

# Age- and Disease-Related Methylation of Multiple Genes in Nonneoplastic Duodenum and in Duodenal Juice

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## ABSTRACT

**Purpose:** Methylation of CpG islands contributes to gene silencing during cancer development, and although some methylation alterations are promising diagnostic markers of cancer, some CpG islands are also methylated in normal tissues. We have previously observed that some normally unmethylated CpG islands that undergo methylation in pancreatic cancers are normally methylated in the adjacent duodenum. Because duodenal methylation patterns are an important consideration when sampling pancreatic tissues for pancreatic cancer methylation alterations, we determined the DNA methylation patterns of 24 genes in the normal duodenum of patients with pancreatic disease and related these patterns to demographic factors.

**Experimental Design:** The nonneoplastic duodenal mucosa of 158 patients with pancreatic carcinoma and 41 patients with chronic pancreatitis was analyzed using methylation-specific PCR and combined bisulfite restriction analysis. Secretin-stimulated pancreatic/duodenal juice from 15 individuals undergoing endoscopic investigation for upper gastrointestinal disease was also analyzed.

**Results:** Low-level methylation was detectable by methylation-specific PCR in the nonneoplastic duodenum of many patients with pancreatic cancer and chronic pancreatitis as well as in the pancreaticoduodenal secretions of patients without pancreaticobiliary disease. For many genes, the prevalence of methylation increased with age and was more prevalent in patients with pancreatic cancer than in age-matched patients with chronic pancreatitis.

Our results indicate that strategies to detect pancreatic cancer using aberrantly methylated genes should rely on analysis of pure pancreatic juice rather than on pancreatic juice collected within the duodenal lumen. Patients who develop pancreatic cancer may have a greater propensity to methylate CpG islands than age-matched controls.

## INTRODUCTION

Aberrant hypermethylation of promoter CpG islands (1) can substitute for mutations to inactivate tumor suppressor genes and other cancer-causing genes such as *p16* (2, 3), *E-cadherin* (4), *VHL* (5), *p14<sup>arf</sup>* (6), and the mismatch repair gene, *hMLH1* (2, 3). The CpG islands of several genes such as the *estrogen receptor* (*ER*; refs. 7–9), *N33*, *myogenic factor 3* (*MYOD1*; ref. 7), and *insulin-like growth factor II* (*IGFII*; ref. 10) undergo increasing methylation with age in both neoplastic and in nonneoplastic tissues (1, 11, 12). In pancreatic and colonic neoplasms, CpG island methylation of some genes increases with histologic grade of neoplasia (13, 14) and even with tumor size (11), suggesting that a propensity to methylate with age could fuel neoplastic development and progression. Some studies, but not others, have suggested that other demographic factors such as cigarette use are associated with DNA methylation in cancer tissues (15). However, aging is the only demographic factor that has been shown to influence methylation patterns in nonneoplastic tissues.

We have previously identified numerous genes that are methylated in pancreatic adenocarcinoma and are rarely, if ever, methylated in nonneoplastic pancreatic tissues. These genes include *p16* (13, 14), *ppENK* (16, 17), *Cyclin D2* (12), *SOCS1* (18), *SPARC* (19), *TSLC1* (20), and others (17, 21). These data indicate that the detection of such aberrant methylation using methylation-specific PCR (MSP)<sup>3</sup> could be a sensitive and specific marker of pancreatic cancer. Indeed, the detection of aberrantly methylated genes is being explored as a cancer-detection strategy in many tissues [plasma (22), saliva (23), sputum (24), mammary lavage fluid (25), bile, urine sediment, and biopsy samples (26)]. The inability of serum markers to accurately diagnose pancreatic cancer has led to interest in using pancreatic juice as a source of molecular markers. Pancreatic juice is thought to have a high concentration of DNA and proteins released from pancreatic cancers compared with other clinical sources such as serum. Pancreatic juice can be collected in the duodenal lumen during routine upper gastrointestinal endoscopy after secretin stimulation, without the need for pancreatic duct cannulation at endoscopic retrograde cholangiopancreatography (ERCP; ref. 16). Indeed, we have previously showed that the detection of several methylated genes in pure pancreatic juice obtained intraoperatively during pancreatic resection is a highly accurate predictor of a diagnosis of pancreatic adenocarcinoma (12, 16, 21). However, when we applied the same strategy to DNA isolated from secretin-stimulated

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pancreatic juice collected endoscopically in the duodenum, methylation of *ppENK* was also frequently detected in patients without pancreatic cancer. The source of this methylated *ppENK* methylation was normal duodenal tissue (16). These results suggest that a better understanding of normal duodenal methylation patterns is necessary to determine if pancreatic juice collected for molecular diagnostic purposes will need to be collected directly from the pancreatic duct using the more involved endoscopic procedure, ERCP or if pancreatic juice can be collected from the duodenum using the safer upper gastrointestinal endoscopy. The ability to use less invasive endoscopy is particularly important if DNA methylation markers are to be used to screen individuals at high risk of developing pancreatic cancer (27). Several genes have been shown to be variably methylated in normal duodenum (2, 3), but these studies have not been designed to investigate the demographic factors that influence normal tissue methylation patterns. Knowledge of normal duodenal methylation patterns would also help determine the utility of using methylation-based detection strategies for the detection of other cancers of the gastrointestinal tract.

We therefore profiled the DNA methylation patterns of 24 genes in duodenal mucosa obtained from pancreaticoduodenectomy specimens. Our study population included a large number of patients with pancreatic cancer and chronic pancreatitis and enabled us to analyze demographic and disease-specific patterns associated with methylation.

## MATERIALS AND METHODS

**Human Tissues.** Macroscopically normal duodenal mucosa was analyzed from two groups of patients that underwent pancreaticoduodenectomy: patients with pancreatic adenocarcinoma and patients with chronic pancreatitis. Pancreaticoduodenal juice was collected endoscopically from the duodenum of a third group of patients undergoing upper gastrointestinal endoscopy and endoscopic ultrasound. Finally, peripheral blood lymphocytes were analyzed from a group of healthy controls. The duodenal tissues consisted of frozen and formalin-fixed paraffin embedded tissue that were obtained from the grossly normal duodenal mucosa distal to the ampulla of Vater from pancreaticoduodenectomy specimens done at the Johns Hopkins Medical Institutions between the years 1991 and 2002. Frozen tissues were obtained from 158 patients with pancreatic adenocarcinoma (81 men and 77 women; age,  $63.9 \pm 13.3$  years; range, 34-89 years). Formalin-fixed, paraffin-embedded duodenal tissue was also obtained from 31 of these patients, as well as from 41 patients with chronic pancreatitis (25 men and 16 women; age,  $49.1 \pm 11.7$  years; range, 21-67 years). The duodenal tissues from patients with chronic pancreatitis were all formalin fixed and paraffin embedded. Pancreaticoduodenal juice samples were also collected via endoscopic aspiration from the duodenum after i.v. injection of secretin from 15 disease controls (9 men and 6 women; age,  $57.9 \pm 17.9$  years; range, 27-81 years) who underwent upper gastrointestinal endoscopy for a variety of reasons such as investigation of abdominal pain, hypoglycemia, monitoring of Barrett esophagus, assessment of pancreatic pseudocysts, and for suspected neoplasms of the upper gastrointestinal tract. These subjects were enrolled as study controls for the purposes

of collecting duodenal juice after secretin injection in a clinical research study to investigate the diagnostic utility of pancreatic juice markers (recombinant secretin was kindly provided by Repligen Corp, Waltham, MA). After investigation, the 15 patients were diagnosed as normal (8), gastritis (1), Barrett's esophagus (1), esophageal cancer (3), gastric cancer (1), and gastrointestinal stromal tumor (1).

