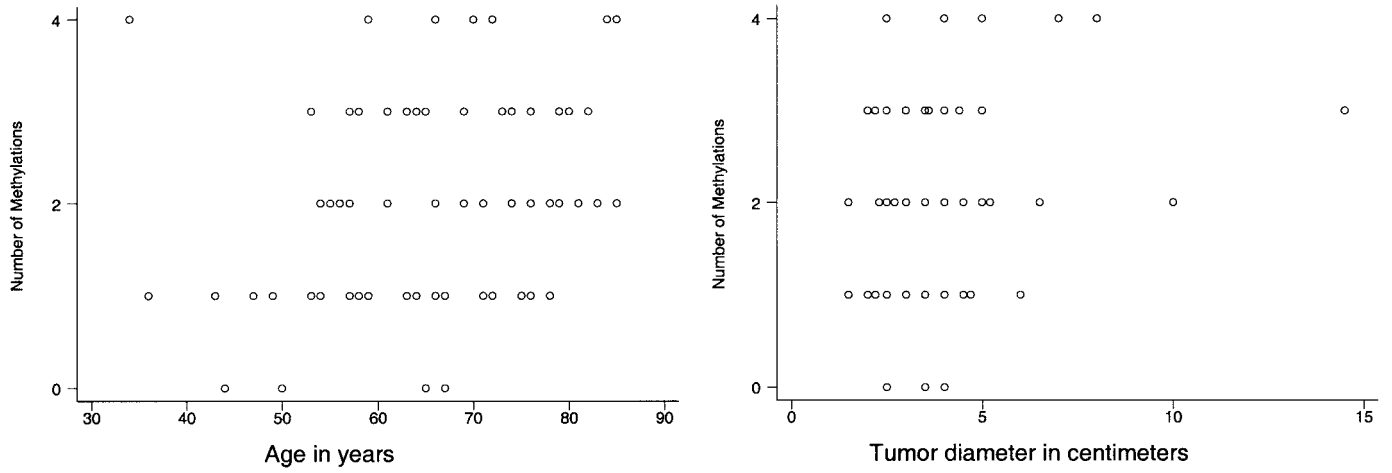


Fig. 5. Aberrant methylation of MICPs and clinicopathological variables. Multivariate linear regression analysis was used to determine the relationship between the number of aberrantly methylated MICPs in a pancreatic adenocarcinoma and the variables patient age (*left plot*) and tumor diameter (*right plot*). For each decade increase in age, the average number of methylated loci increases by 0.28, regardless of the tumor diameter. For each increase in tumor diameter (in cm), the number of methylated loci increases by 0.156, regardless of patient age.

Table 2 *MSP analysis of 7 CpG islands in a panel of 75 pancreatic adenocarcinomas*

