DNA hypermethylation and histone hypoacetylation of the *HLTF* gene are associated with reduced expression in gastric carcinoma

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The SWI/SNF proteins are ATP-dependent chromatin remodeling enzymes that have been implicated in the regulation of gene expression. Recent studies have shown that members of the SWI/SNF superfamily can function as tumor suppressor genes. DNA methylation and transcriptional inactivation of the *HLTF* gene, which is a homologue to the *SWI/SNF* genes, have been observed in colon cancer. In the present study, we studied the DNA methylation status of the *HLTF* gene by methylation-specific PCR in 50 gastric carcinoma tissues, and seven gastric carcinoma cell lines and compared the methylation status with the levels of *HLTF* mRNA expression. DNA methylation of the *HLTF* gene was found in 25 (50%) of 50 gastric carcinomas, and levels of *HLTF* mRNA were associated with methylation status of *HLTF* (P=0.027; Mann-Whitney U test). No correlations were found between *HLTF* mRNA levels and DNA methylation and T grade, N grade, tumor stage, or histological type. In corresponding non-neoplastic mucosae, DNA methylation of the *HLTF* gene was found in 1 (7%) of 15 samples. The methylated allele was not detected in any of 10 normal gastric mucosae from 10 healthy volunteers. Among seven gastric carcinoma cell lines, the KATO-III cell line showed loss of *HLTF* mRNA expression associated with DNA methylation. This loss was rectified by treatment with both Aza-2′-deoxycytidine, a demethylating agent, and trichostatin A, a histone deacetylase inhibitor. Chromatin immunoprecipitation assay revealed that the acetylation levels of histones H3 and H4 in the 5′ CpG island of the *HLTF* gene were inversely associated with DNA methylation status. These results suggest that transcriptional inactivation of *HLTF* by aberrant DNA methylation and histone deacetylation may be involved in stomach carcinogenesis through down-regulation of *HLTF* expression. (Cancer Sci 2003; 94: 692–698)

A variety of genetic and epigenetic alterations are associated with gastric carcinomas.[1] Alterations in DNA methylation patterns, such as hypermethylation of CpG islands, are common changes observed in human cancers.[2] Hypermethylation of CpG islands in promoters is associated with silencing of some tumor suppressor genes.[3–5] Methylation and inactivation of various genes have been reported in gastric carcinoma.[6–18] We have also reported DNA methylation of *MGMT*,[19] $p16^{INK4a}$, *RAR-β*, *CDH1*,[20] and *TSP1*.[21]

Promoter hypermethylation and inactivation of the *Helicase-like transcription factor* (*HLTF*) gene have been reported in human colon cancers.[22] *HLTF* contains a DNA-binding domain, a RING finger domain, and seven helicase domains and is a homologue to SWI/SNF proteins. SWI/SNF proteins are ATP-dependent chromatin remodeling enzymes that have been implicated in regulation of gene expression in yeast and higher eukaryotes.[23,24] Members of the SWI/SNF superfamily are characterized by the presence of DNA-dependent ATPase motifs and use of energy from ATP hydrolysis to alter the position or spacing of nucleosomes. Recent studies have shown strong links between the misregulation of remodelers and cancer. A mutation in the *hSNF5/INI1* gene was found to be inactivated frequently in pediatric malignant rhabdoid tumors,[25] meningiomas,[26] and myeloid leukemia.[27] *BRG1* inactivation mutations and deletions have been detected in cancer cell lines from prostate, lung, breast, and pancreas,[28] suggesting that members of the SWI/SNF superfamily, such as *HLTF*, can function as tumor suppressor genes. It was recently reported that DNA methylation of *HLTF* occurred in a certain proportion of human colon, gastric, and esophageal carcinomas.[29] However, associations of *HLTF* methylation and gene expression as well as histone acetylation were not studied in gastric carcinomas.

In the present study, we examined DNA methylation status of the 5′ CpG island of the *HLTF* gene by methylation-specific polymerase chain reaction (MSP) in 50 primary gastric carcinoma tissues and compared the results with *HLTF* mRNA levels determined with a quantitative reverse transcription (RT)-PCR method. Moreover, to confirm that DNA methylation of *HLTF* gene induces transcriptional inactivation, we performed an *in vitro* study of gastric carcinoma cell lines in combination with treatment with aza-2′-deoxycytidine (Aza-dC), a demethylating agent. Because histone deacetylation has been shown to play an important role in methylation-associated gene inactivation,[30,31] we treated gastric carcinoma cell lines with trichostatin A (TSA), a histone deacetylase (HDAC) inhibitor, and performed chromatin immunoprecipitation (ChIP) assays with antibodies against acetylated histones H3 and H4. We report here that acetylation levels of histones in the 5′ CpG island of the *HLTF* gene were inversely correlated with DNA methylation status, which is associated with gene silencing.