Duodenal epithelial cells from crypts or villi were microdissected manually or using a laser capture microdissection device (MicroBeam IP-MS, PALM Microlaser Technologies, Bernried, Germany) from a hematoxylin-stained section. Peripheral blood cells obtained from 35 healthy controls (spouses of patients with pancreatic cancer, 18 men and 17 women; age,  $61.7 \pm 11.6$  years; range, 44-85 years). Patients were classified as Caucasian, African American, and other ethnicities. Approximately 90% of the patients with pancreatic cancer were Caucasian and most of the remaining patients were of African American ethnicity. About 80% of the patients with chronic pancreatitis were Caucasian, 10% African American, and the rest other ethnicities. Cigarette and alcohol consumption was obtained by chart review from 154 of 158 patients with pancreatic cancer and from 39 of 41 patients with chronic pancreatitis. These studies were carried out with the approval of the Johns Hopkins Committee for Clinical Investigation.

**DNA Extraction.** DNA was extracted from either 10- $\mu$ m paraffin-embedded, formalin-fixed tissue sections or from  $\sim 1$  mg of frozen tissue from each duodenal specimen using TK buffer as previously described (13). DNA from duodenal juice and peripheral blood cells was extracted using a DNeasy tissue kit (Qiagen, Valencia, CA).

**Genes Analyzed for Promoter CpG Methylation.** The genes selected were those that have been previously described as aberrantly methylated in pancreatic cancer [*Cadherin 3* (*CDH3*; ref. 21), *Claudin5* (*CLDN5*; ref. 21), *Cyclin D2* (12), *Cyclin G* (11), *epithelial cadherin* (*E-cad*; ref. 28), *preproenkephalin* (*ppENK*; refs. 11, 13), *forkhead box E1* (*FOXE1*; ref. 21), *LIM homeobox protein 1* (*LHX1*; ref. 21), *neuronal pentraxin II* (*NPTX2*; ref. 21), *p53-induced protein* (*PIG1*; ref. 21), *reprimin* (21), *secreted apoptosis-related protein 2* (*SARP2*; ref. 21), *suppression of tumorigenicity 14* (*ST14*; ref. 21), *suppressor of cytokine signaling-1* (*SOCS1*; ref. 18), *thrombospondin-1* (*THBS1*; ref. 28), *tight junction protein 2* (*TJP2*; ref. 21), *ubiquitin carboxyl-terminal esterase L1* (*UCHL1*; ref. 21), *wingless-type MMTV integration site family, member 7A* (*WNT7A*; ref. 21)] or identified as aberrantly methylated in other cancers [*BRCA1* (29), *death-associated protein kinase* (*DAPK*; refs. 28, 30), *helicase-like transcription factor* (*HLTF*; ref. 31), *human mutL homologue 1* (*hMLH1*; ref. 28), *hyperplastic polyposis protein* (*HPP1*; ref. 32), *O<sup>6</sup>-methylguanine-DNA methyltransferase* (*MGMT*; ref. 28), *p16* (13, 16), *serine/threonine kinase11* (*STK11/LKB1*; refs. 33, 34), *serum deprivation response factor* (*sdr*)–*related gene product that binds to c-kinase* (*SRBC*; ref. 35), and *von Hippel-Lindau* (*VHL*; ref. 5)]. *Claudin4* (*CLDN4*; ref. 36) was included as a gene previously identified as methylated in normal pancreas and hypomethylated in pancreatic cancer.

**Methylation Analysis.** The methylation status of the 5'CpG islands of each gene was determined by MSP as previously described (5). After treatment with sodium bisulfite

(Sigma, St. Louis, MO) for 16 hours at 50°C and purification with the Wizard DNA cleanup system (Promega, Madison, WI), 20 ng of DNA were amplified in 15 µL of PCR solution. MSP was done using DNA controls previously characterized as unmethylated and methylated, and approximately. The primer sequences for 29 genes analyzed in this study are available at our Web site (<http://pathology2.jhu.edu/pancreas/primer.pdf>). Many of these primers were used to characterize pancreatic cancer methylation in previous studies. The primer sites were chosen to interrogate CpG islands in the 5' regions of genes where methylation is thought to inhibit RNA transcription. Twenty-seven of the primers used were the same as in the previous studies: [*BRC1* (methylated MSP primer location: -153 to +32; ref. 29), *CDH3* (+50 to +150; ref. 21), *CLDN4* (+101 to +209; ref. 36), *CLDN5* (-137 to -53; ref. 21), *Cyclin D2* (-914 to -757; ref. 12), *Cyclin G* (-1530 to -1411; ref. 11), ref. 11), *DAPK* (+5 to +102; ref. 28), *E-cad* (-176 to -62; ref. 28), *FOXO1* (+13 to +100; ref. 21), *HLTF* (ref. 31), *hMLH1* (-282 to -177; ref. 28), *LHX1* (+569 to +675; ref. 21), *MGMT* (28), *NPTX2* (-145 to -34; ref. 21), *p16* (-66 to +54; ref. 13), *PIG11* (21), *ppENK* (-217 to -140; ref. 13) *reprim0* (+149 to +266; ref. 21), *SARP2* (-156 to -68; ref. 21), *SOCS1* (+224 to +355; ref. 18), *ST14* (-33 to +103; ref. 21), *STK11/LKB1* (34), *THBS1* (-41 to +33; ref. 28), *TJP2* (+26 to +143; ref. 21), *UCHL1* (-141 to -36; ref. 21), *VHL* (-101 to +59; ref. 5), and *WNT7A* (+74 to +196; ref. 21)]. Primers for *HPPI* (+105 to +286) and *SRBC* (-495 to -394) were designed for this study. PCR conditions were as follows: 95°C for 3 min; then 40/45 cycles of 95°C for 20 seconds; optimum annealing temperature for each primer setting for 20 seconds and 70°C for 30 seconds; and final extension for 4 minutes at 72°C. Forty cycles of PCR were done for DNA isolated from frozen tissue, duodenal juice, and peripheral blood cells and 45 cycles for DNA isolated from paraffin-embedded tissue. Five microliters of PCR product were loaded onto 3% agarose gel and visualized by ethidium bromide.

