Methylation of Cyclin D2 Is Observed Frequently in Pancreatic Cancer but Is Also an Age-related Phenomenon in Gastrointestinal Tissues[1]

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ABSTRACT

Purpose: **Hypermethylation of CpG islands in the promoters of selected genes is a common feature of neoplasia. Aberrant methylation of cyclin D2 has been observed in several cancers. We investigated the methylation of cyclin D2 in aging and pancreatic neoplastic development, and the utility of cyclin D2 methylation as a marker of pancreatic adenocarcinoma.**

Experimental Design: **Methylation-specific PCR was performed on DNA from 165 resected pancreatic exocrine neoplasms [109 adenocarcinomas, 46 intraductal papillary-mucinous neoplasms (IPMNs), and 10 mucinous cystic neoplasms], 14 pancreatic intraepithelial neoplasms, 13 microdissected-normal pancreatic ductal epithelia, 25 normal pancreatic parenchyma, 51 specimens of pancreatic juice obtained perioperatively, 15 pancreatic cancer xenografts, 22 pancreatic cancer cell lines, 59 specimens of normal duodenum, and 49 gallbladders affected by cholecystitis. Cyclin D2 RNA expression was determined in pancreatic cancer cell lines, before and after 5-AZA-2′-deoxycytidine treatment, by reverse transcription-PCR.**

Results: **Methylation of cyclin D2 was identified in 65.1% (71 of 109) of primary pancreatic adenocarcinomas, in 50% (23 of 46) of IPMNs, and in 70% (7 of 10) of mucinous cystic neoplasms, but was detected infrequently in microdissected samples of normal pancreatic epithelia [7.7% (1 of 13)] and in pancreatic intraepithelial neoplasms [14.3% (2 of 14)]. Cyclin D2 methylation was also recognized in 10 of 15 (66.7%) pancreatic cancer xenografts and in 19 of 22 (86.4%) pancreatic cancer cell lines. All of 10 pancreatic cancer cell lines completely methylated at cyclin D2 showed no expression by reverse transcription-PCR. Four of these 10 cell lines were treated with 5-AZA-2′-deoxycytidine, and all 4 showed increased RNA expression of cyclin D2 after treatment. In pancreatic juice, cyclin D2 methylation was detected in 9 of 22 (40.9%) samples from patients with pancreatic cancer and in 6 of 9 (66.7%) patients with IPMNs, but in none of 20 non-neoplastic controls, respectively ($P = 0.0013$ and $P < 0.0001$, respectively). Methylation of cyclin D2 was also observed more in non-neoplastic tissues and with increasing age ($P = 0.041$ in the pancreas, $P = 0.047$ in the duodenum, and $P = 0.0008$ in the gallbladder).**

Conclusions: **The promoter region of cyclin D2 undergoes age-related methylation in multiple tissues, but aberrant methylation is more often detected in tissues and juice samples of pancreatic cancer than in normal tissues. The detection of cyclin D2 methylation in pancreatic juice may aid in the diagnosis of pancreatic adenocarcinoma.**

INTRODUCTION

Pancreatic cancer is a deadly disease. Currently, the most effective treatment for infiltrating pancreatic ductal carcinomas of the pancreas is surgical resection, but the disease is usually advanced at diagnosis, and surgery is only an option for ~15% of patients with pancreatic cancer (1). Whereas most patients present with advanced disease, some patients develop vague symptoms suspicious for pancreatic disease early in the course of their illness, but even these patients may have a delay in their diagnosis, because small lesions (<2 cm) can be difficult to diagnose preoperatively (2, 3) and because of the lack of suitable molecular markers. Existing tumor markers are not yet sufficiently accurate to differentiate benign from malignant diseases of the pancreas (4).

