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Clinical Studies

Cancer Research

Pancreatic Cancers Epigenetically Silence *SIP1* and Hypomethylate and Overexpress *miR-200a/200b* in Association with Elevated Circulating *miR-200a* and *miR-200b* Levels

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Abstract

Aberrant DNA methylation and microRNA expression play important roles in the pathogenesis of pancreatic cancer. While interrogating differentially methylated CpG islands in pancreatic cancer, we identified two members of *miR-200* family, *miR-200a* and *miR-200b*, that were hypomethylated and overexpressed in pancreatic cancer. We also identified prevalent hypermethylation and silencing of one of their downstream targets, *SIP1 (ZFHX1B, ZEB2)*, whose protein product suppresses *E-cadherin* expression and contributes to epithelial mesenchymal transition. In a panel of 23 pancreatic cell lines, we observed a reciprocal correlation between *miR-200, SIP1*, and *E-cadherin* expression, with pancreatic cancer–associated fibroblasts showing the opposite expression pattern to most pancreatic cancers. In Panc-1 cells, which express *SIP1*, have low *E-cadherin* expression, and do not express *miR-200a* or *miR-200b*, treatment with *miR-200a* and *miR-200b* downregulated *SIP1* mRNA and increased *E-cadherin* expression. However, most pancreatic cancers express *miR-200a* and *miR-200b*, but this expression does not affect *SIP1* expression, as the *SIP1* promoter is silenced by hypermethylation and in these cancers *E-cadherin* is generally expressed. Both *miR-200a* and *miR-200b* were significantly elevated in the sera of pancreatic cancer and chronic pancreatitis patients compared with healthy controls ($P < 0.0001$), yielding receiver operating characteristic curve areas of 0.861 and 0.85, respectively. In conclusion, most pancreatic cancers display hypomethylation and overexpression of *miR-200a* and *miR-200b*, silencing of *SIP1* by promoter methylation, and retention of E-cadherin expression. The elevated serum levels of *miR-200a* and *miR-200b* in most patients with pancreatic cancer could have diagnostic utility. *Cancer Res; 70(13); 5226–37. ©2010 AACR.*

Introduction

Pancreatic ductal adenocarcinoma is the fourth leading cause of cancer death in the United States and it has the lowest survival rate for any solid cancer. In 2009, it is estimated that 42,470 Americans will be diagnosed with pancreatic cancer (1) and only about 4% of patients will live 5 years after diagnosis. One important reason for this poor survival is that most patients present with advanced disease; only ~15% to 20% of patients with pancreatic cancer present with small, resectable cancers. Furthermore, pancreatic cancer remains unresponsive to most chemotherapeutic agents. Hence, there is a great need to understand the biological mechanisms that contribute to pancreatic cancer development and progression so as to develop effective therapies, and to identify more effective markers of pancreatic neoplasia so as to more effectively detect pancreatic cancer and its precursors.

Recent studies show that some precursors to pancreatic cancer, pancreatic cystic neoplasms, can be visualized by pancreatic imaging. Pancreatic imaging is being used to detect preinvasive neoplasms in patients with a significant family history of pancreatic cancer and those who have an inherited predisposition to develop the disease because of germline mutations in genes such as *BRCA2, p16*, and *PALB2* (2–4). A limitation of current screening tests is that pancreatic imaging does not reliably detect the microscopic pancreatic cancer precursors known as pancreatic intraepithelial neoplasias (5). In addition, most patients with pancreatic cancer do not have major risk factors for the disease and are not candidates for screening. Hence, there is a need for sensitive, specific, and accurate tests that would facilitate the rapid diagnosis of pancreatic cancer and its precursors. New candidate markers have been described in recent years that have been evaluated in serum (6) and in pancreatic secretions (7) and ductal brushings (8) to detect local pancreatic neoplasia, but more accurate markers are needed.

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Although many genes are mutated in pancreatic cancer (9), mutant *KRAS* is the only gene to be extensively studied as a diagnostic marker (10, 11). Aberrantly methylated genes also have potential as diagnostic markers (7, 8). Aberrant CpG island methylation is an important cause of altered gene function in pancreatic and other cancers (12–17). Alterations in the expression of microRNAs (miRNA) have also been identified in pancreatic and other cancers and their deregulation seems to contribute to cancer development and progression (18). Profiles of miRNAs can discriminate cancer from normal tissue and even distinguish different tumor types (19). MiRNAs are measurable in the circulation (20, 21), raising hopes that the detection of certain overexpressed miRNAs could be useful noninvasive tests for the early detection of cancer. For example, elevated circulating levels of miR-21, miR-141, and miR-92 have been found in patients with certain cancers (20–22).

Recently, we used Agilent microarrays to identify differentially methylated CpG islands in pancreatic cancers (23), and using this method, we now report that the 5′ region of miRNA-200 is aberrantly methylated in pancreatic cancer. *MiR-200a* and *miR-200b* are encoded within a single noncoding polycistronic transcript (24). The *miR-200* family targets two E-box-binding transcription factors, SIP1 (also known as ZFHX1B and ZEB2) and ZEB1, which are negative regulators of *E-cadherin*. Downregulation of *E-cadherin* such as through SIP1 is essential for epithelial-mesenchymal transition (EMT), a program by which cells lose their adhesive phenotype and become more mobile, and the *miR-200* family has recently been implicated as a pivotal regulator of the EMT (25–27). EMT has been implicated in the progression of pancreatic and other cancers (28–30), although some investigators suggest that true EMT is not a general feature of cancers, rather that cancers often retain some features of EMT and should be termed EMT-like (31). Genetic and epigenetic mechanisms are important causes of *E-cadherin* inactivation but occur only occasionally in pancreatic cancers (9, 15). Interestingly, pancreatic cancers lacking *E-cadherin* expression have an undifferentiated phenotype (32). Although some investigators suspect that EMT is associated with genetic alterations in cancer-associated stromal cells (33), we find no evidence that pancreatic cancer–associated fibroblasts have chromosomal alterations (34).

In addition to reporting on the aberrant methylation of *miR-200a/miR-200b* in pancreatic cancer, we find that these miRNAs are overexpressed and that the downstream target of *miR-200, SIP1*, is commonly methylated in pancreatic cancers. We also investigate the influence of *miR-200a* and *miR-200b* on *E-cadherin* and *SIP1* expression and show that *miR-200a* and *miR-200b* are potential markers of pancreatic cancer.

Materials and Methods

Cell lines and tissue samples

Fifteen human pancreatic cancer cell lines including A32-1, A38-5, AsPC1, BxPC3, Capan1, Capan2, CFPAC1, MiaPaCa2, Panc-1, Panc2.5, Panc2.8, Panc3.014, Panc215, PL3, and PL8 and seven pancreatic cancer–associated fibroblasts including CAF15, CAF16, CAF18, CAF19, CAF20, CAF21, and CAF22 were studied and grown under recommended conditions. An immortalized cell line derived from normal human pancreatic ductal epithelium (HPDE) and human pancreatic Nestin-expressing cells (HPNE) were provided by Dr. Ming-Sound Tsao (University of Toronto, Toronto, Ontario, Canada) and Dr. Michel Ouellette (University of Nebraska Medical Center, Omaha, NE), respectively.