Materials and Methods

Tissue samples. Fifty gastric carcinoma tissue samples from 50 patients were studied. Tumors and corresponding non-neoplastic mucosae were removed surgically, frozen immediately in liquid nitrogen, and stored at −80°C until use. We confirmed microscopically that the tumor-tissue specimens consisted mainly (>80%) of carcinoma tissue and that non-neoplastic mucosa did not exhibit any tumor-cell invasion or show significant inflammatory involvement. Histological classification and tumor staging were done according to the Lauren classification system[32] and the TNM stage grouping.[33] In addition, a total of 10 normal gastric mucosae from 10 healthy volunteers who had no clinical symptoms were included in this study. These normal gastric mucosae were removed endoscopically, frozen immedi-

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ately in liquid nitrogen, and stored at −80°C until use. Endoscopic examination showed no significant changes. According to institutional guidelines, all of the patients gave informed consent before collection of the samples.

Cell lines. Seven cell lines derived from human gastric carcinomas were used. The TMK-1 cell line was established in our laboratory from poorly differentiated adenocarcinoma.[34] Five gastric carcinoma cell lines of the MKN series (MKN-1, adenosquamous cell carcinoma; MKN-7, MKN-28, and MKN-74, well-differentiated adenocarcinomas; and MKN-45, poorly differentiated adenocarcinoma) were kindly provided by Dr. T. Suzuki. KATO-III cell line, which was established from signet ring cell carcinoma, was kindly provided by Dr. M. Sekiguchi.[35] All cell lines were maintained in RPMI 1640 (Nissui Pharmaceutical Co., Ltd., Tokyo) containing 10% fetal bovine serum (Whittaker, Walkersville, MA) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. To analyze restoration of *HLTF* gene expression, MKN-28 and KATO-III cells were incubated for 5 days with 1 μ*M* Aza-dC (Sigma, St. Louis, MO) or for 24 h with 300 n*M* TSA (Wako, Tokyo).

Genomic DNA extraction and MSP. To examine DNA methylation patterns in the 5′ CpG island of the *HLTF* gene, we extracted genomic DNAs with a genomic DNA purification kit (Promega, Madison, WI) and performed MSP.[36] In brief, 2 μg of genomic DNA was denatured by treatment with NaOH and modified with 3 *M* sodium bisulfite for 16 h. DNA samples were purified with Wizard DNA purification resin (Promega), treated with NaOH, precipitated with ethanol, and resuspended in 25 μl of water. PCR was performed with 2 μl aliquots of the above DNA preparations as templates. Sequences of primers and annealing temperature for *HLTF* MSP were described previously (Table 1).[22] Each target sequence was amplified in a 25 μl reaction volume containing 0.2 μ*M* dNTPs, 10 m*M* Tris-HCl (pH 8.3), 50 m*M* KCl, 2 m*M* $MgCl_2$, 0.3 μ*M* of each primer, and 0.75 units of Ampli*Taq* Gold (Applied Biosystems, Foster City, CA). PCR amplification consisted of 35 cycles after the initial *Taq* Gold activation step. Each PCR product (15 μl) was loaded onto 8% nondenaturing polyacrylamide gels, separated by electrophoresis, stained with ethidium bromide, and visualized under UV light. All PCRs were performed with positive controls (methylated alleles) and negative controls (no template DNA). CpG sites of genomic DNA isolated from cell line MKN-28 were methylated by treatment with Sss I methylase (New England Biolabs, Inc., Beverly, MA) and served as the positive control for *HLTF* methylation assays. It is important to note that this methylation assay detects methylation of the regions of primer sequences.

Sequencing analysis of methylated and unmethylated PCR products. The PCR products were purified and cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA). Plasmid DNA was extracted from individual clones by alkaline lysis plasmid miniprep preparation. The inserted PCR fragments obtained from each sample were sequenced with M13 forward primer using the PRISM Ampli*Taq* DNA polymerase FS Ready Reaction Dye Terminator Sequencing kit (Applied Biosystems). Reamplified DNA fragments were purified with CENTRI-SEP COLUMNS (Applied Biosystems) and were sequenced with an ABI PRISM 310 genetic analyzer (Applied Biosystems).

RT-PCR analysis of gastric carcinoma cell lines. Total RNA was extracted with an RNeasy Mini Kit (QIAGEN, Hilden, Germany), and 1 μg of total RNA was converted to cDNA with a first strand cDNA synthesis kit (Amersham Pharmacia Biotech, Uppsala, Sweden). We performed RT-PCR to analyze the expression of the *HLTF* gene in gastric carcinoma cell lines. Sequences of primers and annealing temperature were described previously (Table 1).[22] RT-PCR products were then analyzed by 1% agarose gel electrophoresis, stained with ethidium bromide, and examined under UV light. *ACTB*-specific PCR products were amplified from the same RNA samples and served as internal controls.