Aberrant methylation of cytosine residues in the promoter region of growth regulatory genes is now recognized as an epigenetic mechanism of gene inactivation in pancreatic and other cancers (5–7). An increasing number of genes have been identified as aberrantly methylated in cancers using the candidate gene approach and a variety of other methods. One novel approach, to use serial analysis of gene expression to identify genes of which the expression was down-regulated in breast cancer, led to the identification of *cyclin D2* as a commonly methylated gene in breast cancer (8–10).

Cyclin D2 is one of the D-type cyclins that function as rate-limiting controllers of G_1 phase progression in mammalian

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Table 1 Clinicopathological characteristics of pancreatic tumors and juice samples[a]

Sample	Number of cases	Gender (M:F)	Mean and range of age (years)	Histology			Clinical stage[b]		
				Ad.[c]	Int.	Inv.	Ia	Ib	III or IV
Tissue									
PDA	109	57:52	61 (34–88)	0	0	109[d]	5	20	84
IPMN	46	25:21	67 (32–81)	19	9	18	32	4	10
MCN	10	4:6	61 (34–86)	4	3	3	9	1	0
Pancreatic juice									
PDA	22	8:14	66 (38–83)] a	0	0	22	1	4	17
IPMN	9	4:5	67 (56–81)] a	2	3	4	6	1	2
Control	20	10:10	55 (31–81)] a	—	—	—	—	—	—

[a] Control juice samples consisted of 10 cases of chronic pancreatitis, 5 cases of islet cell tumors, 2 cases of serous cystadenoma, 2 cases of duodenal tumor, and 1 case of malignant lymphoma.

[b] Clinical stage was according to Union International Contre le Cancer classification.

[c] Ad., adenoma; Int., intraductal carcinoma; Inv., invasive carcinoma; PDA, pancreatic ductal adenocarcinoma; —, not applicable; a, $P = 0.0177$.

[d] Of 109 invasive pancreatic ductal adenocarcinomas, histological differentiation was well differentiated in 8 cases, moderately differentiated in 64 cases, and poorly differentiated in 37 cases.

cells. Their primary role is to sense the readiness of the cell to replicate DNA and to enforce the commitment to enter S phase, which is mediated by the ability of cyclins to activate cyclin-dependent kinase 4 and cyclin-dependent kinase 6, leading to the phosphorylation of retinoblastoma. Depending on cell lineage, various combination of three D-type cyclins (D1, D2, and D3) are induced by mitogens during G_1, and their continued synthesis throughout the cycle depends on persistent growth factor stimulation (11). Despite the putative function of cyclin D2 as a cell cycle proliferation promoter, D-type cyclins were also shown to have growth-inhibitory effects based on their ability to induce a senescence-like phenotype and to inhibit cell proliferation (12, 13). In fact, cyclin D2 was reported to be up-regulated many-fold under the conditions of growth arrest in phenotypically normal human and murine fibroblasts. Similarly, ectopic expression of cyclin D2 effectively blocks cell cycle progression, suggesting that cyclin D2 alternatively functions to promote exit from cell cycle and to maintain in nonproliferative state (12).

Recently, an investigation of global gene expression patterns in IPMN[3] of the pancreas demonstrated that cyclin D2 expression is reduced several fold relative to a non-neoplastic pancreatic epithelial cell line (14). Therefore, we analyzed the methylation status of cyclin D2 in pancreatic neoplasia, in non-neoplastic pancreas, and in other gastrointestinal tissues, and determined the usefulness of cyclin D2 methylation as a marker of pancreatic adenocarcinoma.

