Frequent promoter methylation and gene silencing of *CDH13* in pancreatic cancer

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It has recently been reported that *CDH13* expression is silenced by aberrant methylation of the promoter region in several cancers. We examined the methylation status of the *CDH13* gene in pancreatic cancer using methylation-specific PCR (MSP), and detected aberrant methylation of *CDH13* in all 6 pancreatic cancer cell lines examined. To confirm the status of the *CDH13* gene in relation to the methylation pattern, we next examined *CDH13* expression in these cell lines using reverse transcription (RT)-PCR. As expected, no *CDH13* expression was detected in any of the 6 pancreatic cancer cell lines. Moreover, 5-aza-2′-deoxycytidine (5-aza-dC) treatment of *CDH13*-methylated cell lines led to restoration of *CDH13* expression. Among primary pancreatic cancers, 19 of 33 (58%) cases exhibited *CDH13* methylation, while no cases exhibited it in corresponding normal pancreatic tissues. *CDH13* methylation was detected even in relatively early pancreatic cancers, such as stage II cancers and cancers less than 2 cm in diameter. Our results suggest that the aberrant methylation of *CDH13* occurs frequently in pancreatic cancer, even at a relatively early stage. (Cancer Sci 2004; 95: 588–591)

Pancreatic cancer is one of the most aggressive cancers, and is the fifth leading cause of cancer mortality in the Western population.[1)] The prognosis is poor, and 5-year survival is rare.[2)] Pancreatic carcinogenesis is a multi-stage process resulting from the accumulation of genetic changes in the somatic DNA of normal cells.[3)] Mutations in proto-oncogenes such as *K-ras* and tumor suppressor genes such as *p16*, *p53*, and *DPC4* are well known.[4–7)] The accumulation of these genetic changes leads to a profound disturbance in cell cycle regulation and normal growth. However, further studies of the genetic alterations are needed to clarify fully the biological character of pancreatic cancer.

In recent years, there has been increasing interest in a large family of transmembrane glycoproteins, the cadherins. Cadherins are prime mediators of calcium-dependent cell-cell adhesion in normal cells and are also involved in contact inhibition of cell growth by inducing cell cycle arrest.[8, 9)] Loss of their expression has been described in many epithelial cancers and may play a role in tumor cell invasion and metastasis.[10, 11)] Recently, aberrant methylation of the *CDH13* (*H-cadherin*) gene associated with gene silencing has been reported in several primary tumors including breast, lung, colorectal, and ovarian cancers, and myeloid leukemia.[12–16)] These reports suggested that *CDH13* may be an important gene for the progression of cancer and prompted us to examine *CDH13* status in pancreatic cancer.

In this study, we first examined the methylation status and gene expression of *CDH13* in pancreatic cancer cell lines using methylation-specific PCR (MSP) and reverse transcription-PCR (RT-PCR), respectively. We then examined the methylation status of the *CDH13* gene in primary pancreatic cancers and corresponding normal pancreatic tissues derived from 33 patients, and evaluated the correlation between the methylation status and the clinicopathological findings.

Materials and Methods

Sample collection and DNA preparation. Six pancreatic cancer cell lines (AsPC-1, BxPC-3, Capan-1, Capan-2, MIA PaCa-2, and SW1990) and 1 colorectal cancer cell line (SW480) were obtained from American Type Culture Collection (Manassas, VA). They were grown in RPMI 1640 supplemented with 10% fetal bovine serum and incubated in 5% CO_2 at 37°C.

Thirty-three primary tumors and corresponding nonmalignant pancreatic tissues were collected at the Nagoya University School of Medicine from pancreatic cancer patients who had been diagnosed histopathologically. All pancreatic cancers were invasive ductal carcinomas. These samples were obtained during surgery. Written informed consent, as required by the institutional review board, was obtained from all patients. All samples were quickly frozen in liquid nitrogen and stored at −80°C until analysis. Genomic DNA was obtained from these samples by digestion with proteinase K, followed by phenol/chloroform extraction as described previously.[17)]