Table 1. Primer sequences for MSP, RT-PCR, ChIP assay

Primer sequence	Annealing temperature	Size
MSP (Methylated)		
F: 5′-TGGGGTTTCGTGGTTTTTTCGCGC-3′	66°C	255 bp
R: 5′-CCGCGAATCCAATCAAACGTCGACG-3′		
MSP (Unmethylated)		
F: 5′-ATTTTTGGGGTTTTGTGGTTTTTTTGTGT-3′	66°C	264 bp
R: 5′-ATCACCACAAATCCAATCAAACATCAACA-3′		
RT-PCR of gastric carcinoma cell lines (HLTF)		
F: 5′-CGATGGTCTATGAAACTTGGA-3′	53°C	2175 bp
R: 5′-GAAATTGTGTCAGTAATACCTCTTCAC-3′		
RT-PCR of gastric carcinoma cell lines (ACTB)		
F: 5′-CTGTCTGGCGGCACCACCAT-3′	55°C	254 bp
R: 5′-GCAACTAAGTCATAGTCCGC-3′		
Quantitative RT-PCR of gastric carcinoma tissues (HLTF)		
F: 5′-TTTTCTGAGAAGGACCGACCAG-3′	55°C	87 bp
R: 5′-TGCAATGGCCGTAAGAGTTTT-3′		
Quantitative RT-PCR of gastric carcinoma tissues (ACTB)		
F: 5′-TCACCGAGCGCGGCT-3′	55°C	60 bp
R: 5′-TAATGTCACGCACGATTTCCC-3′		
ChIP (5′ CpG island of HLTF)		
F: 5′-AAAGTCCCCACGGTTCACC-3′	55°C	76 bp
R: 5′-GCTCCACGGTTTACGAGACC-3′		
ChIP (Coding region of HLTF)		
F: 5′-GAGAACTTGCAGCAGGAGCCT-3′	55°C	81 bp
R: 5′-TTCTGATTTCATTAATTTTGGCTTGT-3′		
ChIP (5′ region of ACTB)		
F: 5′-CCCACCCGGTCTTGTGTG-3′	55°C	72 bp
R: 5′-GGGAAGACCCTGTCCTTGTCA-3′		

Quantitative RT-PCR analysis of gastric carcinoma tissues and gastric carcinoma cell lines. Total RNA was extracted with an RNeasy Mini Kit (QIAGEN), and 1 μg of total RNA was converted to cDNA with a first strand cDNA synthesis kit (Amersham Pharmacia Biotech). To analyze expression of the *HLTF* gene in gastric carcinoma tissues specimens and gastric carcinoma cell lines, we performed real-time RT-PCR. Sequences of primers and annealing temperatures are shown in Table 1. PCRs were performed with the SYBR Green PCR Core Reagents kit (Applied Biosystems). Real-time detection of the emission intensity of SYBR green bound to double-stranded DNAs was done with the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). *HLTF* cDNA and *ACTB* cDNA (internal control) were amplified separately. Relative gene expression was determined from the threshold cycles for the *HLTF* gene and the *ACTB* gene. Reference samples (gastric carcinoma cell line, MKN-1) were included on each assay plate to verify plate-to-plate consistency. Plates were normalized to each other with these reference samples. The PCR amplification was performed with 96-well optical trays and caps according to the manufacturer's instructions. Quantitative PCRs were performed in triplicate for each sample and primer set, and the mean of the three experiments was used as the relative quantification value. At the end of 40 PCR cycles, reaction products were separated electrophoretically on 8% nondenaturing polyacrylamide gels, stained with ethidium bromide, and visualized under UV light for visual confirmation of PCR products.