**Combined Bisulfite Restriction Analysis.** To quantify methylation, combined bisulfite restriction analysis (COBRA) (37) was done on three genes: *hMLH1* (-337 to -175), *HPPI* (+163 to +353), and *UCHL1* (-206 to -36). The PCR conditions were similar to those used for MSP (<http://pathology2.jhu.edu/pancreas/primer.pdf>). PCR products were digested with *Bst*UI (*hMLH1* and *HPPI*) and *Nru*I (*UCHL1*). The percentage of digested PCR product was quantified by image analysis on 3% agarose gels with an image analyzer (ImageJ 1.24, NIH). Because COBRA is a semiquantitative assay, each result was classified as (a) no detectable methylation, (b) <10% methylation, or (c) ≥10% methylation.

**Statistical Analysis.** Statistical analysis was done using the StatView 5.0 statistical software package (SAS Institute Inc., Cary, NC) and Stata Version 8.0. Comparisons of the prevalence of methylation with gender, alcohol, smoking history, and tissue source (frozen or paraffin embedded) were determined by  $\chi^2$  test or Fisher's exact test. The percentage of genes methylated in each individual was calculated as a proportion of the number of genes that showed any evidence of methylation over the total number of genes analyzed. The association between age and the percentage of methylated genes was described using a regression test. Linear regression

was done to estimate the average change in percent methylated genes between ages grouped by decade. The association between the prevalence of methylation for each gene and disease status adjusting for age was estimated using multivariate logistic regression. Power calculations were determined to estimate the power of our sample size to detect differences in methylation between the two patient groups. With a sample size of 147 with pancreatic cancer and 41 with chronic pancreatitis, the power to detect a 4-fold difference in methylation between the two groups for a gene with a prevalence of methylation of at least 20% is 61%, assuming a two-sided  $\alpha$  of 0.05. The power to detect methylation differences among between patient groups increases as the prevalence of methylation increases. For each disease group, the relationship between age and methylation was determined by comparing the mean age of patients with methylation of each gene compared with those without methylation using the Student's *t* test. The quantity of methylation of each gene was grouped by age and compared using the Kruskal-Wallis test and Student's *t* test depending on the numbers of groups compared. A two-sided *P* value of <0.05 was considered statistically significant.

## RESULTS

**DNA Methylation in Duodenum, Pancreaticoduodenal Juice, and Peripheral Blood.** A summary of methylation profiles in these tissues is shown in Fig. 1. We divided the 24 genes affected by methylation into a tumor suppressor gene group (genes genetically inactivated in human cancer) and a "cancer-methylated" gene group (genes previously identified as aberrantly methylated in human cancer but not genetically inactivated in cancer). The CpG islands of most of the genes analyzed were partially methylated in the duodenum of patients with both pancreatic carcinoma and chronic pancreatitis, as determined by MSP. This was true even for tumor suppressor genes/mismatch repair genes such as *hMLH1*, *E-cadherin*, and *p16*. For example, partial *hMLH1* methylation was observed in the duodenum of 20.2% (17 of 84) of patients with pancreatic cancer and in 25% (9 of 36) with chronic pancreatitis (Fig. 1; Table 1). In contrast, methylation of these genes in DNA from peripheral blood was uncommon for most genes except for *DAPK* (35%), *UCHL1* (40.9%), *ppENK* (43.5%), *PIG11* (87.5%), *SRBC* (95%), and *CLDN4* (100%; Table 1). *DAPK* has been recently described as methylated in normal lymphocytes (30).

We also compared the methylation profiles of patients with pancreatic cancer with those of patients with chronic pancreatitis. Because only archival tissue DNA was available from patients with chronic pancreatitis, whereas frozen-tissue DNA was available from most of the patients with pancreatic adenocarcinoma, we first compared the prevalence of duodenal methylation detectable by MSP in the frozen and matching paraffin-embedded tissues of 31 patients with pancreatic adenocarcinoma to determine if methylation detection by our MSP assays was affected by the use of archival compared with frozen tissue. To ensure that our MSP assays were sensitive for identifying methylated DNA, we used 45 cycles of PCR in our MSP assays of DNA obtained from formalin fixed paraffin embedded tissue

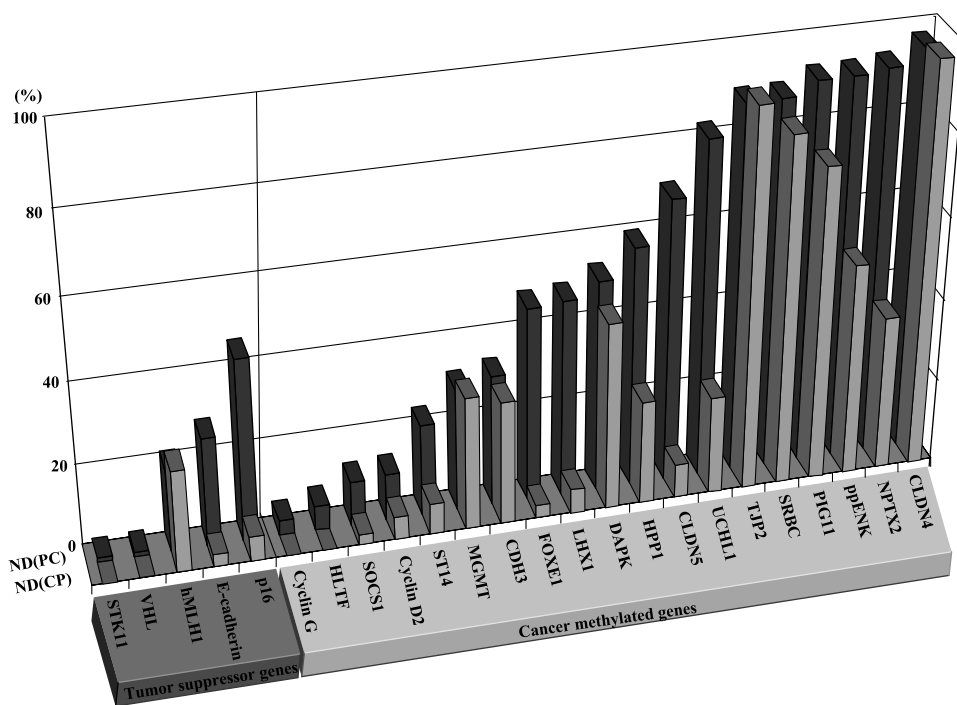


Fig. 1 The prevalence of CpG island methylation of 24 genes in normal duodenum. A bar graph illustrates the percentage of individuals with methylation of each gene in each patient group. Black bars, patients with pancreatic cancer; gray bars, patients with chronic pancreatitis.