Bisulfite modification and MSP. DNA from tumor and normal specimens was subjected to bisulfite treatment as described previously.[18)] Briefly, 2 μg of DNA was denatured with NaOH and modified with sodium bisulfite. DNA samples were then purified using the Wizard purification resin (Promega Corp., Madison, WI), treated again with NaOH, precipitated with ethanol, and resuspended in water. The modified DNA was used as a template for MSP. Primer sequences of CDH13 for the unmethylated reaction were: CDH13 UMS (sense), 5′-TTGTGGGGT-TGTTTTTTGT-3′, and CDH13 UMAS (antisense), 5′-AACTTTTCATTCATACACACA-3′, which amplify a 242-base pair product. Primer sequences of CDH13 for the methylated reaction were: CDH13 MS (sense), 5′-TCGCGGGGT-TCGTTTTTCGC-3′, and CDH13 MAS (antisense), 5′-GACGTTTTCATTCATACACGCG-3′, which amplify a 243-base pair product. These primer sequences were described previously.[14, 19)] The PCR amplification of modified DNA samples consisted of 1 cycle of 95°C for 5 min; 1 cycle of 78°C for 10 min, 30 cycles of denaturing at 95°C for 30 s, 1 min of annealing at specific temperature, 1 min of extension at 72°C and a final extension step of 10 min at 72°C. Modified DNAs obtained from SW480 and Capan-1 were used as positive controls for unmethylated and methylated alleles, respectively. Controls without DNA were included in each assay. Ten microliters of each PCR product was loaded directly onto nondenaturing 6% polyacrylamide gels, stained with ethidium bromide, and visualized under UV illumination. Each MSP was repeated at least twice.

RT-PCR. Expression of the *CDH13* gene was analyzed by RT-PCR. Total RNA was extracted from pancreatic and colorectal cancer cell lines with "ISOGEN" (Nippon Gene, Tokyo) following the manufacturer's instructions. First-strand cDNA was generated from RNA as described previously.[20)] cDNA was amplified by means of a primer set that was specific for the

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CDH13 gene. Primer sequences were: CDH13 S (sense), 5′-TTCAGCAGAAAGTGTTCCATAT-3′, and CDH13 AS (antisense), 5′-GTGCATGGACGAACAGAGT-3′. The PCR amplification consisted of 1 cycle of 94°C for 2 min; 33 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s; and 1 cycle of 72°C for 5 min. Expression of β-actin was used as a control to confirm the success of the RT reaction. PCR products were visualized on 2% agarose gels stained with ethidium bromide.

5-Aza-2′-deoxycytidine (5-aza-dC) treatment. Methylated cell lines of pancreatic cancer were treated with 5-aza-dC (Sigma-Aldrich, St. Louis, MO), a demethylating agent. Cells (1.5×10^6) were grown for 6 days in the presence of different concentrations of 5-aza-dC (0, 1, 3, and 10 μ*M*), with medium changes on days 1, 3, and 5.

Statistical analysis. The associations between *CDH13* promoter methylation and clinicopathological parameters were analyzed by using Fisher's exact test.

Results

We first examined the methylation status of the *CDH13* promoter in 6 pancreatic cancer cell lines using the MSP technique. Aberrant methylation of *CDH13* was detected in all 6 cell lines (Fig. 1A). To confirm the status of the *CDH13* gene according to the methylation pattern, we next examined *CDH13* expression in these cell lines using RT-PCR. As expected, no *CDH13* expression was detected in any of the 6 pancreatic cancer cell lines, whereas *CDH13* expression was distinctly detected in a colorectal cancer cell line (SW480) with unmethylated *CDH13* promoter (Fig. 1B).

To confirm that the promoter methylation was responsible for silencing of the *CDH13* expression, we treated methylated pancreatic cancer cell lines with different concentrations of 5-aza-dC (0, 1, 3, 10 μ*M*), a demethylating agent, and examined *CDH13* expression by RT-PCR. *CDH13* expression in all cell lines was restored in a dosage-dependent manner by the 5-aza-dC treatment (Fig. 1C).

Subsequently, we examined the methylation status of the *CDH13* promoter in primary pancreatic cancers and corresponding normal tissues. Aberrant methylation of the *CDH13* gene was detected in 19 of 33 (58%) primary pancreatic cancers, but not in the corresponding normal pancreatic tissues (Fig. 2). We also examined the methylation status of the *CDH13* gene in 24 primary hepatocellular carcinomas. In contrast to the frequent methylation detected in pancreatic cancer, no aberrant methylation of the *CDH13* gene was detected in these hepatocellular carcinomas (data not shown).

Table 1 shows the *CDH13* methylation status and the clinicopathological findings in 33 pancreatic cancer patients. The *CDH13* methylation was detected even in relatively early pancreatic cancers such as stage II cancers and cancers less than 2 cm in diameter. We then examined the correlation between the *CDH13* methylation status and the clinicopathological findings (Table 2). We found no significant correlation between aberrant methylation in the primary tumors and age, sex, tumor size, histological type, presence of lymph node involvement, stage of the disease, serum CA19-9 level, perineural invasion, venous invasion, or lymphatic invasion.