ChIP assay. ChIP assay was performed as described previously with a modification.[37] In brief, chromatin proteins were cross-linked to DNA by addition of formaldehyde directly to the culture medium to a final concentration of 1%. After a 10-min incubation at room temperature, the cells were washed and scraped off the dishes in ice-cold phosphate-buffered saline containing protease inhibitors. Cells were pelleted and then resuspended in SDS lysis buffer (1% SDS, 10 m*M* EDTA, 50 m*M* Tris-HCl, pH 8.1, protease inhibitors) for 10 min on ice. The lysate was subjected to sonication to reduce the size of the DNA to 300–1000 bp. The sample was centrifuged to remove cell debris and diluted 10-fold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 m*M* EDTA, 16.7 m*M* Tris-HCl, pH 8.1, 167 m*M* NaCl, protease inhibitors). The chromatin solution was pre-cleared with 40 μl of a mixture of salmon sperm DNA-protein A agarose slurry (Upstate Biotechnology, Lake Placid, NY) to reduce non-specific background. After pre-clearing, the solution was centrifuged, and the supernatant was collected. Anti-acetylated histone H3 or H4 antibody (5 μl) (Upstate Biotechnology) was added to the chromatin solution and the mixture was incubated overnight at 4°C with agitation. A no-antibody control was also performed for each ChIP assay. After the overnight antibody incubation, the resulting immune complexes were collected by addition of 60 μl of salmon sperm DNA-protein A agarose slurry and incubated at 4°C with agitation for 1 h. The beads were washed five times, and the attached immune complexes were eluted with a buffer containing 1% SDS and 0.1 *M* $NaHCO_3$. Cross-links were reversed by addition of 5 *M* NaCl followed by incubation at 65°C for 4 h. The samples were then treated with proteinase K for 1 h, and DNA was purified by phenol/chloroform extraction and ethanol precipitation. We performed PCR analysis of immunoprecipitated DNA using primers specific for the 5′ region of the *ACTB* gene, and each PCR product (15 μl) was loaded onto 8% nondenaturing polyacrylamide gels, separated by electrophoresis, stained with ethidium bromide, and visualized under UV light to confirm that there was no genomic DNA contamination of the no-antibody control. For quantitative PCR analysis of immunoprecipitated DNAs, we performed real-time PCR. Sequences of primers and annealing temperatures are shown in Table 1. PCRs were performed with the SYBR Green PCR Core Reagents kit. Real-time detection of the emission intensity of SYBR green bound to double-stranded DNAs was done with the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Relative histone acetylation level was determined from the threshold cycles for the 5′ CpG island of the *HLTF* gene and the 5′ region of the *ACTB* gene. Reference samples (genomic DNA from MKN-1) were included on each assay plate to verify plate-to-plate consistency. Plates were normalized to each other with these reference samples. The PCR amplification was performed in 96-well optical trays with caps according to the manufacturer's instructions. Quantitative PCRs were performed in triplicate for each sample primer set, and the mean of the three experiments was used as the relative quantification value. At the end of 40 PCR cycles, reaction products were separated electrophoretically on 8% nondenaturing polyacrylamide gels, stained with ethidium bromide, and visualized under UV light for visual confirmation of PCR products. Values for enrichment were calculated as the average from at least three independent ChIP experiments.

Statistical methods. Statistical analyses were performed with Fisher's exact and Mann-Whitney *U* tests. *P* values less than 0.05 were regarded as statistically significant.

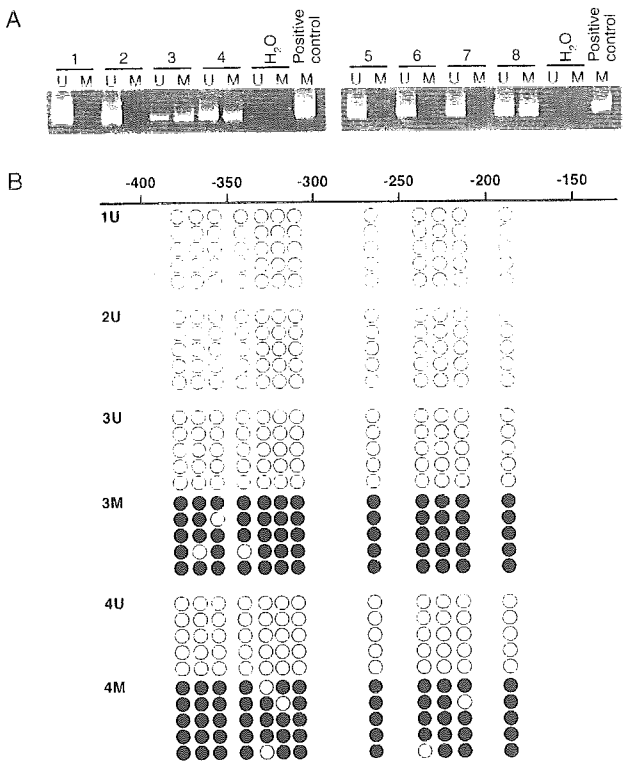

Fig. 1. Methylation status of *HLTF* gene in gastric carcinoma tissues. (A) MSP of *HLTF*. Unmethylated (U), methylated (M). The methylated allele was detected in specimens 3, 4, and 8. (B) Sequencing analysis of 5 cloned MSP products of *HLTF*. Except for primer complementary sequences, *HLTF*-MSP products have 12 CpG sites. Each row of circles represents a single cloned allele, and each circle represents a single CpG site (open circle, non-methylated; filled circle, methylated cytosines). The numbering in this scheme corresponds to position relative to the translation initiation site.