Table 1 Methylation frequency of normal duodenum, duodenum juice, and peripheral blood cells in 24 genes with reference to the previous report on pancreatic carcinoma

Gene	Percentage of methylation (no. of cases)				Statistical difference* between ND (PC) and ND (CP)	Percentage of methylation in PC tissue reference	Ref.
	ND (PC)	ND (CP)	DJ	PBC			
<b>Tumor suppressor genes</b>							
<i>STK11</i>	1.1 (1/91)	0 (0/37)	0 (0/15)	0 (0/27)	No	0 (0/18)	(33)
<i>VHL</i>	1.2 (1/86)	0 (0/32)	0 (0/15)	0 (0/20)	No		
<i>hMLH1</i>	20.2 (17/84)	25.0 (9/36)	6.7 (1/15)	8.7 (2/23)	No	4 (2/45)	(28)
<i>E-cad</i>	26.7 (24/90)	3.1 (1/32)	0 (0/15)	10.0 (2/20)	Yes	7 (3/45)	(28)
<i>p16</i>	44.7 (38/85)	6.1 (2/33)	6.7 (1/15)	10.0 (2/20)	Yes	18 (8/45)	(28)
<b>Cancer methylated genes</b>							
<i>Cyclin G</i>	3.6 (3/84)	0 (0/36)	0 (0/15)	8 (2/25)	No	3 (2/75)	(11)
<i>HLTF</i>	6.0 (5/83)	0 (0/31)	0 (0/15)	0 (0/24)	No		
<i>SOCS1</i>	10.8 (11/102)	2.6 (1/39)	0 (0/15)	0 (0/20)	No	22 (13/60)	(18)
<i>Cyclin D2</i>	11.3 (11/97)	5.6 (2/36)	26.7 (4/15)	5.7 (2/35)	No	65 (71/109)	(12)
<i>ST14</i>	22.2 (18/81)	7.5 (3/40)	0 (0/15)	10.0 (2/20)	No	10 (4/42)	(21)
<i>MGMT</i>	29.8 (25/84)	32.4 (11/34)	13.3 (2/15)	14.3 (3/21)	No	0 (0/45)	(28)
<i>CDH3</i>	31.8 (27/85)	30.0 (12/40)	0 (0/15)	10.0 (2/20)	No	19 (8/42)	(21)
<i>FOXE1</i>	47.1 (41/87)	3.3 (1/33)	6.7 (1/15)	9.5 (2/21)	Yes	69 (29/42)	(21)
<i>LHX1</i>	47.8 (44/92)	5.9 (2/34)	26.7 (4/15)	14.3 (3/21)	Yes	76 (32/42)	(21)
<i>DAPK</i>	51.4 (57/111)	45.5 (15/33)	33.3 (5/15)	35.0 (7/20)	No	2 (2/45)	(28)
<i>HPP1</i>	58.2 (53/91)	25.0 (9/36)	26.7 (4/15)	6.9 (2/29)	No		
<i>CLDN5</i>	68.5 (63/92)	8.1 (3/37)	53.3 (8/15)	3.7 (1/27)	Yes	93 (39/42)	(21)
<i>UCHL1</i>	81.5 (66/81)	23.7 (9/38)	73.3 (11/15)	40.9 (9/22)	Yes	100 (42/42)	(21)
<i>TJP2</i>	88.5 (85/96)	91.8 (34/37)	93.3 (14/15)	5.0 (1/20)	No	64 (27/42)	(21)
<i>SRBC</i>	88.6 (78/88)	84.2 (32/38)	100.0 (15/15)	95.0 (19/20)	No		
<i>PIG11</i>	91.7 (100/109)	75.8 (25/33)	93.3 (14/15)	87.5 (21/24)	No		
<i>ppENK</i>	91.8 (89/97)	51.4 (19/37)	86.7 (13/15)	43.5 (10/23)	Yes	93 (70/75)	(11)
<i>NPTX2</i>	92.6 (75/81)	37.5 (15/40)	80.0 (12/15)	9.5 (2/21)	Yes	98 (41/42)	(21)
<i>CLDN4</i>	96.3 (79/82)	97.3 (36/37)	100.0 (15/15)	100.0 (26/26)	No	85 (17/20)	(36)

NOTE. The percentage methylation of each gene is listed for each tissue type. The prevalence of methylated genes is indicated by a percentage followed in parenthesis by the numbers of samples methylated/analyzed.

Abbreviations: ND, normal duodenum tissue; PC, pancreatic carcinoma; CP, chronic pancreatitis; DJ, duodenal juice, PBC, peripheral blood cells.

\*Statistical analysis was done after age adjustment between PC and CP groups.



and 40 cycles for amplification of DNA obtained from fresh frozen duodenal tissue. The prevalence of methylation by MSP analysis was similar whether analyzed in the frozen or in the archival duodenal tissues from the same patient for 24 of the 29 genes tested. For 4 genes, methylation was detected less often in the archival than in the frozen tissues (*reprim0*, *SARP2*, *THBS1*, and *WNT7A*). For *BRCA1*, methylation was detected more often in the archival tissue DNA (data not shown). Given these findings, we did not include data from these 5 genes in comparing archival and frozen tissues. Thus, 24 genes were analyzed in 147 patients with pancreatic adenocarcinoma and 41 patients with chronic pancreatitis.

Duodenal methylation as measured by MSP analysis was more prevalent in our population compared with other studies. For example, the prevalence of *p16* and *hMLH1* methylation was higher than in previous studies (2, 3), which may relate to differences in primer location (12), the sensitivity of our MSP assays, or to differences in patient age or disease state between our study population and those of other studies.

Methylation of many of these genes was readily detectable in secretin-stimulated pancreatic/duodenal juice collected endoscopically from the duodenal lumen of patients without pancreaticobiliary disease (Table 1). Overall, methylation

patterns of duodenal juice were similar to those of duodenal tissue, although the percentage of duodenal juice samples with methylation was mostly lower than that observed in duodenal tissue (Table 1).

**Relationships between Demographic Factors and Duodenal DNA Methylation.** On average, older patients had more genes methylated in their duodenum than younger patients, but this relationship was not observed among the five genes (*p16*, *E-cadherin*, *STK11*, *VHL*, and *hMLH1*) of the group analyzed that are genetically inactivated in human cancer (tumor suppressor genes and the mismatch repair gene, *hMLH1*). The increasing methylation with age was observed among both patients with pancreatic cancer and patients with pancreatitis (Fig. 2; Table 2). The pattern of methylation to increase with each decade is presented in Table 3. When analyzed by individual gene, the association of greater methylation with age was significant for many of the genes analyzed (*HLTF*, *ST14*, *CDH3*, *LHX1*, *UCHL1*, *SRBC*, *SOCS1*, *MGMT*, and *HPPI*; Table 2). Similarly, among individuals without an upper gastrointestinal cancer, methylation was more often found in the duodenal juice samples of patients  $\geq 50$  years (36.8%) than in those  $< 50$  years of age (24%;  $P < 0.05$ ,  $\chi^2$  test). Patients ages  $\geq 50$  years with an upper gastrointestinal