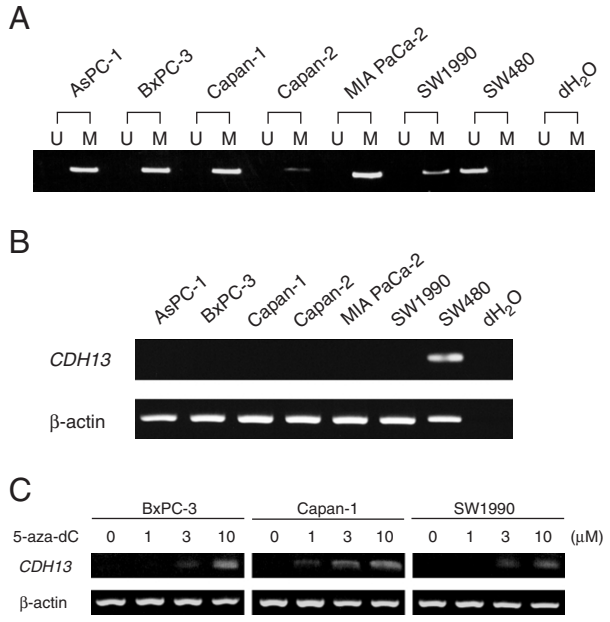

Fig. 1. Methylation and expression status of *CDH13* in pancreatic cancer cell lines. (A) MSP of *CDH13* promoter. The presence of a visible PCR product in lanes U indicates the presence of unmethylated genes; the presence of product in lanes M indicates the presence of methylated genes. All 6 pancreatic cancer cell lines show *CDH13* promoter methylation, whereas a colorectal cancer cell line (SW480) has unmethylated *CDH13* promoter. (B) Analysis of *CDH13* expression by RT-PCR. No *CDH13* expression was detected in any of the pancreatic cancer cell lines, whereas *CDH13* expression was distinctly detected in the case of SW480, whose *CDH13* promoter is unmethylated. Expression of β-actin was used as a control to confirm the success of the RT reaction. (C) The effect of 5-aza-2′-deoxycytidine (5-aza-dC) treatment on *CDH13* expression in pancreatic cancer cell lines with methylation of *CDH13* promoter. Recovery of *CDH13* expression was restored dosage-dependently by 5-aza-dC treatment.

Discussion

Aberrant methylation of the promoter regions associated with gene silencing is one of the major mechanisms for the inactivation of tumor suppressor genes, and has been observed in various cancers,[21] including pancreatic cancer.[22–24] Ueki *et al.* examined the methylation status of several tumor suppressor genes in pancreatic cancer using MSP and defined the "CpG island methylator phenotype (CIMP)" in this disease.[25] CIMP+ phenotype was observed in 14% of pancreatic cancers and aberrant methylation of tumor suppressor genes, such as *RAR*b, *p16*, and *E-cadherin*, appeared at a rate of 5–20% for each gene. These results suggested that aberrant methylation might be a common mechanism for the inactivation of the tumor suppressor gene in pancreatic cancer.

Recently, the *CDH13* gene has been identified as a new member of the cadherin superfamily. Furthermore, it has been reported that *CDH13* gene expression was silenced by aberrant methylation of the promoter region in several primary tumors.[12–16] However, the methylation status of the *CDH13* gene has not yet been studied in pancreatic cancer. In the present study, we examined the methylation status of the *CDH13* gene and found that the *CDH13* promoter was methylated in all 6 pancreatic cancer cell lines and in 19 of 33 primary pancreatic cancers (58%). This result suggests that *CDH13* promoter methylation is a fairly frequent event, and that it plays a role in the progression of pancreatic cancers. *CDH13*, as a member of the cadherin family, would be a cell

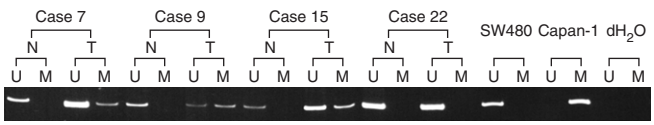

Fig. 2. Representative MSP of *CDH13* promoter in primary pancreatic cancers and the corresponding normal tissues. In primary pancreatic cancers, cases 7, 9, and 15 exhibited *CDH13* promoter methylation.

Table 1. *CDH13* methylation status and clinicopathological findings in 33 pancreatic cancer patients