Results

DNA methylation status and mRNA expression levels of *HLTF* gene in gastric carcinoma tissues. To investigate the methylation status of the 5′ CpG island of the *HLTF* gene in gastric carcinoma tissues, we performed MSP of 50 gastric carcinoma tissues. Rep-

Table 2. Association between *HLTF* methylation status and clinicopathological features in gastric carcinomas

		HLTF methylation status		*P* value[1]
		Methylated	Unmethylated	
T grade	T1/2	12	10	0.776
	T3/4	13	15	
N grade	N0	9	5	0.345
	N1/2/3	16	20	
Stage	Stage I/II	14	7	0.084
	Stage III/IV	11	18	
Lauren classification	Intestinal	14	10	0.3961
	Diffuse	11	15	

1) Fisher's exact test.

Table 3. *HLTF* mRNA expression level

		Case number	*HLTF* mRNA expression level[1] (Mean±SE)	*P* value[2]
5′ CpG island	Methylated	25	0.040±0.012	0.027
	Unmethylated	25	0.090±0.020	
T grade	T1/2	22	0.085±0.022	0.162
	T3/4	28	0.050±0.013	
N grade	N0	14	0.075±0.026	0.469
	N1/2/3	36	0.061±0.014	
Stage	I/II	21	0.085±0.022	0.216
	III/IV	29	0.051±0.013	
Lauren classification	Intestinal	24	0.053±0.018	0.109
	Diffuse	26	0.076±0.017	

1) The units are arbitrary, and we calculated the *HLTF* mRNA expression level by standardization with 1 μg of total RNA of the MKN-1 gastric carcinoma cells, taken as 1.0.
2) Mann-Whitney *U* test.

resentative results are shown in Fig. 1A. The methylated allele was detected in three cases (cases 3, 4, and 8). In total, DNA methylation of the *HLTF* gene was found in 25 (50%) of the 50 gastric carcinomas. Bisulfite genomic DNA sequencing of representative methylated PCR products (cases 3 and 4) and unmethylated PCR products (cases 1, 2, 3, and 4) of the *HLTF* gene showed that all cytosines at non-CpG sites were converted to thymine. This excluded the possibility that successful amplification could be attributable to incomplete bisulfite conversion. All methylated PCR products of the *HLTF* gene showed extensive methylation of CpG sites that are located inside the amplified genomic fragments, whereas all unmethylated PCR products of the *HLTF* gene showed no methylation of CpG sites (Fig. 1B). The results of bisulfite sequencing analyses were thus consistent with those of MSP, indicating that it is appropriate to infer the methylation status of the *HLTF* gene from the results of MSP assay. As shown in Table 2, *HLTF* methylation status was not significantly associated with T grade (depth of tumor invasion), N grade (degree of lymph node metastasis), tumor stage, or histological type. In corresponding non-neoplastic mucosa, DNA methylation of the *HLTF* gene was found in 1 (7%) of 15 samples, though the band that corresponds to the methylated form was faint (data not shown). The methylated allele was not detected in any of 10 normal gastric mucosae from 10 healthy volunteers (data not shown).

We then used quantitative RT-PCR analysis to determine whether DNA methylation of the *HLTF* gene affects expression of the mRNA. As shown in Table 3, levels of *HLTF* mRNA in tumor tissues with DNA methylation (0.040±0.012) were significantly lower than those in tumor tissues without DNA methylation (0.090±0.020, P=0.027, Mann-Whitney U test). Moreover, levels of *HLTF* mRNA were significantly lower in tumor tissues with DNA methylation (0.040±0.012) than in corresponding non-neoplastic mucosae (0.066±0.009, P=0.020, Mann-Whitney U test). *HLTF* mRNA levels were not associated significantly with T grade, N grade, tumor stage, or histological type (Table 3).

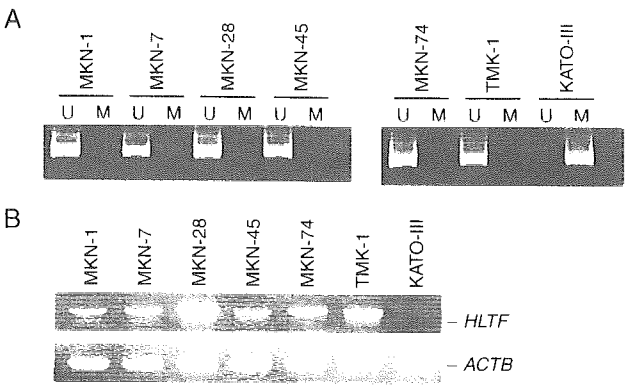

Fig. 2. DNA methylation status and mRNA expression levels of *HLTF* in gastric carcinoma cell lines. (A) MSP of *HLTF*. Unmethylated (U), methylated (M). The methylated allele was detected only in the KATO-III cell line. (B) RT-PCR analysis of gastric carcinoma cell lines. Expression of *HLTF* was absent in KATO-III cells.