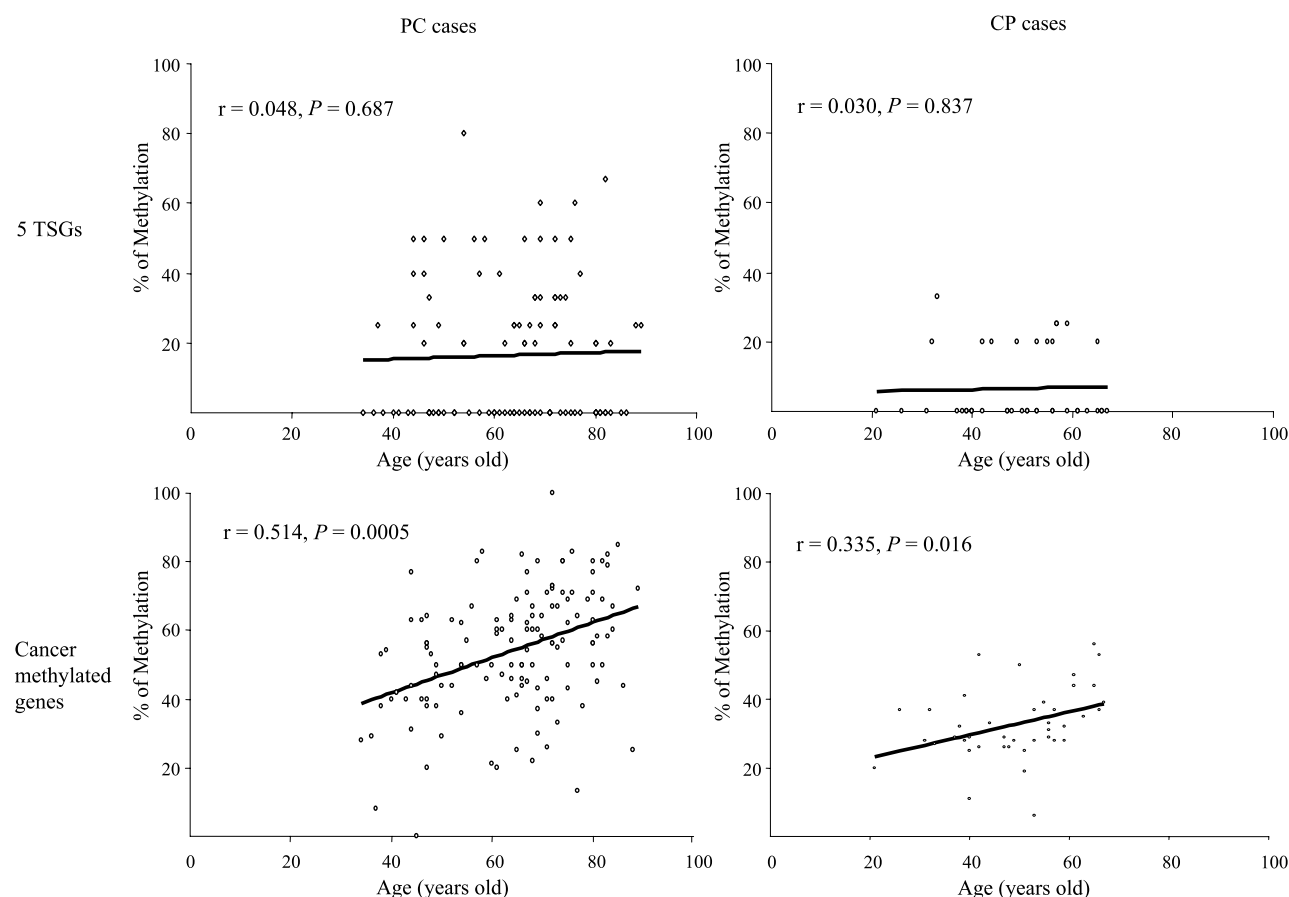


Fig. 2 The relation between age and DNA methylation of normal duodenum.  $\circ$ , number of genes methylated (%) by MSP in a given individual. For patients with both pancreatic carcinoma (PC) and chronic pancreatitis (CP), the number of cancer methylation genes methylated in duodenum increases significantly with patient age, but tumor suppressor gene methylation does not.

Table 2 Age of patients (years) with and without methylation of each of 24 genes in their duodenum

Gene	PC		CP	
	Unmethylated	Methylated	Unmethylated	Methylated
Tumor suppressor genes				
<i>STK11</i>	63.6 ± 13.5	66	49.3 ± 11.8	NA
<i>VHL</i>	64.6 ± 14.1	54	50.2 ± 11.9	NA
<i>hMLH1</i>	65.6 ± 13.8	60.9 ± 12.3	48.4 ± 11.3	50.2 ± 11.7
<i>E-cadherin</i>	62.3 ± 15.1	65.0 ± 9.4	49.9 ± 11.7	53
<i>p16</i>	63.0 ± 13.7	63.9 ± 14.5	50.9 ± 10.4	48.5 ± 9.2
Cancer methylated genes				
<i>Cyclin G</i>	63.5 ± 14.3	66.3 ± 17.0	50.3 ± 10.8	NA
<i>HLTF</i>	64.3 ± 13.4*	79.2 ± 6.1*	50.8 ± 10.8	NA
<i>SOC31</i>	63.0 ± 14.1†	72.1 ± 13.2†	49.8 ± 11.6	56
<i>Cyclin D2</i>	63.3 ± 13.5	65.8 ± 15.5	50.0 ± 11.4	41.5 ± 21.9
<i>ST14</i>	60.9 ± 14.5*	71.8 ± 9.7*	48.7 ± 10.5†	64.3 ± 16.6†
<i>MGMT</i>	65.0 ± 14.4‡	70.3 ± 10.4‡	49.1 ± 11.6	45.5 ± 13.0
<i>CDH3</i>	60.7 ± 14.3*	70.1 ± 11.3*	48.1 ± 11.9	52.4 ± 11.3
<i>FOXE1</i>	63.8 ± 14.2	64.2 ± 13.7	52.0 ± 10.6	31
<i>LHX1</i>	62.5 ± 15.3*	68.1 ± 12.3*	50.6 ± 10.6	63.0 ± 2.8
<i>DAPK</i>	62.4 ± 12.8	65.4 ± 14.5	47.7 ± 12.3	50.1 ± 11.9
<i>HPP1</i>	61.4 ± 12.8†	67.3 ± 12.9†	47.7 ± 11.3	53.1 ± 12.9
<i>CLDN5</i>	62.1 ± 14.6	65.7 ± 12.3	50.8 ± 10.6	50.0 ± 16.7
<i>UCHL1</i>	53.5 ± 15.3§	67.1 ± 12.2§	48.8 ± 11.0	55.4 ± 10.0
<i>TJP2</i>	65.2 ± 14.6	65.2 ± 13.4	45.0 ± 7.0	51.2 ± 11.1
<i>SRBC</i>	68.1 ± 14.8	65.5 ± 13.4	38.7 ± 13.6‡	52.1 ± 10.3‡
<i>PIG11</i>	57.9 ± 17.7	63.9 ± 13.5	47.4 ± 15.1	50.8 ± 11.8
<i>ppENK</i>	58.8 ± 14.4	66.7 ± 13.1	48.6 ± 10.6	52.7 ± 11.1
<i>NPTX2</i>	63.0 ± 6.1	63.9 ± 12.7	47.5 ± 12.1	52.7 ± 10.9
<i>CLDN4</i>	65.3 ± 16.0	63.3 ± 13.6	57	48.6 ± 12.4

Abbreviation: NA, not applicable.

\* $P < 0.005$ .