Normal and neoplastic tissues were obtained from pancreatic adenocarcinomas resected at the Johns Hopkins Hospital. Fresh-frozen pancreatic tissues from 9 patients who underwent a pancreatic resection for an intraductal papillary mucinous neoplasm (IPMN) without associated invasive adenocarcinoma, 7 patients for neuroendocrine tumors, and 19 patients for invasive ductal adenocarcinoma were microdissected to obtain normal pancreatic tissue for DNA analysis. Their mean age ± SD was 64.0 ± 13.0 years (18 female). DNA was analyzed from 36 pancreatic cancer xenografts (32 pancreatic, 3 distal bile duct, and 1 duodenal cancer; mean age, 67.3 ± 8.8 y; 24 females) established from primary carcinomas (15). Fresh-frozen pancreatic cancer tissues (n = 7; mean age, 62.4 ± 15.4 y; 4 females) and normal pancreas from patients who had undergone resection for a pancreatic neuroendocrine neoplasm, IPMN, and mucinous cystadenoma (n = 13; mean age, 55.5 ± 20.6 y; 5 females) were microdissected, placed in RNAlater immediately after dissection, and stored at −80C°. Frozen pancreatic tissues were microdissected using the PALM microlaser system as previously described (9). We also microdissected formalin-fixed paraffin-embedded tissues for *SIP1* and *E-cadherin* methylation analysis as previously described (35).

Eighty-eight serum samples from Johns Hopkins Hospital were analyzed, including 45 preoperative samples from patients with pancreatic ductal adenocarcinoma (64.4 ± 10.0 y; 19 females), 11 preoperative samples from patients with chronic pancreatitis undergoing surgical resection (64.0 ± 12.3 y; 3 females), and 32 healthy controls (44.3 ± 10.2 y; 18 females). All sera were collected using standard procedures and stored at −80°C until analysis. All specimens were collected and analyzed with the approval of the Johns Hopkins Committee for Clinical Investigation.

Bisulfite treatment, methylation-specific PCR, and bisulfite-modified sequencing

These were done as previously described (23).

RNA isolation and real-time PCR

Total RNA was extracted using *mir*Vana miRNA isolation kit (Ambion) for tissues and *mir*Vana PARIS kit (Ambion) for serum (100–400 μL) according to the manufacturer's instructions, and reverse transcription-PCR (RT-PCR) was done as previously described (36). Real-time PCR was done in triplicate. MiRNAs were amplified after specific reverse transcription using TaqMan miRNA assays and TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions (Applied Biosystems) and normalized against 18S rRNA for cell lines and against *miR-16* for serum and microdissected

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tissues (20, 22, 37, 38). Relative expression was determined using the ΔΔCt method, and a >35 Ct value indicated negative amplification. Primers are shown in Supplementary Table S1.

5-Aza-2′-deoxycytidine and Trichostatin A treatment

The treatment was done as we previously described (17). For anti-miRNA transfection analysis, 2 days after being treated with 1 μmol/L 5-aza-2′-deoxycytidine (5-aza-dC), cells were transfected for 1 day and then retreated with 5-aza-dC for another 24 hours.

Transfection of miRNA precursors and inhibitors

Cells were plated at 5×10^4 per well in 12-well plates and transfected with pre-miR miRNA precursors or anti-miR miRNA inhibitors (has-*miR-200a* and has-*miR-200b*, Ambion) at 40 nmol/L for *miR-200a, miR-200b*, and the combination (each 20 nmol/L) using siPORT NeoFX Transfection Agent (Ambion). Cy3 dye–labeled Pre-miR Negative Control #1 and Cy3 dye–labeled Anti-miR Negative Control #1 (Ambion) were used as controls for pre-miRNA and anti-miRNA transfection, respectively. Cells were collected for RT-PCR 48 hours after transfection. For the multiple transfection experiment, cells were split and retransfected with additional pre-miRNA every 3 to 4 days for 18 days.

Immunohistochemistry

The expression of E-cadherin protein was examined by immunohistochemical labeling of formalin-fixed, paraffin-embedded tissue microarray sections of pancreatic cancers and normal pancreas from 328 patients who underwent pancreaticoduodenectomy at Johns Hopkins Hospital using previously described methods (32, 39). Tissue sections were incubated with an anti–*E-cadherin* mouse monoclonal antibody (clone HEDC-1, Zymed Laboratories; 1/10 dilution) for 60 minutes at room temperature. The mean age ± SD of these patients was 66.5 ± 10.5 years (46% female). Expression patterns were classified as intact, partial loss, or total loss of expression, with total loss of expression indicating less than 5% of cancer cells expressing E-cadherin and partial loss indicating less than 95% of cells expressing E-cadherin.

Statistics

Statistical analysis was done using the SPSS Statistics 17.0 and Microsoft Excel statistics software. A two-tailed *P* value of <0.05 indicated statistical significance.

Results

MiR-200a and *miR-200b* are hypomethylated in pancreatic cancer

MiR-200a and *miR-200b* were identified as candidate hypomethylated genes after comparing the CpG island methylation profiles of six pancreatic cancer lines to normal pancreas using the methylation restriction assay known as methylation CpG island amplification (MCA) and identifying methylation patterns using the Agilent 244K CpG island array (23). On the array, a CpG island containing 183 CpG dinucleotides [CGI-183; chr1: 1087907–1090447 bp (2,541 bp); %GC = 65.0%; observed/expected CpGs = 69.0%] had probes with elevated log 2 Cy3/Cy5 ratios (normal/cancer) indicating hypomethylation in 5 of 6 pancreatic cancer cell lines relative to normal tissues (Fig. 1A). This CpG island is located upstream of the putative TSS of the *miR-200a/200b* locus (Fig. 1B; ref. 24).

To validate the hypomethylation of *miR-200a* and *miR-200b* in pancreatic cancer, we examined its promoter methylation status in pancreatic cancer and nonneoplastic cell lines using methylation-specific PCR (MSP). Eleven of 15 (73.3%) pancreatic cancer cell lines were unmethylated, whereas two microdissected normal pancreatic duct samples were predominantly methylated (Table 1; Supplementary Fig. S1). The nonneoplastic cell line HPNE was completely methylated, whereas HPDE immortalized by HPV16-E6E7 was unmethylated (Table 1; Supplementary Fig. S1A).