Case No.	Age	Sex	Tumor size (mm)	Histological type[1)]	Lymph node metastasis	TNM stage[2)]	CA19-9 (U/ml)	Perineural invasion[3)]	Venous invasion[3)]	Lymphatic invasion[3)]
Methylated cases for CDH13										
1	66	male	35	poor	–	II	90	–	–	+
5	66	female	20	uncertain	–	II	180	–	–	–
6	83	female	45	well	–	IVA	3302	–	–	–
7	76	male	25	mod	–	II	492	+	–	+
8	73	female	40	well	+	IVB	1476	+	–	+
9	68	male	45	mod	+	IVA	263	–	+	+
11	52	male	45	mod	+	IVB	<5	+	+	+
12	61	male	55	mod	+	IVA	3805	+	+	+
15	47	male	15	mod	–	IVA	unknown	+	–	+
17	58	male	45	poor	+	IVB	186	+	–	+
19	63	male	45	mod	+	IVA	1663	+	–	+
21	66	male	70	poor	+	IVA	83	+	+	+
23	55	female	70	mod	+	IVA	unknown	+	–	+
26	67	female	30	poor	–	IVA	899	+	+	+
27	58	male	30	mod	+	IVB	35	+	+	+
28	68	male	20	well	+	III	104	–	+	+
29	79	female	31	mod	–	II	34	+	–	+
31	66	female	61	well	+	IVA	1030	–	–	–
33	69	male	30	mod	+	III	28	–	+	+
Unmethylated cases for CDH13										
2	51	female	35	mod	–	IVA	1220	+	–	–
3	68	male	30	mod	+	IVA	216	+	+	+
4	63	female	30	mod	+	III	9	–	–	–
10	71	female	30	mod	+	IVA	1101	+	–	+
13	71	male	30	poor	+	III	40	–	–	+
14	48	female	45	mod	+	IVA	126	+	–	+
16	76	male	45	mod	+	IVB	2149	+	–	+
18	57	male	45	mod	+	IVB	210	+	–	+
20	62	male	18	mod	–	II	91	+	–	+
22	50	female	20	mod	+	IVA	407	+	–	+
24	66	female	28	uncertain	+	IVA	285	–	–	+
25	71	female	18	mod	–	IVA	7	+	+	–
30	48	male	25	well	+	III	24	–	–	+
32	73	male	25	poor	+	IVA	279	+	–	+

1) Well, well differentiated adenocarcinoma; mod, moderately differentiated adenocarcinoma; poor, poorly differentiated adenocarcinoma.
2) Classified according to the International Union Against Cancer tumor-node-metastasis classification.
3) Classified according to the classification of pancreatic carcinoma of the Japan Pancreas Society.

surface glycoprotein responsible for cell adhesion. Therefore, it is conceivable that *CDH13* is inactivated in pancreatic cancers by promoter methylation, leading to cancer cell disassociation, which is a characteristic of pancreatic cancer. Recently, it was reported that *E-cadherin* expression in pancreatic tumor cells resulted in arrest of tumor development at the adenoma stage, whereas expression of a dominant negative form of *E-cadherin* induced early invasion and metastasis.[26)] This report supports the notion that the inactivation of cadherin family genes would be a critical event in scattering cancer cells, because they code for proteins responsible for selective cell recognition and adhesion.

In conclusion, our results suggest that the aberrant methylation of *CDH13* occurs frequently in pancreatic cancer, even at a relatively early stage. On the other hand, because of frequent hypermethylation of the *CDH13* gene and the high sensitivity of MSP, which can detect 1 methylated allele among 1000 unmethylated alleles,[27)] it can potentially be used for early detection and monitoring of pancreatic cancer by the detection of the *CDH13* methylation status in clinical samples such as serum, stool, pancreatic juice, and duodenal fluid.[18, 28, 29)]

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Table 2. Correlation between *CDH13* methylation and clinicopathological findings in 33 pancreatic cancer patients

Clinicopathological findings	Variable	Number of cases	*CDH13* methylation [number (%)]		*P* value[1)]
			Methylated (n=19)	unmethylated (n=14)	
Age	<60	10	5 (26)	5 (36)	0.707
	≥60	23	14 (74)	9 (64)	
Sex	male	19	12 (63)	7 (50)	0.497
	female	14	7 (37)	7 (50)	
Tumor size	<3cm	10	4 (21)	6 (43)	0.257
	≥3cm	23	15 (79)	8 (57)	
Histological type[2)]	well-mod	25	14 (78)	11 (85)	>0.999
	poor	6	4 (22)	2 (15)	
Lymph node metastasis	–	10	7 (37)	3 (21)	0.455
	+	23	12 (63)	11 (79)	
TNM stage[3)]	I, II, III	10	6 (32)	4 (29)	>0.999
	IVA, IVB	23	13 (68)	10 (71)	
CA19-9 level (U/ml)	<100	11	6 (35)	5 (36)	>0.999
	≥100	20	11 (65)	9 (64)	
Perineural invasion[4)]	–	11	7 (37)	4 (29)	0.719
	+	22	12 (63)	10 (71)	
Venous invasion[4)]	–	23	11 (58)	12 (86)	0.131
	+	10	8 (42)	2 (14)	
Lymphatic invasion[4)]	–	6	3 (16)	3 (21)	>0.999
	+	27	16 (84)	11 (79)	

1) Analyzed by Fisher's exact test.
2) Well-mod, well or moderately differentiated adenocarcinoma; poor, poorly differentiated adenocarcinoma.
3) Classified according to the International Union Against Cancer tumor-node-metastasis classification.
4) Classified according to the classification of pancreatic carcinoma of the Japan Pancreas Society.

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