MATERIALS AND METHODS

Patients and Tissues. As shown in Table 1, DNA was isolated from resected tissues obtained from each of 109 patients with infiltrating ductal adenocarcinoma of the pancreas, 46 patients with IPMNs, 10 patients with MCNs, and samples of 54 microdissected pancreatic foci (15 acinar and islet tissues, 17 normal pancreatic ductal epithelia, 3 squamous cell metaplasia, 9 PanIN 1, and 10 PanIN 2) from 7 resected pancreata (3 pancreata resected for pancreatic carcinomas, 3 for chronic pancreatitis, and 1 for biliary duct carcinoma), and 25 patients with bulk normal pancreatic parenchyma. In addition, DNA was isolated from normal duodenum from 59 patients, from the gallbladder of 49 patients with chronic cholecystitis, and from samples of human peripheral blood from 35 patients. All of the tissues were obtained from the archives of The Johns Hopkins Hospital Department of Pathology with approval of the Institutional Review Board (Joint Committee on Clinical Investigation). Microdissection was performed manually from four sections of 5-μm thickened consecutive sections of deparaffinized tissues using 30-gauge sterile needles (15). Pancreatic lesions were classified according to criteria agreed upon recently (16, 17), and the clinical stage of pancreatic tumors were determined by Union International Contre le Cancer classification (18).

Pancreatic Juice Specimens. Pancreatic juice specimens, aspirated directly from the main pancreatic duct during pancreatic resection, were obtained from 22 patients with pancreatic carcinoma, 9 patients with an IPMN, and 20 control patients with other periampullary lesions (10 patients with chronic pancreatitis, 5 patients with an islet cell tumor, 2 patients with a serous cystadenoma, 2 patients with duodenal carcinoma that did not involve the ampulla, and 1 patient with a malignant lymphoma). The mean age of these control patients (55 years) was younger than the patients with pancreatic cancer (66 years; $P = 0.0177$; Table 1).

Xenografts and Cell Lines. DNA was also prepared from 15 pancreatic cancer xenografts (19) generated in the laboratory of Dr. Scott Kern (Johns Hopkins University, Baltimore, MD) and from 22 pancreatic cancer cell lines, 4 EBV-transformed lymphocyte cell lines (LCL660, LCL 725, LCL 736, and LCL770), and an immortal cell line from normal pancreatic duct epithelium (HPDE) transfected by the E6 and E7 gene of human papillomavirus 16 (20). The pancreatic cancer cell lines were AsPc1, BxPc3, Capan1, Capan2, Hs766T, MiaPaca2, and Panc1 (all from American Type Culture Collection, Rockville, MD); Colo357 (from European Collection of

[3] The abbreviations used are: IPMN, intraductal papillary-mucinous neoplasm; MSP, methylation-specific PCR; MCN, mucinous cystic neoplasm; 5-AZA-dC, 5-AZA-2′-deoxycytidine; TSA, trichostatin A; PanIN, pancreatic intraepithelial neoplasm; RT-PCR, reverse transcription-PCR.

Animal Cell Cultures, Salisbury, United Kingdom); and 14 pancreatic cancer cell lines (PL1 to PL14) established in the laboratory of Dr. Liz Jaffee (Johns Hopkins University, Ref. 21). HPDE was kindly provided by Ming-Sound Tsao, Ontario Cancer Institute, Toronto, Ontario, Canada (20).

DNA Isolation. Microdissected tissues and pancreatic juice samples were transferred into the sterile tubes containing 50 μl of 1× TK buffer (200 μg/ml of proteinase K and 0.5% of Tween 20) and incubated at 56°C overnight. The tubes were placed in a 100°C block for 10 min to inactivate proteinase K.