† $P < 0.05$ .

‡ $P < 0.01$ .

§ $P < 0.0005$ .

cancer had a similar frequency of methylation (40%) to age-matched patients without cancer (Table 4).

In contrast, gender and ethnicity did not seem to influence methylation levels (data not shown). Methylation patterns were similar in males and females for 23 of the 24 genes analyzed. Only methylation of *ppENK* showed a small difference in the prevalence of methylation between men (97.8%) and women (86%;  $P < 0.05$ ,  $\chi^2$  test). Because this was the only gene among 24 tested for which this pattern was observed, we suspect that

this difference is probably the result of chance arising from multiple comparisons.

Information about smoking history was available for 143 of 147 patients with pancreatic cancer and all of 41 patients with chronic pancreatitis. Overall, there was no significant difference in the prevalence of duodenal DNA methylation among smokers and nonsmokers for any of the genes tested. This was true for patients with pancreatic adenocarcinoma as well as those with chronic pancreatitis, even after adjustment for age. Similarly,

Table 3 Correlation between age and percentage of methylated genes among patients with pancreatic carcinoma and chronic pancreatitis

Age	PC cases			CP cases		
	Prevalence (%)	RC* (95% CI)	$P$	Prevalence (%)	RC* (95% CI)	$P$
20s	—	—	—	21.7	—†	—
30s	37.2	—†	—	26.4	4.72 (−7.80 to 17.2)	—
40s	39.5	2.38 (−11.1 to 15.9)	—	24.5	2.81 (−9.28 to 14.9)	—
50s	45.7	8.58 (−5.37 to 22.5)	—	26.3	4.53 (−7.26 to 16.3)	—
60s	46.2	9.05 (−3.70 to 21.8)	—	37.2	15.5 (3.15-27.8)	—
70s	51.6	14.4 (1.46-27.4)	—	—	—	—
80s	52.1	15.0 (1.33-28.7)	—	—	—	—
Overall	46.8	0.31 (0.08-0.53)	0.008	27.8	0.36 (0.17-0.55)	<0.001

Abbreviation: CI, confidence interval.

\*RC = value of regression coefficient analysis.

†The RC and 95% CI for the patients with PC in their 30s or CP in their 20s were not presented because these groups were used as reference groups for statistical analysis.

**Table 4** Prevalence of promoter CpG methylation in duodenal juice samples from nonneoplastic and neoplastic upper gastrointestinal diseases

Sample no.	Age	Sex	Prevalence of methylation, % (no. of genes)	Endoscopic and histologic diagnosis
Patients without upper GI neoplasm or pancreaticobiliary disease (age <50 y)				
1	27	F	25.0 (6/24)	Normal*
2	27	F	16.7 (4/24)	Normal
3	36	F	33.3 (8/24)	Normal
4	49	F	20.8 (5/24)	Normal
Subtotal (1-4)			24.0 (23/96) <sup>b</sup>	
Patients without upper GI neoplasm or pancreaticobiliary disease (age ≥50 y)				
5	51	F	29.2 (7/24)	Normal
6	53	M	33.3 (8/24)	Normal
7	54	M	37.5 (9/24)	Barrett's esophagus
8	68	M	25.0 (6/24)	Gastritis
9	71	M	41.7 (10/24)	Normal
10	81	F	54.2 (13/24)	Normal
Subtotal (5-10)			36.8 (53/144) <sup>c</sup>	
Patients with upper GI neoplasms				
11	59	M	41.7 (10/24)	Esophageal cancer
12	62	M	37.5 (9/24)	Esophageal cancer
13	71	M	41.7 (10/24)	Gastric cancer
14	78	M	37.5 (9/24)	GIST
15	81	M	41.6 (10/24)	Esophageal cancer
Subtotal (11-15)			40.0 (48/120) <sup>d</sup>	

NOTE. Methylation was analyzed by MSP, b versus c, b versus d ( $P < 0.05$ ,  $\chi^2$  test).

Abbreviations: *GI*, gastrointestinal; *GIST*, gastrointestinal stromal tumor.

\*Upper GI endoscopy was normal; no biopsies were obtained.

methylation levels did not differ among those that had a history of excess alcohol consumption and those without such a history (data not shown).

**Duodenal Methylation in Patients with Pancreatic Carcinoma and Chronic Pancreatitis.** Overall, duodenal DNA methylation averaged for all 24 genes analyzed by MSP was more common in patients with pancreatic carcinoma than in those with chronic pancreatitis. This was true after adjusting for patient age using multiple logistic regression (Fig. 3; Table 1). When we compared the prevalence of methylation of individual genes between patients with pancreatic cancer and patients with chronic pancreatitis, duodenum from patients with pancreatic cancer had significantly higher levels of methylation of *E-cadherin*, *p16*, *FOXE1*, *LHX1*, *CLDN5*, *UCHL1*, *ppENK*, and *NPTX2* than did duodenum from patients with chronic pancreatitis.

**Quantification of Duodenal Methylation by COBRA.** COBRA (37) was done in duodenal tissue to quantify methylation of *hMLH1*, *HPPI*, and *UCHL1* (Table 5). As was observed for MSP analysis, COBRA was similarly able to

identify methylation in both frozen and formalin-fixed, paraffin-embedded tissue from the same patients (data not shown). COBRA had a lower limit of detection of ~3% in our hands as determined by serial dilutions of methylated and unmethylated templates (data not shown). As a result, methylation of these genes was detected less frequently by COBRA than by the more sensitive MSP ( $P < 0.01$ ). Most duodenal tissues with methylation had low levels of methylated DNA, usually ~5% to 15% by densitometry analysis. Methylation of *HPPI* and *UCHL1* by COBRA was more prevalent with increasing age ( $P = 0.005$  for *HPPI* and  $P = 0.006$  for *UCHL1*, Student's *t* test). Similar age-related increases in the methylation of these two genes were found using MSP analysis. There was also a significant increase in the quantity of methylation with increasing age for *UCHL1* ( $P = 0.04$ , Kruskal-Wallis test), and a similar trend for *hMLH1* and *HPPI* (not statistically significant; Table 5). As was observed by MSP analysis, there was also greater duodenal DNA methylation in patients with pancreatic cancer than in those with chronic pancreatitis. Indeed, COBRA analysis of duodenal tissues failed to identify any methylation in any patient with pancreatitis (Table 5), whereas 5.5%, 16.9%, and 27.6% of patients with pancreatic cancer had duodenal methylation of *hMLH1*, *HPPI*, and *UCHL1*, respectively.

**Methylation of the Multiple Genes in Microdissected Duodenal Tissue.** To determine the origin of methylated DNA in duodenal tissues, we did manual and laser-captured microdissection of duodenal tissue and extracted DNA from epithelial and other compartments from a 61-year-old patient with pancreatitis. Methylation was detected in 4 (*p16*, *TJP2*, *PIG11*, and *ppENK*) of 13 genes analyzed in this sample and methylated DNA was amplified not only in whole duodenal mucosa but also within the villi, crypts, and Brunner's gland, but not in muscular propria.