| Sample | age | Tumor Diam.(cm) | nodes | no. methylated | CpG Island ppENK | MICP27 | MICP38 | MICP25 | MICP36 | CyclinG | zbp |
|---|---|---|---|---|---|---|---|---|---|---|---|
| AsPC1 | | | | 3 | ■ | ■ | ■ | | | | |
| BxPC3 | | | | 3 | ■ | ■ | | | ■ | | |
| CAPAN1 | | | | 3 | ■ | ■ | ■ | | | | |
| CAPAN2 | | | | 3 | ■ | ■ | ■ | | | | |
| CFPAC | | | | 2 | ■ | | ■ | | | | |
| Colo357 | | | | 5 | ■ | ■ | ■ | ■ | ■ | | ■ |
| Hs766T | | | | 3 | ■ | ■ | ■ | | ■ | | |
| MiaPaca2 | | | | 4 | ■ | ■ | ■ | ■ | | | ■ |
| Panc1 | | | | 2 | ■ | ■ | ■ | | | | |
| PL3 | | | | 5 | ■ | ■ | ■ | ■ | ■ | | |
| PL8 | | | | 4 | ■ | ■ | ■ | | ■ | ■ | ■ |
| Px17 | 69 | 4.4 | + | 2 | ■ | | | | ■ | | ■ |
| Px29 | 66 | 2.5 | + | 3 | ■ | ■ | | | ■ | | ■ |
| Px30 | 57 | 3.0 | + | 0 | ■ | | | | | | |
| Px64 | 76 | 2.5 | + | 1 | ■ | | | | | | |
| Px65 | 63 | 2.2 | + | 1 | ■ | | | | | | |
| Px74 | 83 | 3.5 | + | 2 | ■ | ■ | | | | | |
| Px75 | 63 | 5.0 | 0 | 2 | ■ | ■ | | | | | ■ |
| Px76 | 64 | 2.2 | + | 3 | ■ | ■ | ■ | | | | |
| Px102 | 47 | 3.0 | 0 | 1 | ■ | | | | | | |
| Px108 | 76 | 3.0 | + | 2 | ■ | ■ | | | | | |
| Px120 | 70 | 5.0 | + | 4 | ■ | ■ | ■ | ■ | | | |
| Px132 | 61 | 3.6 | + | 3 | ■ | ■ | ■ | | | | |
| {x143 | 54 | 2.0 | + | 1 | ■ | | | | | | |
| Px184 | 59 | 4.0 | + | 4 | ■ | ■ | ■ | | ■ | | |
| Px186 | 55 | 4.0 | + | 2 | ■ | ■ | | | | | |
| Px195 | 67 | 4.0 | + | 0 | | | | | | | |
| Px282 | 79 | 3.0 | 0 | 2 | ■ | ■ | | | | | |
| 1 | 64 | 4.7 | 0 | 1 | ■ | | | | | | |
| 2 | 57 | 3.0 | + | 2 | ■ | | ■ | | | | |
| 4 | 54 | 2.7 | + | 2 | ■ | ■ | | | | | |
| 6 | 69 | 4.5 | + | 2 | ■ | | | | ■ | | |
| 9 | 85 | 8.0 | + | 4 | ■ | ■ | ■ | | ■ | | |
| 11 | 65 | 2.5 | + | 0 | | | | | | | |
| 12 | 81 | 10.0 | + | 2 | ■ | | | ■ | | | |
| 13 | 53 | | + | 1 | ■ | | | | | | |
| 14 | 57 | 3.0 | + | 2 | ■ | | | ■ | | ■ | |
| 196 | 84 | 5.0 | + | 4 | ■ | ■ | ■ | | ■ | | |
| 198 | 76 | 4.0 | + | 3 | ■ | ■ | ■ | | | | |
| 240 | 50 | 3.5 | 0 | 0 | | | | | | | |
| 248 | 67 | 2.0 | + | 1 | ■ | | | | | | |
| 281 | 36 | 6.0 | + | 1 | ■ | | | | | | |
| 287 | 72 | 2.5 | 0 | 1 | ■ | | | | | | |
| 666 | 34 | 8.0 | + | 4 | ■ | ■ | ■ | | ■ | | |
| 5350 | 44 | 4.0 | + | 0 | | | | | | | |
| 11632 | 66 | 6.5 | + | 2 | ■ | ■ | | | | | |
| 12616 | 58 | Bx | | 1 | ■ | | | | | | |
| 13671 | 58 | 6.0 | + | 1 | ■ | | | | | | |
| 13653 | 63 | 14.5 | + | 3 | ■ | | ■ | | ■ | | |
| 17496 | 73 | 3.5 | + | 3 | ■ | ■ | | ■ | | | |
| 20852 | 78 | 5.0 | + | 2 | ■ | ■ | | | | | |
| 21190 | 82 | 3.5 | + | 3 | ■ | ■ | | ■ | | | |
| 21889 | 80 | 4.0 | + | 3 | ■ | ■ | ■ | | | | |
| 22384 | 74 | 2.0 | + | 3 | ■ | ■ | ■ | | | | |
| 24494 | 78 | 3.5 | + | 1 | ■ | | | | | | |
| 24721 | 71 | 2.5 | + | 2 | ■ | ■ | | | | | |
| 25392 | 57 | 3.0 | + | 1 | ■ | | | | | | |
| 26183 | 56 | 1.5 | 0 | 2 | ■ | | ■ | | | | |
| 30011 | 65 | 4.0 | + | 3 | ■ | ■ | ■ | | | | |
| 30845 | 76 | 2.5 | + | 3 | ■ | ■ | ■ | | | | |
| 31742 | 66 | 1.5 | 0 | 1 | ■ | | | | | | |
| 32008 | 61 | | 0 | 2 | ■ | | ■ | | | | |
| 35083 | 59 | 1.5 | + | 1 | ■ | | | | | | |
| 35583 | 75 | 3.0 | + | 1 | ■ | | | | | | |
| 36378 | 74 | 5.2 | + | 2 | ■ | | ■ | | | | |
| 37880 | 43 | 4.0 | 0 | 1 | ■ | | | | | | |
| 42946 | 71 | 3.5 | + | 2 | ■ | | ■ | | | | |
| 45885 | 53 | Bx | | 3 | ■ | ■ | ■ | | | | |
| 46391 | 79 | 2.3 | + | 2 | ■ | ■ | | | | | |
| 49126 | 49 | 4.0 | + | 1 | ■ | | | | | | |
| 49390 | 79 | 2.5 | + | 3 | | | ■ | ■ | ■ | | |
| 52150 | 72 | 7.0 | 0 | 3 | ■ | ■ | | ■ | | | ■ |
| 52225 | 58 | 3.5 | + | 3 | ■ | | ■ | ■ | | | |
| 53413 | 85 | 3.5 | + | 2 | ■ | ■ | | | | | |
| 55090 | 71 | 4.5 | + | 1 | ■ | | | | | | |

*Cyclin G* was not methylated in a panel of normal pancreata, and *Cyclin G* was expressed in 4 normal pancreata by RT-PCR. Treatment with 5Aza-dC increased the expression of *Cyclin G* in PL8 (Fig. 4*C*). Because cyclin G was ~50% methylated, we sequenced the coding region of cyclin G in the PL8 cell line. No mutations were found suggesting that cyclin G was not biallelically inactivated in this cell line.