MSP. The methylation status of the cyclin D2 promoter site in normal pancreas was determined at two CpG-rich regions that are ~230 bp apart from each other using two different sets of MSP primers as described previously (22, 23). Primer set 1 was located close to the site of primers used previously by Evron *et al.* (8), but we used a different set of primers, because we wanted to design an MSP primer that would amplify a small PCR product, optimal for amplifying DNA from small amounts of archival tissue. A second MSP primer set located closer to the transcriptional start site was also used to characterize cyclin D2 methylation, because the primer set 1 yielded high levels of methylation in normal pancreas. The extracted DNA was treated with sodium bisulfite and analyzed for methylation status in cyclin D2 promoter CpG island (GenBank accession no. U47284) using MSP. MSP was performed in 15-μl PCR reactions with 1 μl of bisulfite-modified DNA using two sets of MSP. The first primer sets specific for unmethylated DNA were 5′-TTAAGGATGTGTTAGAGTATG-3′ (sense, −1450 to −1430) and 5′-CACCAACCCTCCAATTTCAC-3′ (antisense, −1341 to −1360), yielding a 110-bp PCR product, and those for methylated DNA were 5′-CGTTAGAGTACGTGT-TAGGGTC-3′ (sense, −1441 to −1420) and 5′-TCGCCGAC-CCTCCGATTTCG-3′ (antisense, −1338 to −1357), yielding a 104-bp PCR product. The second primer sets specific for unmethylated DNA were 5′-TGGTGTGGTTATGTTTAGTG-3′ (sense, −1188 to −1169) and 5′-ACAATACAACATCTA-AAACCAC-3′ (antisense, −1041 to −1062), yielding a 148-bp PCR product, and those for methylated DNA were 5′-TCGGT-GTGGTTACGTTTAGC-3′ (sense, −1189 to −1170) and 5′-TAAAACGACGCGATACAACG-3′ (antisense, −1032 to −1051), yielding a 158-bp PCR product. The PCR conditions were as follows: 1 cycle of 95°C for 3 min "hot start," followed by the addition of 1 μl of Taq polymerase; 40–45 cycles of 95°C for 20 s, 59°C for 30 s, and 72°C for 30 s; and 1 cycle of 72°C for 3 min. PCR was performed using 45 cycles of amplification on DNA extracted from paraffin-embedded tissue. In our hands the cyclin D2 MSPs had a lower limit of sensitivity of ≥0.2 ng of DNA. For analysis of normal pancreatic epithelia, DNA extracted from an estimated 50–200 cells was used as a template DNA for each MSP.

Treatment of Cells with 5-AZA-dC and TSA. Ten pancreatic cancer cell lines, 4 with complete methylation, 4 with partial methylation and 2 without methylation of Cyclin D2, were seeded at a density of 1×10^6 cells/100-mm plate, respectively. Cells were treated with 1 μM and 2 μM of 5-AZA-dC (Ref. 24; Sigma) and/or TSA (Ref. 25; Sigma) every day. The cellular DNA and RNA was extracted 24 h after treatment with TSA and 4 days after treatment with 5-AZA-dC.

RT-PCR. Total RNA was treated with RNase-free DNase (Boehringer-Mannheim; 0.5–1 units/μl) for 30 min at 37°C, followed by heat inactivation at 65°C for 10 min. Reverse transcription reactions contained 2 μg of DNase-treated RNA, 0.25 g/liter pdN6 random primers (Pharmacia), 1× first-strand buffer (Life Technologies, Inc.), 1 mM of each deoxynucleotide triphosphate (Pharmacia), and 200 units of Moloney murine leukemiavirus-reverse transcription (Life Technologies, Inc.), were incubated at 37°C for 1 h, followed by heat inactivation at 75°C for 5 min. Thirty cycles of PCR was performed using the primers specific for cyclin D2 and temperature conditions described in the previous literature (12).

Statistical Evaluation. Statistical analyses of the prevalence of cyclin D2 methylation by gender, tumor location, histology, and clinical stage were performed by χ^2 test, and age and tumor size by Student-*t* test.