We next performed bisulfite sequencing to verify the hypomethylation of the *miR-200* promoter. We found that 9 of 11 pancreatic cancer cell lines, 6 of 6 pancreatic cancer xenografts, and the nonneoplastic cell line HPDE were completely unmethylated for the 36 CpG sites sequenced, whereas the nonneoplastic cell line HPNE and two cancer lines, MiaPaCa2 and Panc-1, were methylated in all sequenced CpG sites (Supplementary Fig. S1B), consistent with MSP data. We next expanded our MSP assay to 36 xenografts of primary pancreatic cancers and 35 normal pancreatic tissues and found that 97.2% (35 of 36) of pancreatic cancer xenografts were unmethylated whereas all 35 normal pancreatic tissue samples were methylated. To further analyze normal pancreatic tissue methylation, we performed bisulfite sequencing on 10 normal pancreas samples, 6 of which showed predominant methylation at most CpG sites, 2 with ~50% methylation, and 2 that were predominantly unmethylated (Supplementary Fig. S1C).

MiR-200a and *miR-200b* are overexpressed in pancreatic cancer

To evaluate whether *miR-200a* and *miR-200b* are overexpressed in pancreatic cancers, we measured their expression in pancreatic cell lines and primary pancreatic tissues using quantitative RT-PCR. As shown in Fig. 2, compared with the nonneoplastic cell lines HPNE and HPDE, *miR-200a* and *miR-200b* were overexpressed in 11 of 14 pancreatic cancer cell lines. Ten of 11 overexpressing cell lines were completely unmethylated at 5′CpGs, whereas the 12th cell line, AsPC1, was partially methylated (Table 1). *MiR-200a* and *miR-200b* were also overexpressed in primary pancreatic cancers compared with normal pancreatic tissues ($P < 0.001$; Fig. 3). Among the pancreatic cancer cell lines A38-5, MiaPaCa2 and Panc-1, which have low levels of *miR-200a* and *miR-200b* (Fig. 2), treatment with 5-aza-dC induced *miR-200a* and *miR-200b* by 3-fold or more (Fig. 4). Similarly, 5-aza-dC induced *miR-200a* and *miR-200b* expression in HPNE (Fig. 4). In contrast, apart from A38-5, treatment of pancreatic cancer cell lines with the histone deacetylase inhibitor trichostatin A did not alter *miR-200a/200b* expression (Supplementary Fig. S2).

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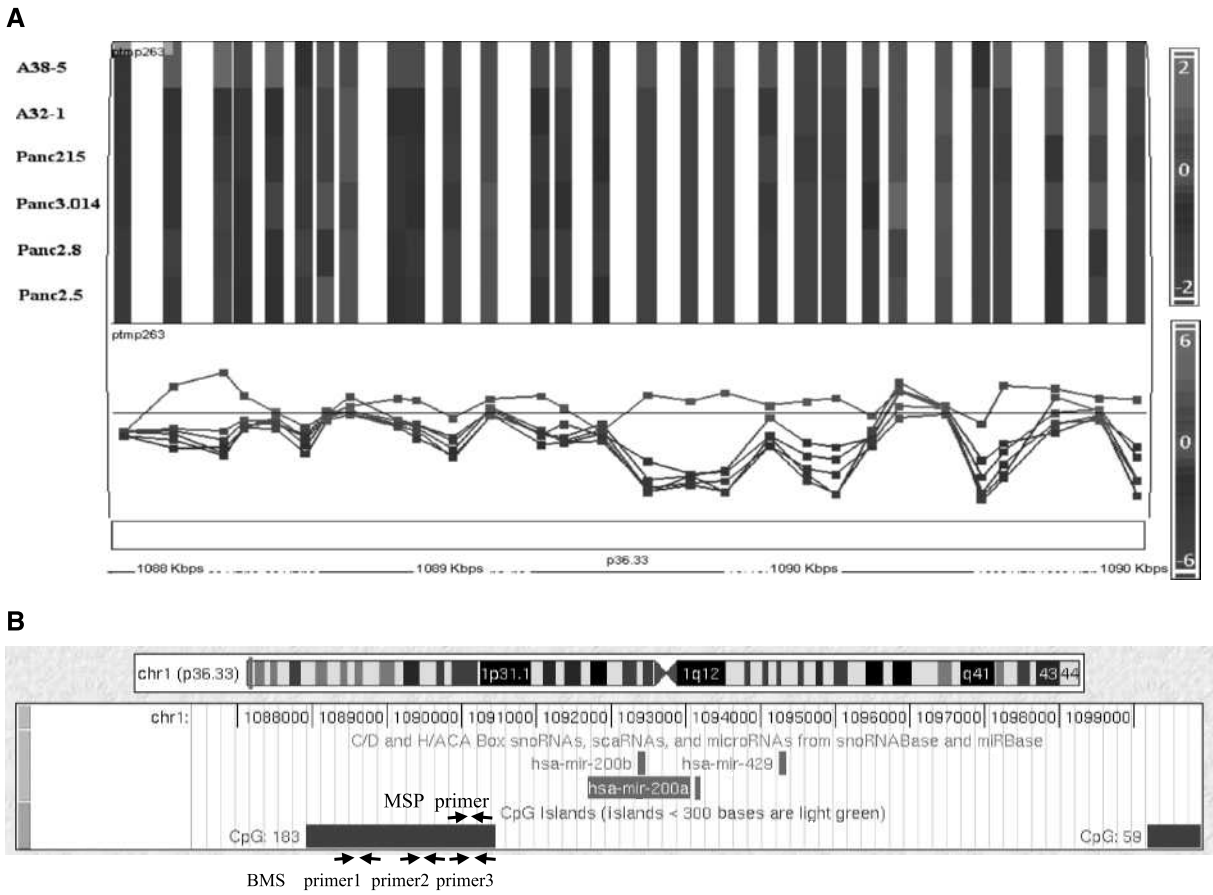

Figure 1. *MiR-200a* and *miR-200b* hypomethylation in pancreatic cancer. A, probes within the *miR-200* CpG island (CGI-183) indicating reduced log 2 Cy3/Cy5 ratios (normal/cancer) in 5 of 6 pancreatic cancer cell lines relative to normal tissues by MCA CpG microarray (23). B, CGI-183 is located upstream of the *miR-200* start site (UCSC database). Primer locations for MSP and bisulfite-modified sequencing are shown.

DNA methylation and expression of *SIP1* and the effects of *miR-200a* and *miR-200b*

We next focused on one of the targets of *miR-200, SIP1*. *SIP1* is an important transcriptional repressor that down-regulates multiple genes, including *E-cadherin*, and shows aberrant expression in several cancers. Because *SIP1* showed hypermethylation in our MCA microarray data, we examined the methylation status of *SIP1*. We found that the *SIP1* promoter was methylated in 73.3% (11 of 15) of pancreatic cancer cell lines and in 97.1% (34 of 35) of pancreatic cancer xenografts, whereas 2 nonneoplastic cell lines and 94.3% (33 of 35) of normal pancreas samples were unmethylated (Table 1). Thirty of 35 pancreatic cancer xenografts had complete *SIP1* methylation. *E-cadherin* was also unmethylated in all but one of these xenografts (data not shown). *SIP1* generally displayed a methylation profile inverse to that of *miR-200* (Table 1). By quantitative RT-PCR (Fig. 2) and by serial analysis of gene expression (SAGE; ref. 9), *SIP1* (ZEB2) was expressed in HPNE but was not expressed in most pancreatic cancer cell lines. Similarly, microdissected pancreatic cancer tissues had significantly lower *SIP1* RNA by quantitative RT-PCR than normal pancreas (Fig. 3). In contrast, we found abundant *SIP1* RNA in 7 of 7 pancreatic cancer–associated fibroblasts, which also displayed inverse expression profiles to *miR-200a* and *miR-200b* (Fig. 2). Because Snail and Slug induce SIP1 expression and repress E-cadherin, we examined their expression in pancreatic cancers by SAGE and found that SNAIL was minimally expressed and there was no evidence that SNAIL or SLUG expression repressed E-cadherin expression (data not shown).