DNA methylation and histone acetylation status of *HLTF* gene in gastric carcinoma cell lines. To confirm that DNA methylation of the *HLTF* gene induces transcriptional inactivation, we performed an *in vitro* study of gastric carcinoma cell lines in combination with Aza-dC or TSA treatment. Among seven gastric carcinoma cell lines, the methylated allele was detected only in the KATO-III cell line by MSP (Fig. 2A). RT-PCR analysis revealed that transcriptional inactivation occurred only in KATO-III cells, whereas other cell lines expressed *HLTF* at various levels (Fig. 2B). To investigate whether transcriptional inactivation of *HLTF* caused DNA methylation in KATO-III cells, we

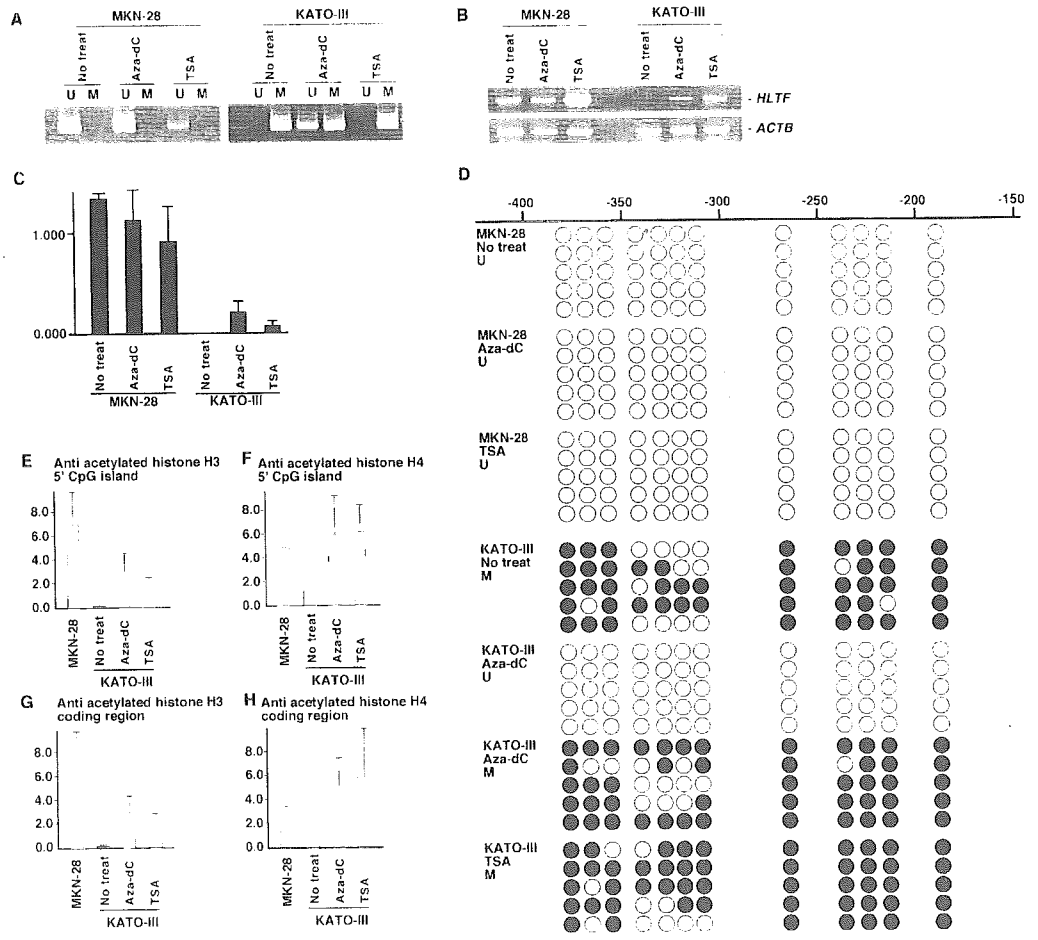

Fig. 3. Effect of Aza-dC and TSA in MKN-28 and KATO-III cells. (A) MSP in Aza-dC-treated and TSA-treated MKN-28 and KATO-III cells. The unmethylated allele was observed only in Aza-dC-treated KATO-III cells. (B) RT-PCR analysis of Aza-dC-treated and TSA-treated MKN-28 and KATO-III cells. Expression of *HLTF* mRNA was observed in both Aza-dC-treated and TSA-treated KATO-III cells. (C) Quantitative RT-PCR analysis of Aza-dC-treated and TSA-treated MKN-28 and KATO-III cells. In KATO-III cells, treatment with both Aza-dC and TSA restored *HLTF* expression, whereas in MKN-28 cells, treatment with both Aza-dC and TSA did not significantly change *HLTF* expression. The value was calculated as the average of three independent quantitative RT-PCR experiments. Error bars indicate SE from the mean. The units are arbitrary, and we calculated the *HLTF* mRNA expression level by standardization with 1 μg of total RNA of the MKN-1 gastric carcinoma cells, taken as 1.0. (D) Sequencing analysis of 5 cloned MSP products of *HLTF*. Each row of circles represents a single cloned allele, and each circle represents a single CpG site (open circle, non-methylated; filled circle, methylated cytosines). The methylated PCR products of the *HLTF* gene showed extensive methylation of CpG sites, whereas the unmethylated PCR products of the *HLTF* gene showed no methylation of CpG sites. The numbering in this scheme corresponds to position relative to translation initiation site. (E–H) ChIP assay of MKN-28 and KATO-III cells. The value was calculated as the average of three independent ChIP experiments. Error bars indicate SE from the mean. The units are arbitrary, and we calculated the value by standardization with the value from 0.1 μg of genomic DNA of MKN-1 gastric carcinoma cells, taken as 1.0.