RESULTS

Cyclin D2 Methylation in Tissue Samples. The frequency of cyclin D2 methylation of normal pancreatic ductal epithelium was 11.8% (2 of 17) in primer 1 and 7.7% (1 of 13) in primer 2, and that of normal acinar and islet tissue was 71.4% (10 of 14) in primer 1 and 13.3% (2 of 15) in primer 2, respectively (primer 1 *versus* primer 2 in acinar and islet tissue, $P = 0.0015$). Cyclin D2 methylation was also observed in all areas of squamous cell metaplasia (3 of 3 foci in primer 1 and 2 of 2 foci in primer 2) and in 14.3–40% of PanINs [PanIN 1, 22.2% (2 of 9) in primer 1 and 14.3% (1 of 7) in primer 2; PanIN2, 40% (4 of 10) in primer 1 and 14.3% (1 of 7) in primer 2]. As we found that methylation in the normal pancreas was lower in using primer set 2, we used primer set 2 to analyze cyclin D2 methylation in all of the subsequent studies (Fig. 1). As shown in Table 2, cyclin D2 methylation was identified in 65.1% of usual infiltrating ductal adenocarcinoma of the pancreas, in 50% of IPMNs, in 70% of MCNs, and in 5.7–80% of nonneoplastic tissues other than pancreas.

Correlation between Age and Cyclin D2 Methylation in Non-Neoplastic Samples. The prevalence of cyclin D2 methylation was 20% (5 of 25) in bulk normal pancreatic parenchyma, 13.6% (8 of 59) in normal duodenal mucosa, and 49% (24 of 49) in chronic cholecystitis. The mean age of patients with methylation in their tissues was higher than in those patients with only unmethylated cyclin D2 [In the pancreas,75 *versus* 63 years ($P = 0.0409$), 71 *versus* 62 years in the duodenum ($P = 0.0470$), and 68 *versus* 56 years in the gall bladder ($P = 0.0008$), respectively; Table 2].

Clinicopathological Correlates of Cyclin D2 Methylation. Despite the finding of age-related methylation in normal pancreas, there was no difference in the mean age of patients with cyclin D2 methylation in their pancreatic cancers compared with those without cyclin D2 methylation in their pancreatic cancer. There was no difference in the male:female ratio with respect to cyclin D2 methylation in patients with usual ductal adenocarcinomas, although cyclin D2 methylation was identified more often in IPMNs from male compared with from female patients [64% (16 of 25) in male *versus* 33.3% (7 of 21) in female; $P = 0.0383$]. Similarly, the prevalence of cyclin D2 methylation did not increase with tumor size in patients with