We found that *ppENK* was expressed in normal pancreata, but in 4 pancreatic cancer cell lines with aberrant methylation of the 5′region no expression was observed (Fig. 4*D*). 5Aza-dC treatment restored *ppENK* expression in all 4 of the cell lines. Thus, hypermethylation of the 5′ CpG island of *ppENK* coincides with absent expression in pancreatic cancers.

## DISCUSSION

Using MCA/RDA we isolated seven CpG islands (*Cyclin G*, *ppENK*, ZBP, MICP25, 27, 36, and 38) aberrantly methylated in pancreatic carcinoma compared with normal pancreas. In particular, we observed aberrant methylation of the 5′ CpG island of *ppENK* in virtually all of the pancreatic carcinomas tested. Methylation was associated with transcriptional silencing of this gene in pancreatic cancer cell lines. *ppENK* encodes opioid growth factor, also known as Met5-enkephalin. This opioid peptide induces apoptosis in lung cancer cell lines (17), delays progression through the cell cycle (23), and has a negative growth regulatory effect on various kinds of cancers, including pancreatic cancer (18). PpENK is currently undergoing Phase I clinical trials in for the treatment of pancreatic adenocarcinoma.[9] Although we also observed a low level of methylation of *ppENK* in other normal mucosae by MSP, these results indicate that *de novo* methylation of the 5′ CpG island of *ppENK* and its transcriptional repression might contribute to pancreatic carcinogenesis.

Having identified by MCA/RDA 7 MICPs aberrantly methylated in pancreatic carcinoma, we examined a population of pancreatic cancers comparing the methylation patterns of these 7 MICPs to the distribution of methylated loci we observed in a previous study, which used a panel of candidate genes that undergo aberrant methylation (7). Pancreatic cancers found previously to have a high prevalence of aberrant methylation of candidate genes also harbored methylation of more MICPs ($P = 0.002$). This data provides additional evidence that some pancreatic carcinomas harbor greater numbers of aberrantly methylated CpG islands. Classifying pancreatic carcinomas into subsets depending on their prevalence of aberrant methylation at CpG islands (the so-called "CIMP" classification) may shed light on the clinical and biological significance of the differences in global methylation patterns seen in pancreatic and other cancers.

We also found that cancers with a high number of aberrantly methylated MICPs were more likely to be larger in size and to have come from older patients than cancers with little or no aberrantly methylated MICPs. The correlations between methylated loci, tumor size, and patient age were not strong suggesting that other factors influence the development of aberrant methylation, but they suggest that aberrant methylation is more likely to be observed with increasing age of the neoplasm. Because pancreatic neoplasms are usually malignant once they reach 1–2 cm in size, these data raise the possibility that some aberrant methylation events may continue to occur after the transition to malignancy. Aberrant methylation of some genes is known to occur in early benign neoplasms (24–26). As shown in Fig. 3, for some genes biallelic methylation was commonly observed (*ppENK*, MICP27, and MICP3 8) suggesting that it arose during carcinogenesis and may have been clonally selected; for others biallelic methylation was not found (such as MICP3 6, *cyclin G*, or *ZBP*). Comparison of methylation patterns in primary carcinomas, their local recurrences, distant metastases and neoplastic precursors (PanINs) should shed light on the timing of methylation in cancer development and evolution. Another possibility for the relationship between tumor size and methylation status is that cancers with high levels of aberrant methylation may be more likely to present when the tumor is larger. These clinicopathological correlations will require confirmation in other studies. Other investigators have attempted to identify the basis of cancer-related methylation by looking for associations between clinicopathological variables and methylation. Salem *et al.* 25 found a relationship between bladder cancer stage and the number of methylated genes among a panel (PAX6, exon 2 of p16, *DBC*, and *TPEF*). Using a panel of candidate genes, Zochbauer-Muller *et al.* (27) observed an association between cigarette smoking and *p16* methylation in lung cancers.

The absence of methylated templates of *ppENK* in normal pancreas and the absence of MICP36 and MICP38 in any normal tissue examined raises the possibility that MSP could be used to detect aberrant methylation of these MICPs in clinical samples such as pancreatic juice, duodenal fluid, stool, or blood for the early detection of pancreatic cancers. This would be valuable for individuals at high risk of developing pancreatic adenocarcinoma such as those with a strong family history of the disease (28).