We found that DNA methylation regulated the expression of *SIP1*, as treatment with 5-aza-dC induced *SIP1* expression in all seven cell lines tested (Fig. 4). Interestingly, in HPNE cells that have methylated *miR-200*, induction of *miR-200a* and *miR-200b* expression by 5-aza-dC (Fig. 4A and B) was associated with a 5-fold reduction of *SIP1* expression, supporting the negative regulation of *SIP1* by *miR-200* (Fig. 4). SIP1 recruits the transcriptional repressor CtBP to inhibit Smad-mediated transcription (40), and because *SMAD4* is mutated

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Table 1. Methylation profiles of *miR-200a, miR-200b*, and *SIP1* in pancreatic tissues by MSP

No.	Cell line	*miR-200*	SIP1	No.	XPC	Age	Gender	Diagnosis
Cancer				1	Panc140	74	F	P.C.
1	A32-1	U	M	2	Panc154	77	F	P.C.
2	A38-5	P	U	3	Panc159	64	F	P.C.
3	AsPC1	P	M	4	Panc163	77	F	P.C.
4	BxPC3	U	M	5	Panc185	60	F	P.C.
5	Capan1	U	M	6	Panc194	57	F	P.C.
6	Capan2	U	P	7	Panc198	69	F	P.C.
7	CFPAC1	U	U	8	Panc215	60	F	P.C.
8	MiaPaCa2	M	M	9	Panc219	55	F	P.C.
9	Panc-1	M	U	10	Panc253	53	F	P.C.
10	Panc2.5	U	P	11	Panc266	59	M	P.C.
11	Panc2.8	U	M	12	Panc281	66	F	P.C.
12	Panc215	U	M	13	Panc294	66	F	P.C.
13	Panc3.014	U	U	14	Panc325	70	F	P.C.
14	PL3	U	M	15	Panc354	81	M	P.C.
15	PL8	U	M	16	Panc363	86	F	P.C.
Normal				17	Panc421	58	M	P.C.
16	HPDE	U	U	18	Panc430	73	F	P.C.
17	HPNE	M	U	19	JHH10	54	F	P.C.
18	midND1	P	N/A	20	JHH11	78	M	P.C.
19	midND2	P	N/A	21	JHH15	69	F	P.C.
				22	JHH21	80	M	P.C.
				23	JHH24	64	M	P.C.
				24	JHH27	70	M	P.C.
				25	JHH34	57	F	P.C.
				26	Px65	63	F	P.C.
				27	Px194	77	F	P.C.
				28	Px352	65	F	P.C.
				29	JHH33	60	M	IPMN
				30	Panc169	75	M	IPMN
				31	Panc410	59	F	IPMN
				32	Panc420	68	M	IPMN
				33	Panc286	69	F	D.C.
				34	Panc247	82	M	B.D.C.
				35	Panc287	65	M	B.D.C.
				36	Panc291	62	F	B.D.C.

(*Continued on the following page*)

in ~55% of pancreatic cancers, we determined if loss of *SIP1* was more likely found in pancreatic cancers with wild-type *SMAD4*, but we did not find any correlation (data not shown).

To test if *miR-200* downregulates *SIP1* expression in pancreatic cancer cells, we transfected the cell line Panc-1, which is fully methylated at *miR-200* and unmethylated at *SIP1*, with pre-*miR-200a* or/and pre-miR *miR-200b* precursors and examined their effects at day 2 and day 18 after infection. Compared with control transfected cells, *SIP1* mRNA was significantly reduced by *miR-200a* ($P < 0.01$, $P < 0.01$), *miR-200b* ($P < 0.05$, $P < 0.01$), and combined *miR-200a/200b* treatment ($P < 0.01$, $P < 0.01$) 2 days after transfection and after 18 days of repeated transfections, respectively (Supplementary Fig. S3).

Although miRNAs are not thought to induce epigenetic silencing, as we observed *miR-200* overexpression and DNA methylation of *SIP1*, we considered the possibility that epigenetic inactivation of the *SIP1* promoter in pancreatic cancers could be caused by aberrant *miR-200* expression. To test this possibility, we maintained the expression of *miR-200* for 2 weeks by transiently transfecting Panc-1 cells every 3 to 4 days. However, induction of *miR-200* expression did not induce any *SIP1* promoter methylation by MSP analysis (data not shown).

We also examined if inhibition of *miR-200* could increase *SIP1* expression. First, we treated AsPC1 cells with antisense inhibitors to *miR-200a, miR-200b*, or both. Because *SIP1* is

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Table 1. Methylation profiles of *miR-200a, miR-200b*, and *SIP1* in pancreatic tissues by MSP (Cont'd)

miR-200	SIP1	No.	NP	Age	Gender	Diagnosis	*miR-200*	SIP1
U	M	1	NP1	58	F	P.C.	P	U
U	M	2	NP2	81	M	P.C.	P	U
U	M	3	NP3	51	M	P.C.	P	P
U	M	4	NP4	53	M	P.C.	P	U
U	M	5	NP5	76	M	P.C.	P	U
U	M	6	NP6	69	M	P.C.	P	U
U	M	7	NP7	75	M	P.C.	P	U
U	M	8	NP8	76	F	P.C.	P	U
U	M	9	NP9	42	F	P.C.	P	U
U	M	10	NP10	53	F	P.C.	P	U
U	M	11	NP11	78	M	P.C.	P	U
U	P	12	NP12	74	F	P.C.	P	U
U	M	13	NP13	62	M	P.C.	P	U
U	M	14	NP14	71	M	P.C.	P	U
U	M	15	NP15	79	M	P.C.	P	U
U	M	16	NP16	54	M	P.C.	P	U
U	M	17	NP17	76	M	P.C.	P	U
U	P	18	NP18	70	F	P.C.	P	U
U	M	19	NP19	74	F	P.C.	P	P
U	M	20	NP20	63	F	P.E.T.	P	U
U	P	21	NP21	42	F	P.E.T.	P	U
U	M	22	NP22	39	M	P.E.T.	P	U
U	M	23	NP23	66	F	P.E.T.	P	U
U	M	24	NP24	56	M	P.E.T.	P	U
U	ND	25	NP25	45	F	P.E.T.	P	U
U	P	26	NP26	41	F	P.E.T.	P	U
U	U	27	NP27	82	M	IPMN	P	U
U	M	28	NP28	63	M	IPMN	P	U
U	M	29	NP29	57	M	IPMN	P	U
U	M	30	NP30	72	F	IPMN	P	U
U	M	31	NP31	78	F	IPMN	P	U
P	M	32	NP32	72	F	IPMN	P	U
U	M	33	NP33	56	F	IPMN	P	U
U	M	34	NP34	81	F	IPMN	P	U
U	M	35	NP35	72	F	IPMN	P	U
U	M							