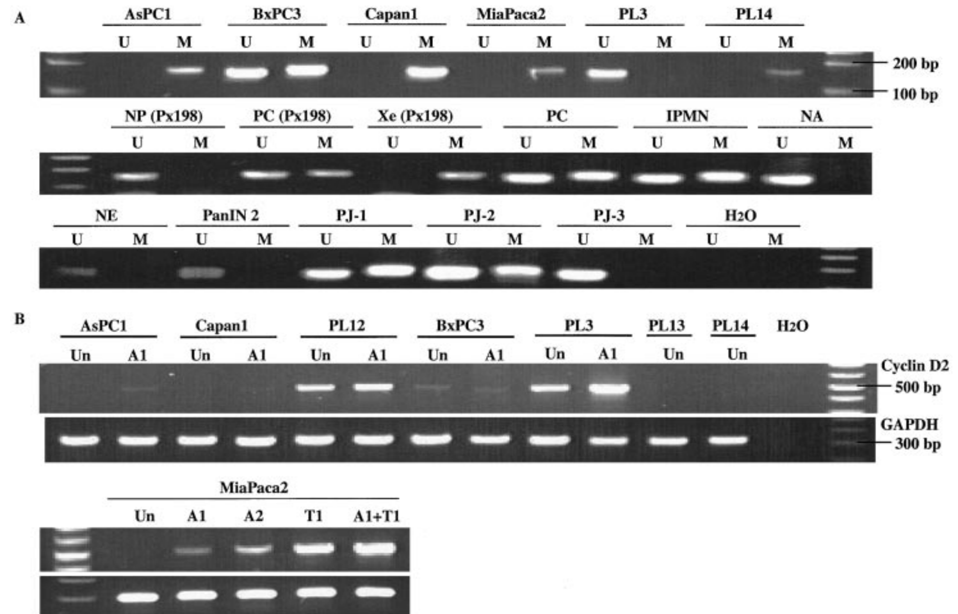

Fig. 1 MSP (*A*) and RT-PCR (*B*) analysis of cyclin D2 in pancreatic samples. In the completely methylated cell lines, AsPC1, Capan1, MiaPaca2, and PL 14, a cyclin D2 RT-PCR product was not observed before 5-Aza-dC treatment, but was detected after 5-Aza-dC treatment (*B*). Abbreviations: *U* and *M*, unmethylated and methylated, respectively. *NP,* normal pancreas; *PC,* primary pancreatic carcinoma; *Xe,* cancer xenograft; *NA,* normal acinar tissue; *NE,* normal pancreatic duct epithelium; *PJ,* pancreatic juice (PJ-1: pancreatic juice from a patient with pancreatic cancer, PJ-2: PJ from a patient with an IPMN, PJ-3: PJ from a patient with chronic pancreatitis); *Un,* untreated by 5Aza-dC nor TSA; *A1,* 1 μM of 5-Aza-dC treated; *A2,* 2 μM of 5-Aza-dC treated; *T1,* 1 μM of TSA treated; *A1+T1,* 1 μM of 5-Aza-dC and TSA treated.

usual ductal adenocarcinomas, but was more frequently detected in larger compared with smaller IPMNs (45.4 ± 18.7 mm *versus* 34.3 ± 18.1 mm; $P = 0.026$). We did not observe any trends between cyclin D2 methylation and tumor histology (adenoma, intraductal carcinoma, and invasive carcinoma), histological differentiation, or clinical stage (Ia, Ib, and III, or IV).

Cyclin D2 Methylation in Pancreatic Juice. In pancreatic juice samples (Table 2), the prevalence of cyclin D2 methylation was significantly higher in patients with pancreatic adenocarcinoma [40.9% (9 of 22)] or IPMN [66.7% (6 of 9)] than in those patients with chronic pancreatitis and other periampullary neoplasms [0% (0 of 20)] ($P = 0.0013$ and $P < 0.0001$, respectively). The mean age of this disease control group (55 years; range, 31–81) was younger than the patients with pancreatic cancer (66 years; range, 38–83; $P = 0.0177$; Table 1).

Methylation and RNA Expression of Cyclin D2 in Xenografts and Cell Lines. Ten of 15 (66.7%) pancreatic ductal adenocarcinoma xenografts and 19 of 22 (86.4%) pancreatic cancer cell lines showed aberrant cyclin D2 methylation, whereas the normal pancreatic epithelial cell line (HPDE) lacked cyclin D2 methylation using MSP (Fig. 1). Of 19 methylated cell lines, 10 were completely methylated (AsPC1, Capan1, Capan2, MiaPaca2, PL2, PL5, PL9, PL10, PL13, and PL14), and 9 were partially methylated (BxPC1, Colo357, Panc1, PL1, PL4, PL6, PL7, PL8, and PL12). By RT-PCR (Fig. 1), cyclin D2 expression was identified in 10 of 22 (45%) pancreatic cancer cell lines and in the HPDE cell line. All of 10 cell lines that harbored complete methylation of cyclin D2 by MSP showed no RNA expression of cyclin D2. In addition, 2 (PL4 and PL7) of 8 cell lines with partially methylated cyclin D2 showed no expression. In contrast, 2 of 3 unmethylated cell lines (HS766T and PL3) showed a strong cyclin D2 band by RT-PCR, whereas the third cell line (PL11) weakly expressed cyclin D2. After 5-AZA-dC treatment, expression was observed in all of 4 (AsPC1, Capan1, Capan 2, and MiaPaca2) cell lines that lacked cyclin D2 expression before treatment.