The identification of several genes that harbor low-level methylation in normal pancreas highlights the need for care when assigning significance to certain cancer-related methylation data. A gene that is methylated in only a small percentage of normal cells may appear to undergo selection during carcinogenesis if it is completely methylated in a cancer. Such a methylation pattern could arise in a cancer if a non-neoplastic cell harboring "normal" methylation undergoes selection and neoplastic transformation as a result of subsequent genetic or epigenetic events. This phenomenon makes it much more difficult to assign causality to methylation phenomena in cancers compared with genetic events such as homozygous deletion. For normally unmethylated genes of which the function is well characterized such as *hMLH1*, for genes that are methylated as a second hit for a tumor suppressor gene (*e.g.*, *VHL* and *E-cadherin*; Ref. 29), or for tumor suppressor genes alternatively targeted by genetic and epigenetic inactivation (*e.g.*, *p16* and *RB*; Ref. 2), the biological significance of "aberrant methylation" is well accepted. Methylation of all of the templates in a neoplastic specimen is often interpreted as indicating that both alleles are methylated. In some cases this can also result from methylation of one allele combined with loss of the other allele by LOH (29). As additional genes are identified that are methylated in pancreatic and other cancers, it will be important to identify low-level methylation in normal tissues using sensitive techniques such as MSP (13) to help determine whether such genes have truly undergone selection through *de novo* methylation.

Methylation of several CpG islands has also been observed in non-neoplastic colorectal (30, 31), bladder, and prostate tissues (11). Methylation in normal tissues that is not the result of imprinting is frequently "age-related." This has been best shown in the colonic mucosa for genes such as *ER* (30, 31). We did not observe a pattern of age-related methylation in 15 histologically normal pancreata (mean age of 62) though we did observe some variability in the methylation of CpG islands in histologically normal pancreas. Our normal tissue population was not large enough to rule out trends in methylation with age. Methylation of *ppENK*, MICP25, and MICP27 in a significant percentage of the DNA templates within normal gastric, duodenal, and colonic mucosae, and its absence in normal pancreas and peripheral blood mononuclear cells highlights the fact that some CpG islands are methylated in a tissue-specific fashion (11).

[9] Internet address: http://www.psu.edu/ur/archives/intercom_1999/Nov11/research.html.

Other genes have been shown to be methylated in histologically normal gastrointestinal tissue. For example, methylation of an *APC* gene promoter occurs in both normal and cancerous gastric and esophageal epithelia (32, 33). This low-level methylation of different normal tissues might also explain some of the tumor type-specific methylation patterns observed by other investigators (8).

We also observed methylation of several MICPs in pancreata affected by chronic pancreatitis. Two of the 5 pancreata with chronic pancreatitis harbored aberrant methylation, 1 of these 2 pancreata contained a PanIN lesion, and this latter sample displayed methylation of 3 MICPs. Previous studies demonstrated that chronic pancreatitis is a significant risk factor for the development of pancreatic cancer (34), and duct lesions (PanIN) often found in chronic pancreatitis are considered precursors to infiltrating pancreatic carcinoma (16). The presence of aberrant methylation in DNA from chronic pancreatitis suggests that *de novo* methylation of CpG islands may be an early event in pancreatic cancer development in this setting.

Several approaches have been used to identify differentially methylated genes in cancer. The subtractive and kinetic enrichment of differentially methylated sequences by RDA may have advantages over other techniques to isolate differentially methylated sequences between normal tissue and cancer (8, 11). RLGS has been used successfully to profile methylation alterations in multiple tissues (8), but it has limitations, because it cannot discriminate between methylation and deletion events, the latter of which is common in pancreatic carcinoma (35). Furthermore, unlike MCA/RDA, RLGS and AP-PCR require the isolation and the subsequent cloning of identified spots or bands. Finally, MCA/RDA, like RLGS and AP-PCR, not only identifies absolute differences in methylation between cancer and normal DNA, it also will identify methylated sequences present both in cancer and normal DNA if there is low-level methylation in normal DNA as was observed for many CpG islands cloned in this study.

In conclusion, our results indicate that *ppENK* is commonly transcriptionally silenced by aberrant methylation in pancreatic adenocarcinomas. We have found that aberrant methylation of CpG islands in pancreatic adenocarcinomas is more common among older patients and those with larger tumors. The biological basis for these clinicopathological correlations and their clinical utility remain to be determined. Detection of methylated *ppENK* and other methylated genes may be a valuable biomarker for the early detection of pancreatic adenocarcinoma among patients at high risk of developing this disease.

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# Identification and Characterization of Differentially Methylated CpG Islands in Pancreatic Carcinoma

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