NOTE: , methylation (M); , partial methylation (P); , unmethylation (U).
Abbreviations: B.D.C., bile duct cancer; D.C., duodenal cancer; IPMN, pancreatic cancer associated with IPMN; midND, microdissected normal duct; NP, normal pancreas; P.C., pancreatic cancer; P.E.T., pancreatic endocrine tumor; XPC, xenograft of P.C.; ND, not determined.

silenced in association with DNA methylation in AsPC1 cells, treatment with *miR-200a* and/or *miR-200b* inhibitors did not induce expression of *SIP1* in AsPC1 cells (Supplementary Fig. S3). To determine if *miR-200a* and *miR-200b* still have the ability to regulate *SIP1* expression in cells in which the *SIP1* promoter is methylated, we pretreated AsPC1 cells with 5-aza-dC to induce *SIP1* expression. Inhibitors of *miR-200a* and *miR-200b*, alone and in combination, increased *SIP1* expression by 31% ($P = 0.39$), 82% ($P < 0.05$), and 128% ($P < 0.05$) compared with negative control transfected cells, respectively (Supplementary Fig. S4).

Effect of *miR-200a* and *200b* on *E-cadherin* expression and EMT

Because *miR-200* and *SIP1* have been identified as regulators of EMT, we examined *E-cadherin* expression in pancreatic cancers and determined if it was upregulated by *miR-200* in pancreatic cancer cells. *E-cadherin* was expressed in 11 of 14 pancreatic cancer cell lines at levels approximately at or above the control pancreatic duct line, HPDE, but was not expressed in 7 CAF lines (Fig. 2). Of the three cell lines lacking *E-cadherin* expression, MiaPaca2 was completely methylated at the *E-cadherin* locus associated with gene silencing

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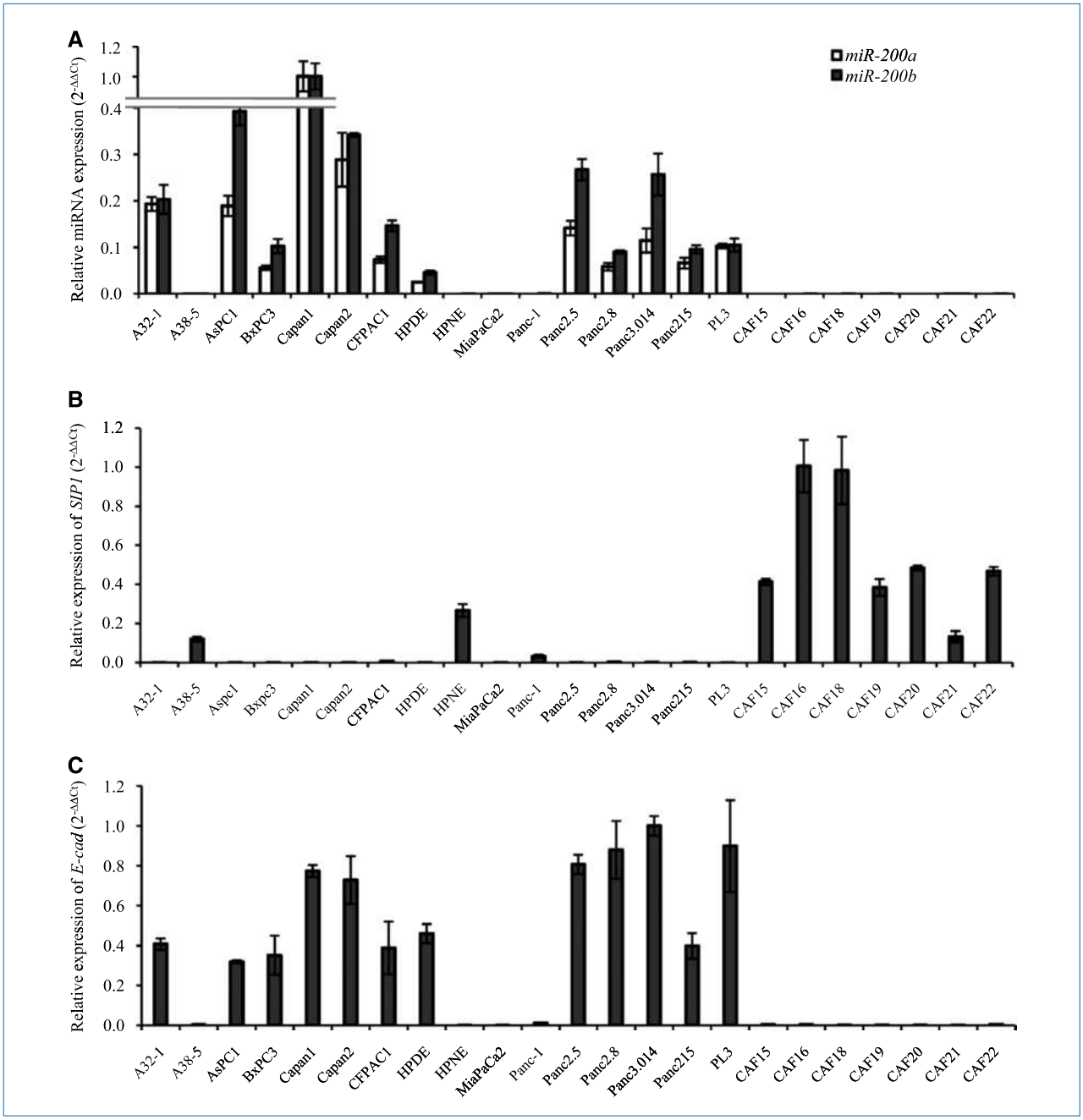

Figure 2. Expression of *miR-200a/miR-200b* (A), *SIP1* (B), and *E-cadherin* (C) in pancreatic cancer cell lines relative to nonneoplastic HPNE and CAF cell lines by real-time PCR. Reference RNAs were used for *miR-200a* and *miR-200b* (18S rRNA), *SIP1*, and *E-cadherin* (*GAPDH*). Columns, mean of triplicates; bars, SD. Representative of two to three independent experiments.

(Fig. 5; ref. 15), whereas Panc-1 and A38-5 expressed *SIP1* and lacked *miR-200* expression, suggesting that these pancreatic cancers have lost *E-cadherin* expression and have undergone EMT as a result of *SIP1* expression. Similarly, by SAGE (9), *E-cadherin* was expressed at or above the levels in pancreatic duct epithelial cells in 21 of 24 pancreatic cancers. Indeed, we found that pre-*miR-200b* and combined pre-*miR-200a/200b* treatment increased *E-cadherin* expression in Panc-1 cells ($P < 0.05$, $P < 0.05$; $P < 0.05$, $P < 0.01$) both at day 2 and at day 18 (after multiple transfections), respectively (Supplementary Fig. S3). E-cadherin expression was also reduced in AsPC1 cells both by anti-miR inhibitors of *miR-200b* and *miR-200a* and by 5-aza-dC treatment (Supplementary Fig. S4).