DISCUSSION

In this study, we find a high prevalence of aberrant hypermethylation of the cyclin D2 promoter in pancreatic neoplasms (Table 2). Because we found that methylation of the upstream CpG island of cyclin D2 is uncommon in normal pancreas, the detection of cyclin D2 methylation may be a useful marker of pancreatic duct neoplasia. In keeping with this notion, we identified cyclin D2 methylation in ~40% of pancreatic juice samples obtained from patients with pancreatic ductal adenocarcinomas but in none of 20 juice samples obtained from patients without a periampullary ductal neoplasm. Because many genes are known to be aberrantly methylated in pancreatic cancer (5, 6), methylated cyclin D2 may be useful as part of a panel of markers that could be used to identify early pancreatic cancer (26, 27). Among those patients in whom we analyzed pancreatic juice, we also observed a significant age difference between those with pancreatic cancer and those with other periampullary diseases raising the possibility that age differences could explain our results. However, we do not believe age is the major reason for these pancreatic juice results, because there was considerable age overlap between the cancer and disease control group, but determination of cyclin D2 methylation in a larger population of patients with pancreatic disease is needed to determine how useful cyclin D2 will be as a marker of pancreatic cancer. In this regard, Evron *et al.* (9) applied a trio of methylation markers including cyclin D2 to breast ductal fluid obtained by irrigation with endoscopy and showed that the panel of markers had an overall sensitivity of 85% for predicting invasive breast cancer. Similarly, the application of a panel of markers to pancreatic juice samples, which can be obtained after secretin stimulation

Table 2 Cyclin D2 methylation and age in the samples of pancreas and other organs[a]

Samples	Cyclin D2 methylation (no. of cases)	Mean and range of age (years)		*P* in age difference
		U	M	
Primary pancreatic neoplasm				
PDA[b]	65.1% (71/109)	64 (34–83)	65 (43–88)	0.5794
IPMN	50% (23/46)	65 (36–81)	66 (32–79)	0.6788
MCN	70% (7/10)	68 (55–86)	59 (34–70)	0.4455
Normal pancreatic parenchyma	20% (5/25)	63 (36–84)	75 (70–84)	0.0409
Normal duodenal mucosa	13.6% (8/59)	62 (34–84)	71 (53–80)	0.0470
Gallbladder mucosa affected by chronic cholecystitis	49% (24/49)	56 (37–80)	68 (41–86)	0.0008
Normal peripheral blood cell	5.7% (2/35)	NA	NA	NA
Normal gastric mucosa	80% (4/5)	NA	NA	NA
Normal colonic mucosa	50% (1/2)	NA	NA	NA
Pancreatic juice				
PDA	40.9% (9/22)]b	60 (38–83)	73 (58–80)	0.0073
IPMN	66.7% (6/9)]a	68 (66–71)	65 (56–81)	0.6853
Control	0% (0/20)]a]b	55 (31–81)	—	—

[a] Control juice samples consisted of 10 cases of chronic pancreatitis, 5 cases of islet cell tumors, 2 cases of serous cystadenoma, 2 cases of duodenal tumor, and 1 case of malignant lymphoma.

[b] PDA, pancreatic ductal adenocarcinoma; U, cyclin D2 unmethylated; M, cyclin D2 methylated; NA, not analyzed; a, $P < 0.0001$; b, $P = 0.0013$.

during routine upper endoscopy (28), may be a useful approach for detecting pancreatic cancer.

We also demonstrated that methylation of cyclin D2 occurs in normal tissues with increasing age. We observed some degree of age-related cyclin D2 methylation in approximately 15–20% of normal pancreatic and duodenal samples, with even higher levels in the gallbladder. This higher level of methylation in gallbladders with chronic cholecystitis may be the result of chronic inflammation, but we did not include normal gallbladders in our study to determine whether this is the case. Promoter hypermethylation related to aging (29, 30) and chronic inflammation (29, 31) has been described, and may contribute to the increase in cancer observed with aging. Thus far, age-related hypermethylation of CpG islands located in promoters has been reported in many genes (30–34). The fact that cyclin D2 methylation occurs with aging in some tissues limits its potential as a tumor marker and indicates that future studies analyzing cyclin D2 methylation as a tumor marker in breast and other tissues should also determine whether methylation in these tissues is a normal feature of aging. The aberrant methylations seen in chronic cholecystitis suggest that pancreatic juice analysis may be more specific than analysis of duodenal fluid, because the latter may contain bile from the gallbladder.