treated KATO-III cells and MKN-28 cells, as an unmethylated control, with both Aza-dC and TSA and then performed MSP (Fig. 3A) and RT-PCR (Fig. 3B) analysis. *HLTF* expression was restored in KATO-III cells by treatment with both Aza-dC and TSA. We also performed quantitative RT-PCR analysis of the same samples (Fig. 3C). In KATO-III cells, treatment with both Aza-dC and TSA restored *HLTF* expression, whereas in MKN-28 cells, treatment with both Aza-dC and TSA did not significantly alter *HLTF* expression. In MSP analysis, the unmethylated allele was detected in KATO-III cells after Aza-dC, but not TSA treatment. We confirmed that the methylated PCR products of the *HLTF* gene (KATO-III, Aza-dC-treated KATO-III, and TSA-treated KATO-III) showed extensive methylation of CpG sites, whereas the unmethylated PCR products of the *HLTF* gene (MKN-28, Aza-dC-treated MKN-28, TSA-treated MKN-28, and Aza-dC-treated KATO-III) showed no methylation of CpG sites by sequence analysis (Fig. 3D). ChIP assay was carried out in MKN-28 and KATO-III cells to investigate the acetylation status of histones H3 and H4 in the *HLTF* gene (Fig. 3E, F, G, and H). Levels of acetylation of histones H3 and H4 in both the 5′ CpG island and coding region of *HLTF* in KATO-III cells were significantly lower than those in untreated MKN-28 cells. After treatment with both TSA and Aza-dC, the levels of acetylation of histones H3 and H4 in both the 5′ CpG island and coding region of *HLTF* were significantly enhanced in KATO-III cells. No significant changes were observed in MKN-28 cells (data not shown).

Discussion

Recent data suggest that the SWI/SNF superfamily may function as tumor suppressors.[25–27] However, the relation between SWI/SNF complex and gastric carcinoma is not well understood. We previously reported that *BRG1*, a component of the SWI/SNF complex, is not mutated in gastric carcinoma.[38] In the present study, we found that in 50% of gastric carcinoma tissues, the 5′ CpG island of the *HLTF* gene was methylated, and this methylation was associated with reduced expression. In

the gastric carcinoma cell lines. DNA methylation of *HLTF* was detected in KATO-III cells, which did not appear to express *HLTF* mRNA. Aza-dC treatment induced demethylation of the 5′ CpG island of *HLTF* and restored expression of the *HLTF* mRNA in KATO-III. Treatment with Aza-dC did not induce *HLTF* gene expression significantly in MKN-28 cells by quantitative RT-PCR analysis, suggesting that the induction of *HLTF* gene expression is due to demethylation of the 5′ CpG island of the *HLTF* gene in KATO-III. These results suggest that DNA methylation of the *HLTF* gene plays an important role in inactivation of *HLTF* in gastric carcinoma.

On the other hand, Hibi *et al.* reported that *HLTF* gene methylation occurred in 17% of gastric carcinomas,[29] while in this study, we found the methylation in 50% of gastric carcinomas. Several possible explanations may underlie these different methylation frequencies: (a) The sequences of primers for MSP we used are different. (b) The cycle number of the PCR amplification in our study (35 cycles) was higher than that in their study (33 cycles). (c) In our study, DNA methylation of the *HLTF* gene was found in 1 of 15 corresponding non-neoplastic mucosa. Because detection of methylation by MSP shows only the presence of some methylated DNA molecules in the sample analyzed, we cannot completely exclude the possibility that the higher frequency of *HLTF* hypermethylation may be due in some cases to contamination with adjacent non-neoplastic mucosa in which methylation of the *HLTF* gene occurred. However, because in our study the *HLTF* gene methylation was significantly associated with reduced expression in gastric carcinoma tissues and cell lines, the region of MSP primers we used corresponds to the target site of DNA methylation for transcriptional silencing.