However, we did not observe any reversal of EMT morphology (i.e., reversal of fibroblastoid spindles or increased

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cell-to-cell contacts) in Panc-1 cells even after sustained suppression of *E-cadherin* by repeated transfection of *miR-200a* and *miR-200b* precursors (Supplementary Fig. S3C).

E-cadherin protein expression in primary pancreatic adenocarcinomas

Our results indicate that only a minority of pancreatic cancer cell lines lack *E-cadherin* expression. Furthermore, we also find that most xenografts of primary pancreatic adenocarcinomas have complete methylation of *SIP1*, suggesting that *SIP1* is not expressed in most pancreatic cancers. However, because other mechanisms of EMT could be active in pancreatic cancers, we examined the prevalence of low E-cadherin expression by immunohistochemistry in the resected pancreatic cancer tissues of 329 patients who had undergone pancreaticoduodenectomy. We found intact expression in 188, partial loss in 134, and total loss in 7 of E-cadherin expression in 329 pancreatic ductal adenocarcinomas (Supplementary Fig. S5). Loss of E-cadherin expression was observed in 2 of 8 well-differentiated (25%), 65 of 175 moderately differentiated (37%), and 74 of 146 poorly differentiated (51%) carcinomas ($P = 0.03$, χ^2 test).

To examine if DNA methylation was responsible for the loss of E-cadherin, we obtained additional pancreatic cancer tissues from 6 of the 7 primary pancreatic cancers with total loss of E-cadherin. These pancreatic cancers were microdissected and assayed for methylation of *E-cadherin* and *SIP1* by MSP. *E-cadherin* was methylated in 5 (83.3%) of these 6 pancreatic cancers. In contrast, *E-cadherin* was partially methylated in only 1 of 6 pancreatic cancer xenografts that had retained E-cadherin expression in its primary pancreatic cancer. *SIP1* methylation was detected in 11 of 12 of these pancreatic cancers. These results implicate *E-cadherin* methylation as the likely cause of absent E-cadherin expression in most of these pancreatic cancers. We found that 1 of the 6 pancreatic cancers with absent E-cadherin expression had unmethylated *SIP1*, suggesting that in this case, SIP1 expression likely caused *E-cadherin* silencing (Supplementary Table S2).

Elevated serum *miR-200a* and *miR-200b* in patients with pancreatic cancer

We next determined if *miR-200a* and *miR-200b* could be detected in serum and if they were more abundant in patients with pancreatic cancer. We measured *miR-200a* and *miR-200b* concentrations in 45 patients with pancreatic cancer and 32 healthy controls. Both *miR-200a* and *miR-200b* were significantly elevated in sera of patients with pancreatic cancer compared with healthy controls ($P < 0.001$ and $P < 0.001$, respectively, Mann-Whitney; Fig. 5; *miR-16* was used as a reference). The receiver operating characteristic (ROC) curve indicated that serum levels of *miR-200a* and *miR-200b* could differentiate patients with pancreatic cancer from healthy controls, with ROC curve areas of 0.861 (95% confidence interval, 0.774–0.949) and 0.85 (95% confidence interval, 0.763–0.938), respectively (Fig. 5C and D). With the cutoff at 0.28 (relative expression value), *miR-200a* had an 84.4% sensitivity for pancreatic cancer and a specificity of 87.5% compared with healthy controls. At a cutoff of 0.5 (relative expression value), *miR-200b* had a sensitivity of 71.1% and a specificity of 96.9%. The serum levels of *miR-200a* and *miR-200b* in serum samples were significantly correlated ($R^2 = 0.745$, $P < 0.0001$, Spearman).

Pancreatitis can mimic pancreatic cancer, and many markers of pancreatic cancer are also abnormal in patients with chronic pancreatitis. We measured *miR-200a* and *miR-200b* in the serum of 11 patients with chronic pancreatitis and found that serum levels were not significantly different from those observed in patients with pancreatic cancer ($P = 0.322$ and $P = 0.933$, respectively, Mann-Whitney test; Fig. 5).

Discussion

In this study, we find that most pancreatic cancers harbor hypomethylation and overexpression of *miR-200a* and *miR-200b* and epigenetic silencing of *SIP1*. In contrast, in many other cancer types, SIP1 is overexpressed (41, 42) and *miR-200* is silenced (25–27, 43, 44). DNA methylation has

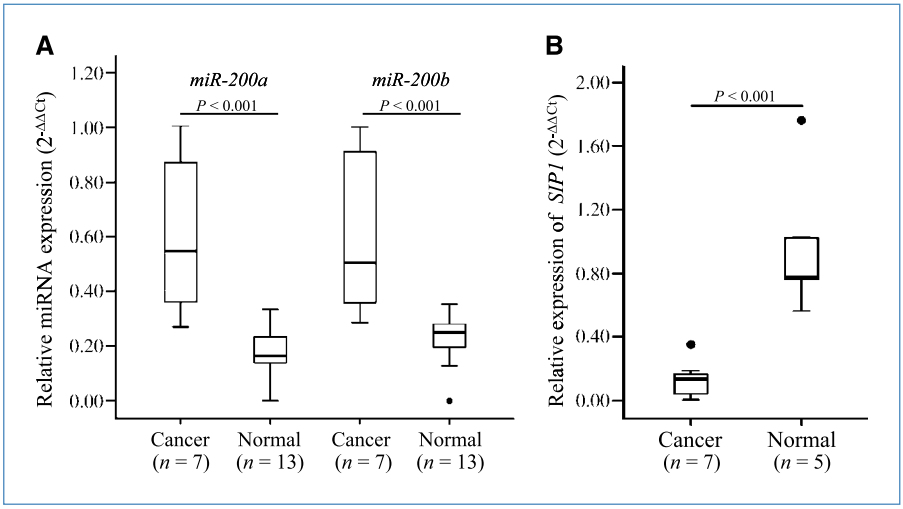

Figure 3. Box plot of *miR-200a* and *miR-200b* (A) and *SIP1* (B) expression in pancreatic cancer tissues by real-time PCR. Boxes represent the interquartile range and lines indicate the median value. Reference RNAs were used for *miR-200a/miR-200b* (*MiR-16*) and *SIP1* (*GAPDH*).