## DISCUSSION

In this study, we report that normal duodenal tissues frequently harbor low-level CpG island methylation of genes important for cancer development such as *E-cadherin*, *hMLH1*, and *p16* (13, 23, 24, 38). The percentage of patients with methylation in their duodenum ranged from very few (~1% for *STK11* and *VHL*) to 10% to 20% of patients (for genes such as *hMLH1*, *E-cadherin*, and *p16*), to the majority of patients for other genes (*DAPK*, *CLDN5*, and others). Methylation was also detected in the pancreaticoduodenal secretions of patients without upper gastrointestinal neoplastic disease. We chose to measure duodenal DNA methylation alterations by MSP at CpG islands in the 5' regions of genes in which hypermethylation has been previously described as present in certain cancers, but generally not in their corresponding normal tissues, and at loci in which methylation has sometimes although not always been implicated with gene silencing. In general, the prevalence of methylation in patients' duodenal tissues was similar to that previously observed in pancreatic cancer tissues (Table 1). A few genes such as *Cyclin D2* and *LHX1* were methylated more often in pancreatic carcinoma tissue than in their normal duodenum or duodenal juice (data not shown). Overall, these results suggest

Table 5 Correlation between age and methylation quantity\* in the normal duodenum

Gene	Disease	Methylation (%)	Proportion of cases	Age, mean $\pm$ SD (y)	
<i>hMLH1</i>	PC	0	94.5% (69/73)	64.8 $\pm$ 11.4	
		~ 10	2.7% (2/73)	59.0 $\pm$ 11.4	
		$\geq 10$	2.7% (2/73)	74.5 $\pm$ 10.6	
<i>HPP1</i>	CP	0	100% (32/32)	50.3 $\pm$ 12.0	
		PC	0	83.1% (59/71)	63.8 $\pm$ 12.2*
		~ 10	11.3% (8/71)	70.0 $\pm$ 7.3	
<i>UCHL1</i>	CP	$\geq 10$	5.6% (4/71)	74.5 $\pm$ 10.6	
		0	100% (34/34)	49.7 $\pm$ 11.8	
		PC	0	72.4% (55/76)	63.3 $\pm$ 11.6†
	PC	~ 10	13.2% (10/76)	70.9 $\pm$ 7.7	
		$\geq 10$	14.5% (11/76)	71.0 $\pm$ 8.8	
		CP	0	100% (33/33)	49.9 $\pm$ 11.8

NOTE. Methylation was quantified by COBRA.

\* $P = 0.005$ .

† $P = 0.006$ .

that caution is needed when considering a strategy of detecting aberrantly methylated genes in lumen-derived gastrointestinal tract specimens using methylation specific PCR. This conclusion is likely to be true not only in the context of collecting and analyzing pancreatic juice collected in the duodenum as a source of pancreatic cancer DNA (16) but also when using other clinical samples such as stool as a source of colon cancer DNA. However, we believe these findings should not prevent the use of

methylation-based markers for the detection of pancreatic cancer for several reasons. First, we did observe that some tumor suppressor genes are rarely methylated in normal duodenum, suggesting that there are likely to be some genes methylated in cancers that are not normally methylated in the gastrointestinal tract. MSP primer location is an important consideration when designing assays that distinguish cancer and normal DNA methylation patterns. We generally chose to locate our MSP primers at CpG islands in 5' gene regions in which methylation was previously identified as occurring in pancreatic cancer tissues but not in normal tissues. However, further investigation of the methylation profiles of some of the genes in our panel could identify regions of these CpG islands that are more protected from methylation in normal tissues. Given the large number of genes methylated in pancreatic cancer, it is likely that some genes exist whose methylation is specific for pancreatic cancer and not normal pancreas or duodenum or other upper gastrointestinal tissues. In addition, the level of DNA methylation of normal duodenum was generally of a low level as measured by COBRA raising the possibility that quantification of methylated DNA in certain gastrointestinal secretions could be used in some instances to distinguish low-level methylation of adjacent normal tissues from higher levels observed in cancers. For example, because pancreatic secretions can also be obtained endoscopically by direct cannulation of the pancreatic duct during ERCP, analysis of "pure" pancreatic juice for the detection of aberrantly methylated genes is likely to still be a useful diagnostic strategy, particularly because many of the

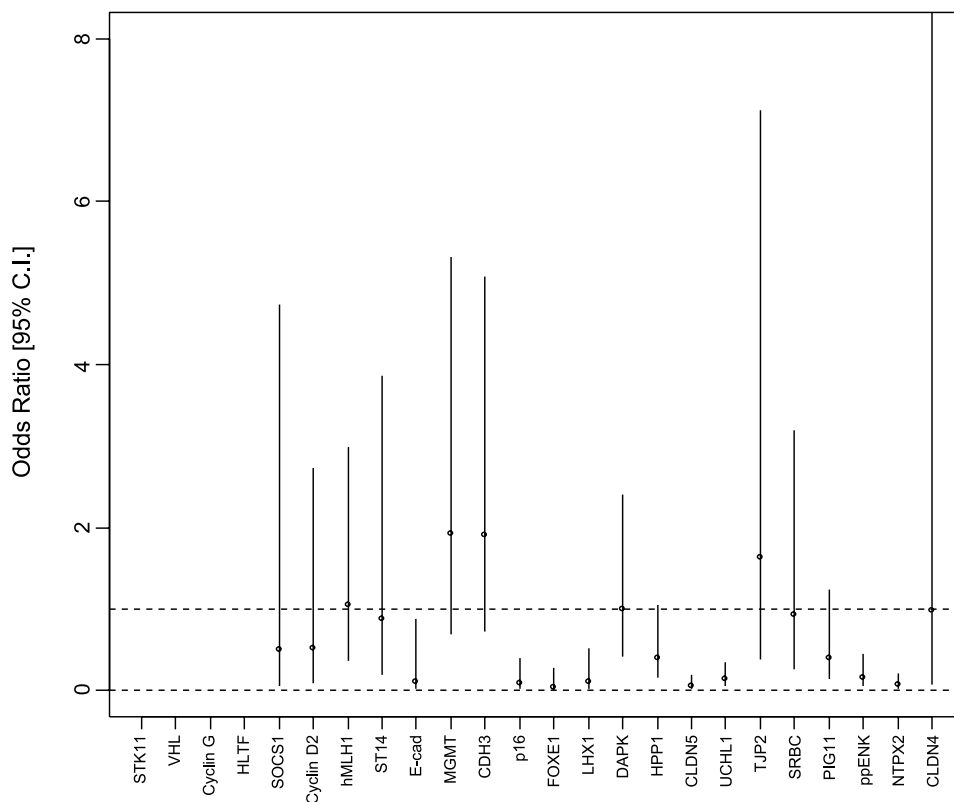


Fig. 3 Comparison of duodenal DNA methylation in patients with pancreatic carcinoma and chronic pancreatitis. The methylation results between the two groups were adjusted for patient age. An odds ratio favoring patients with pancreatic carcinoma trended toward 0 and that associated with chronic pancreatitis toward 1. Several genes had an odds ratio whose 95% confidence intervals were  $<1.0$ , demonstrating that they were statistically more commonly methylated in patients with pancreatic carcinoma than in those with chronic pancreatitis.



genes aberrantly hypermethylated in pancreatic cancer perform well as diagnostic markers when detected in pancreatic juice obtained intraoperatively by direct aspiration of the pancreatic duct (12, 16). Alternatively, because methylation of a given gene can be observed in the duodenal tissues of some individuals but not others, it may be possible to compare the methylation profiles of duodenal secretions before and after secretin stimulation. Methylation of a gene that was absent in the baseline pure duodenal secretions of an individual but present in the pancreaticoduodenal juice obtained after secretin stimulation would likely reflect methylation changes arising from the pancreas.