We also demonstrated an inverse correlation between promoter methylation of cyclin D2 and RNA expression of cyclin D2 in pancreatic cancer cell lines, supporting a role for transcriptional silencing by methylation. It is likely that methylation of cyclin D2 explains the recent finding that the expression of cyclin D2 is reduced in IPMNs using global gene expression profiling of IPMNs (14). The functional significance, if any, of cyclin D2 methylation and the associated reduction of cyclin D2 expression in infiltrating ductal adenocarcinoma of the pancreas is unknown. It is thought that D-type cyclins are involved in regulation of cell cycle through phosphorylation, leading to transition of the cell from G_1 to DNA synthesis, and are implicated in cell differentiation and neoplastic transformation. Interestingly, overexpression of cyclin D2 has been reported in gastric carcinoma (35), ovarian granulose cell tumors (36), and hematopoietic cell cancers (37), and has been shown to correlate with disease aggressiveness and poor prognosis in gastric cancer (35). By contrast, reduced or lack of cyclin D2 expression has been reported in the majority of breast cancers (8, 38). In one report, pancreatic cancer also lacked cyclin D2 expression (40), whereas other studies have revealed that pancreatic cancer expresses high levels of cyclin D1 (39) and cyclin D3 (40). Our finding of a high prevalence of methylation in pancreatic cancers in the context of age-related methylation of cyclin D2 in normal pancreas raises the possibility that cyclin D2 methylation in pancreatic and perhaps other cancers simply reflects an acceleration of age-related methylation of this gene during neoplastic development. However, we did not find that cyclin D2 methylation was more common among older patients with pancreatic cancer, IPMNs, or MCNs.

Importantly, as a marker of pancreatic neoplasia, methylated cyclin D2 in pancreatic juice was highly specific, because it was not detected in patients without pancreatic ductal neoplasia. This is despite finding that a low level of cyclin D2 methylation was evident in some normal pancreata. One reason for the lack of any cyclin D2 detection in the pancreatic juice of our disease control group may lie in the sensitivity of the MSP assay. We found that the lower limit of detection of cyclin D2 MSP is 10–20 templates; similar results have been shown by other investigators using MSP for other genes (41). It is not possible to achieve better sensitivity using MSP at present, because bisulfite modification for MSP leads to significant DNA degradation. Thus, it is possible that MSP is not sensitive enough to pick up very low levels of methylated cyclin D2 in patients without pancreatic ductal neoplasia but can easily identify higher levels of cyclin D2 methylation in the pancreatic juice samples with a pancreatic neoplasm. If future studies identify methylated cyclin D2 in pancreatic juice samples from individuals without cancer, quantitative methylation analysis using real-time PCR could quantify the level of cyclin D2 methylation in pancreatic juice samples and may facilitate the

differentiation low levels of methylation from high levels of methylation.

In conclusion, we find that cyclin D2 methylation is commonly present in the primary cancers, and in the pancreatic juice of patients with pancreatic ductal adenocarcinoma and with IPMNs compared with those patients without pancreatic ductal neoplasms, suggesting that cyclin D2 methylation is a useful marker of the pancreatic ductal neoplasia. However, because methylation of cyclin D2 is more common in normal tissues with increasing age, future studies determining the utility of cyclin D2 as a marker of cancer will have to determine the effect of age on the methylation cyclin D2 in corresponding nonneoplastic tissues.

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