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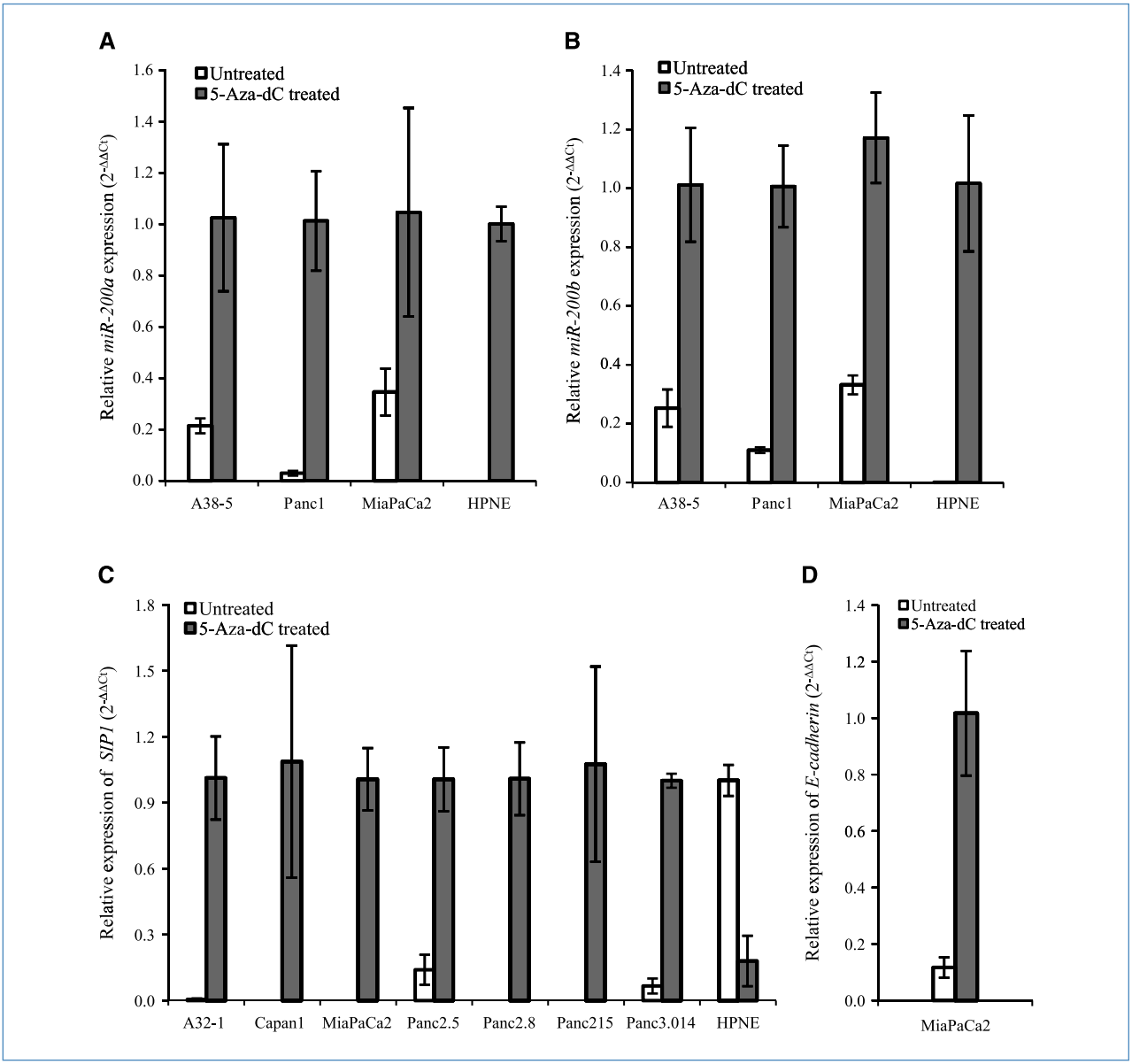

Figure 4. Quantitative PCR expression of *miR-200a, miR-200b, SIP1*, and *E-cadherin* before and after 5-aza-dC. Columns, mean of triplicates; bars, SD. Representative of two independent experiments. Reference RNAs were used for *miR-200a* and *miR-200b* (18S rRNA), *SIP1*, and *E-cadherin (GAPDH)*. A, *MiR-200a*; B, *miR-200b*; C, *SIP1*; D, *E-cadherin*.

been implicated in the regulation of several miRNAs, with most studies identifying hypermethylation of transcriptionally silenced miRNAs (45–47), rather than hypomethylation and overexpression as we found with *miR-200*. After observing the hypomethylation and overexpression of miR-200 in pancreatic cancers, we chose to focus on the relationship between *miR-200* and *SIP1* to highlight that this relationship is complicated in pancreatic cancers and distinct from the pattern observed in other cancers. Our evidence indicates that for most pancreatic cancers, *miR-200* expression is not required to suppress *SIP1* because the gene is already silenced by methylation. In many cancer types, *SIP1* represses the expression of *E-cadherin* and other genes and is implicated in EMT. The lack of *SIP1* expression in most pancreatic cancers indicates that *SIP1* does not suppress *E-cadherin* or mediate EMT in affected pancreatic cancers.

Our results are also consistent with reports showing that only a minority of pancreatic cancers completely lack E-cadherin expression (32). However, we find that many primary pancreatic cancers have focal areas of E-cadherin expression loss, and this loss is more common in poorly differentiated cancers, consistent with reports that undifferentiated pancreatic cancers lack E-cadherin expression (32). Such focal loss of E-cadherin expression could represent focal areas of EMT perhaps reflecting tumor microenvironment influences on EMT, and because EMT is suspected in the chemoresistance of

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pancreatic and other cancers, the focal loss of E-cadherin in these cancers could have therapeutic implications (28, 30). Although E-cadherin loss and its functional consequences are a central feature of EMT, other measures of the EMT phenotype such as reorganization of actin filaments could help clarify the significance of pancreatic cancers with weak or focal loss of E-cadherin expression. Our results also indicate that the prevalence of E-cadherin expression loss is greater than the combined prevalence of genetic (9) and DNA methylation–induced inactivation of *E-cadherin* (15) or expression of *SIP1*, suggesting that there are additional mechanisms for *E-cadherin* silencing in pancreatic cancers. Indeed, one recent report found that loss of FOXA1/A2 expression could induce EMT in pancreatic cancers (48). In pancreatic cancer cells expressing *SIP1*, we found that treatment with *miR-200a* and *miR-200b* or epigenetic induction of *miR-200a* and *miR-200b* expression can downregulate *SIP1* expression. These results suggest that in cancers with epigenetic silencing of *miR-200* and expression of *SIP1*, it may be possible to reverse an EMT phenotype with DNA methyltransferase inhibitors. And in cell lines expressing *miR-200a* and *miR-200b* and having a methylated and silenced *SIP1* promoter, *SIP1* expression could be induced by inhibiting DNA methylation with 5-aza-dC and further induced by blocking *miR-200* expression with antisense to *miR-200*. These results are consistent with recent studies showing that members of the *miR-200* family can inhibit EMT (25–27, 49). However, although prolonged pre-*miR-200* treatment of Panc-1 cells reversed *E-cadherin* expression, surprisingly, it did not alter the morphologic features of EMT. Perhaps, *miR-200a/200b* treatment of Panc-1 cells reversed the subtle features of EMT besides morphology, or alternatively, additional factors could be