We also found that patients with pancreatic cancer have a greater propensity to methylate normal duodenum than patients with pancreatitis. This difference held true after adjusting for age and was observed not only for the number of genes methylated in an individual but also in the quantity of methylation in duodenum. Indeed, using COBRA, which requires DNA methylation in ~5% of templates for detection, we did not detect duodenal DNA methylation in any patients with pancreatitis. Another consideration is tumor contamination of the duodenal tissues. However, we do not believe contamination from pancreatic cancer DNA is a likely reason for the difference in duodenal tissue methylation between patients with pancreatitis and pancreatic cancer. In theory, contamination could occur either by undetected microscopic involvement of the duodenal tissue by infiltrating cancer or by pancreatic juice secretions coating the duodenal mucosa with pancreatic cancer cells. However, methylation was analyzed in grossly normal duodenal tissue carefully chosen by a pathologist to be away from the invasive adenocarcinoma to minimize the possibility of contamination. Second, we believe that the number of pancreatic cancer cells in pancreatic juice secretions that would adhere to the duodenal mucosa is likely to be relatively few compared with the many millions of duodenal cells present in the frozen samples of bulk duodenum epithelia used to characterize duodenal methylation. Indeed, it is quite likely that even if some pancreatic cancer cells released into the duodenum adhere to the duodenal wall, it is likely such contamination would be below the level of detection of MSP assays (the level of detection of MSP is ~1 methylated DNA molecule per 1,000 unmethylated templates). Third, the methylation profile of normal-appearing duodenal tissue from patients with pancreatic cancer differed from that of pancreatic cancer. For example, *MGMT* and *DAPK* were methylated in the duodenum of 30% and 51% of patients with pancreatic cancer, two genes that have previously shown to be unmethylated in pancreatic cancers (28). Although the differences in duodenal methylation between patients with pancreatitis and pancreatic cancer were evident for some genes (*E-cadherin*, *p16*, *FOXO1*, *LHX1*, *CLDN5*, *UCHL1*, *ppENK*, and *NPTX2*) but not others (Fig. 3; Table 1), these data suggest that an individual's overall propensity to methylate certain CpG islands may result in a greater likelihood of developing pancreatic cancer and that this propensity is reflected in the level of CpG island methylation in normal tissues. If this is true, then determining methylation of a selected set of CpG islands in nonneoplastic tissues such as the duodenum could be a useful biomarker of future cancer risk. Additional studies are needed to confirm our observation that

cancer patients have higher levels of CpG island methylation in their normal tissues. In addition, it will be important to identify if there are CpG islands whose level of methylation are most predictive of future cancer risk.

Despite the frequent finding of CpG island methylation in nonneoplastic duodenum, it is an organ in which cancer rarely develops. This is despite the many similarities in histology and epidemiology between small and large intestinal neoplasms. The reasons for the low prevalence of duodenal cancer are unknown, but hypotheses include luminal factors such as an alkaline pH, the liquid nature of the intestinal content, and a relative absence of bacteria, as well as tissue factors such as a rapid turnover of epithelial cells and an abundant immune system. When they do develop, duodenal cancers are thought to arise because of multiple genetic and epigenetic changes (2, 39, 40). Indeed, Kim et al. (2) recently reported promoter CpG methylation of a panel of genes, including *p14* and *p16*, in duodenal cancers. Duodenal cancers have also been shown to harbor mutations in *K-ras*, *p53*, and other genes but also have some genetic differences from colorectal cancer (39–41). The high prevalence of CpG island methylation in the nonneoplastic duodenum of patients not predisposed to developing duodenal cancer suggests that methylation of many of the genes analyzed in this study are probably not critical for cancer development. Notwithstanding, our observation of a prevalent CpG island methylation in nonneoplastic duodenum and methylated genes detected in epithelial components may explain some of the molecular profiles of duodenal adenocarcinoma such as a high frequency of microsatellite instability (39) and frequent methylation of *p14*, *p16*, and *hMLH1* (2).

Our results also highlight our incomplete understanding of the factors that influence overall differences in CpG island methylation in normal tissues. In the colon, methylation of crypt cells increases with aging but varies between crypts and at least for certain genes shows a mosaic pattern within a single crypt (42). Aging has been shown to be a risk factor for methylation for several genes and in multiple nonneoplastic tissues (3, 10, 12). For instance, promoter CpG methylation of *ER* is very high in aged liver (7), intermediate in colon (43), and barely detectable in lung (8). An unexplained feature of age-related methylation is the high degree of patient-to-patient variability one observes within a relatively homogeneous group of patients (9). One explanation for the cancer- and age-related methylation of some normally unmethylated CpG islands that has been observed at certain gene loci is that it arises by gradual spreading of methylation by DNA methyltransferases from adjacent normally methylated CpG islands (1). However, the factors responsible for the initiation and progression of such aberrant methylation are not understood.

We did not find any evidence that gender, ethnicity, alcohol, or smoking habit influenced duodenal methylation. Our data are in contrast to previous studies demonstrating that smokers differed in their lung cancer methylation patterns (15) and that gender seemed to influence methylation of the *hMLH1* gene in colon cancers (38). Chronic inflammation has been also implicated as a cause of higher levels of CpG island methylation in the colon in the setting of ulcerative colitis

(44) and in the liver in the setting of chronic liver disease (45), but not in the pancreas in the setting of chronic pancreatitis (46). Environmental exposures in certain tissues were also found to affect subsequent cancer development and associate with DNA methylation status in exposed tissues (8, 45). Genetic factors such as *MTHFR* gene status has been thought to influence global DNA methylation levels in normal cells (47), suggesting that methyl group availability can influence DNA methylation, thereby linking folic acid and other dietary intakes to DNA methylation. Other dietary constituents such as polyphenols (48) found in green tea have been shown to inhibit or reverse methylation at promoter CpGs by inhibiting DNA methyltransferases. These results suggest that using dietary inhibitors of DNA methyltransferases to reduce CpG island methylation could be an effective chemopreventive strategy.

In conclusion, we have shown that methylation of CpG islands in nonneoplastic duodenal tissue is common, increases with age, may be a biomarker of cancer risk, and should be taken into account when using methylation-based assays to detect cancer in the gastrointestinal tract.

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# Clinical Cancer Research

## Age- and Disease-Related Methylation of Multiple Genes in Nonneoplastic Duodenum and in Duodenal Juice

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