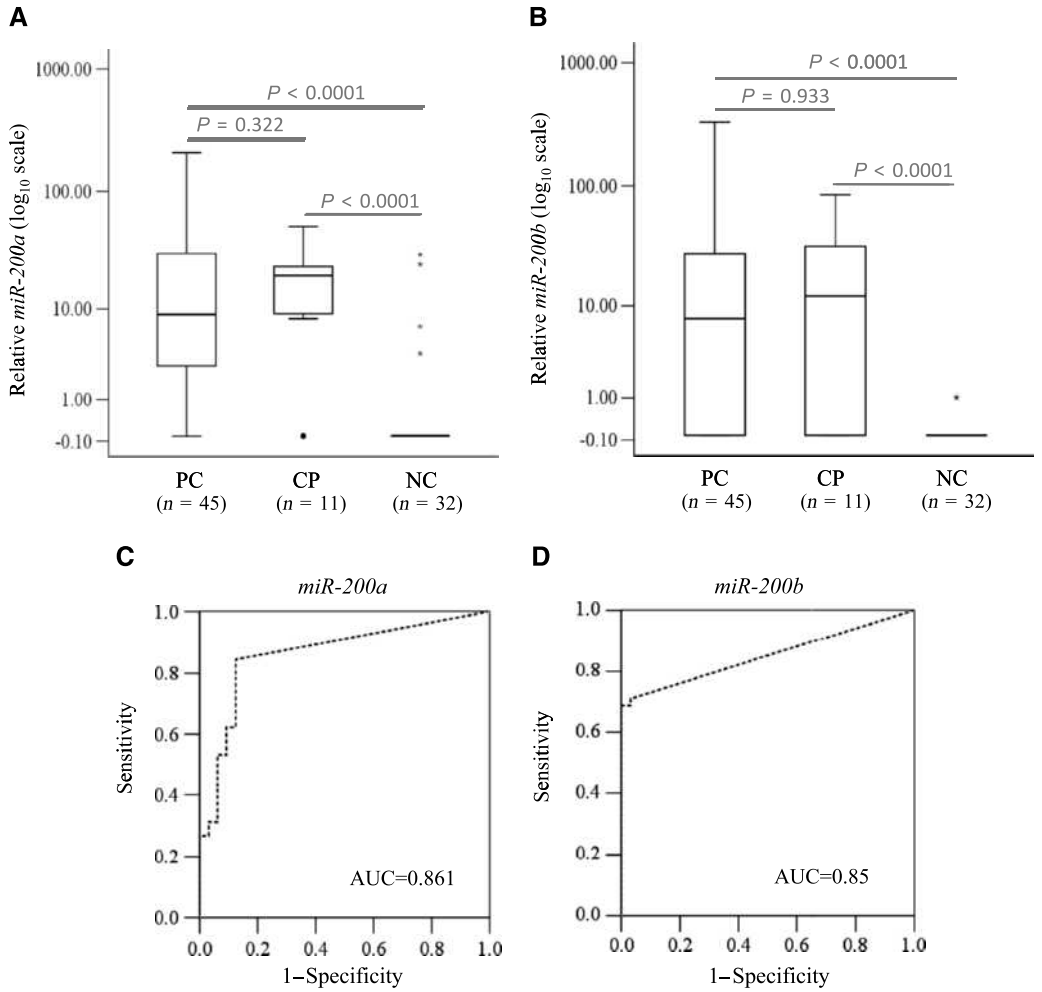

Figure 5. Serum levels of *miR-200a* and *miR-200b* in patients with and without pancreatic disease. Box plots of serum *miR-200a* (A) and *miR-200b* (B) levels in patients with pancreatic cancer (PC), patients with chronic pancreatitis (CP), and normal controls (NC). MiRNA expression levels are normalized to *miR-16*. ROC areas for serum *miR-200a* (C) and *miR-200b* (D).

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required to induce EMT in these cells (31). Because *SIP1* promoter hypermethylation rather than miR-200 expression is the mechanism for silencing of *SIP1* expression in most pancreatic cancers, our results point to as yet unidentified functional consequences of *miR-200* overexpression in pancreatic cancers that merit further investigation.

Previous studies have emphasized the expression of *SIP1* in pancreatic cancer cells (9, 50), but we find that only a minority of pancreatic cancers express *SIP1*. Our results differ from those of Imamichi and colleagues (42), who described elevated *SIP1* expression in primary pancreatic cancer tissues, which may reflect high levels of *SIP1* in contaminating stromal fibroblasts in pancreatic cancer samples. Instead, our results point to a potential advantage to pancreatic cancers of silencing *SIP1* perhaps by releasing the transcriptional repression of as yet unidentified downstream targets.

We also found that methylated *SIP1* has promising characteristics as a marker of pancreatic neoplasia. By MSP, methylation was detected in more than 90% of pancreatic cancers and in less than 10% of pancreatic tissues. We have shown that several aberrantly methylated genes including *SPARC, NPTX1, FOXE1*, and *ppENK* are sensitive and specific markers of pancreatic cancer when detected in pancreatic juice and in pancreatic and biliary brushings (7, 8). Further studies are needed to evaluate the role of methylated *SIP1* as a marker of pancreatic neoplasia.

We find that *miR-200a* and *miR-200b* are significantly elevated in the serum of patients with resectable pancreatic cancer. Indeed, *miR-200a* and *miR-200b* are only occasionally detectable in the serum of healthy controls, suggesting that they have potential as a diagnostic marker of pancreatic cancer. The elevated serum *miR-200a* and *miR-200b* levels in pancreatic cancer are consistent with the elevated levels in pancreatic cancer tissues. *MiR-200a* and *miR-200b* levels were also elevated in chronic pancreatitis sera compared with sera from healthy controls, which may reflect some expression in normal pancreas released with pancreatitis. Chronic pancreatitis can mimic pancreatic cancer in its clinical presentation and is a common obstacle for pancreatic cancer markers. However, often the clinical suspicion of pancreatic cancer is much higher than that of chronic pancreatitis. In this setting, an elevated serum *miR-200a/200b* level would increase the suspicion of pancreatic cancer and warrants further investigations such as pancreatic imaging. Similarly, patients with a strong family history of pancreatic cancer who do not have an increased predisposition to chronic pancreatitis and who undergo screening for pancreatic cancer (4) might benefit from measurement of markers such as *miR-200a* and *miR-200b* or other markers that are highly sensitive for pancreatic cancer but not elevated in most other conditions. However, further studies are needed, such as in high-risk populations, to evaluate the performance of these markers.

In conclusion, we find that most pancreatic cancers hypomethylate and overexpress *miR-200a* and *miR-200b*, silence *SIP1* by promoter methylation, and retain expression of E-cadherin. We also find that most patients with pancreatic cancer have elevated serum levels of *miR-200a* and *miR-200b* compared with healthy controls. Further evaluation of the diagnostic utility of these markers is warranted.

Disclosure of Potential Conflicts of Interest

M. Goggins: ownership interest, Palb2 discovery, other methylated genes. The other authors disclosed no potential conflicts of interest.

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