Histone deacetylation plays an important role in methylation-associated gene inactivation.[30, 31] In the present study, we investigated the histone acetylation status of the 5′ CpG island of *HLTF* using ChIP assay. The methylated 5′ CpG island was hypoacetylated, whereas the unmethylated 5′ CpG island was hyperacetylated. In KATO-III cells, after treatment with Aza-dC, the 5′ CpG island was unmethylated and hyperacetylated, resulting in induction of gene expression. These findings support a model in which methyl-CpG-binding domain proteins act as anchors on methylated DNA, recruiting accessory proteins, such as HDAC, that can modulate chromatin structure and transcriptional activity of the gene. Similar phenomena have been reported in transcriptional regulation of the *COX-2*, *p57^{KIP2}*, and *DAP kinase* genes.[6, 39, 40] We also showed that the histone acetylation status of the coding region is associated with gene expression. This is consistent with the data that transcript elongation and histone acetylation are needed to form and maintain, respectively, an unfolded structure of transcribing nucleosomes.[41] Taken together, these findings indicate that DNA methylation and histone deacetylation are deeply involved in inactivation of the *HLTF* gene in gastric carcinoma.

We showed that treatment with TSA alone induced *HLTF* gene expression in KATO-III cells, but not in MKN-28 cells, suggesting that the induction of *HLTF* gene expression by treatment of TSA may be associated with DNA methylation. Although in general, treatment with TSA alone does not induce expression of genes silenced by DNA methylation, such as *hMLH1* and *p16^{INK4a}*,[42] previous studies have indicated that in MDA-MB-231 breast cancer cells, *RAR-β* can be reactivated by treatment with TSA alone in the presence of a methylated *RAR-β* promoter.[43, 44] TSA induction of gene expression silenced by DNA methylation may be gene-specific. Demethylation at *HLTF* does not seem to be an absolute requirement for *HLTF* gene expression in KATO-III cells.

The significance of reduced expression of the *HLTF* gene remains unclear. It has been reported that transfection of HLTF expression vector into HLTF-deficient colon cancer cells suppresses growth,[22] suggesting that *HLTF* silencing may confer a growth advantage to some colon cancers and that HLTF may be a tumor suppressor in colon cancer. Studies of the growth suppression effect of HLTF in gastric carcinoma cells are needed. HLTF has been reported to be involved in expression of the *plasminogen activator inhibitor-1* (*PAI-1*) gene, and over-expression of *HLTF* caused a threefold induction of *PAI-1* transcription in HeLa cells,[45] suggesting that silencing of *HLTF* by DNA methylation may reduce *PAI-1* expression. PAI-1 controls the activity of urokinase-type plasminogen activator (uPA), and inhibition of uPA activity leads to inhibition of invasion in several experimental systems.[46, 47] It has been proposed that PAI-1 plays a role in protecting the tumor, and is a potentially important prognostic factor in breast carcinoma.[48, 49] However, in gastric carcinoma, PAI-1 antigen levels are higher in tumor tissue than in normal tissue.[50] Identification of the target genes of HLTF is needed to understand the mechanism of HLTF involvement in stomach carcinogenesis.

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DNA methylation of multiple genes in gastric carcinoma: Association with histological type and CpG island methylator phenotype

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Hypermethylation of CpG islands is associated with silencing of various tumor suppressor genes. Recent studies on colorectal and gastric cancer have identified a CpG island methylator phenotype (CIMP), which involves the targeting of multiple genes by promoter hypermethylation. For determination of association between DNA methylation pattern or histological type and CIMP status in gastric carcinoma, CpG islands in the promoters of *hMLH1* and *CDH1* genes, CpG islands overlapping exon 1 of *MGMT* and *p16^{INK4a}* genes, and a non-CpG island in exon 1 of the *RAR-β* gene were studied. The presence of the CIMP was determined by monitoring five *methylated in tumor* (*MINT*) loci in 103 gastric carcinomas. Among the 103 gastric carcinomas, DNA hypermethylation was detected in the following frequencies: 14 (14%) for *hMLH1*, 26 (25%) for *MGMT*, 26 (25%) for *p16^{INK4a}*, 54 (52%) for *CDH1*, and 53 (52%) for *RAR-β*. Forty-two (41%) of 103 gastric carcinomas were positive for the CIMP. CIMP and hypermethylation of *p16^{INK4a}* gene were found more frequently in intestinal and diffuse-adherent types than in diffuse-scattered type (P=0.013 and 0.017, respectively). In contrast, hypermethylation of the *CDH1* and *RAR-β* genes was more common in the diffuse-scattered type than in the other types (P=0.008 and 0.007, respectively). In intestinal- and diffuse-adherent-type gastric carcinomas, we found significant associations between the presence of the CIMP and hypermethylation of several genes: *hMLH1* (P=0.006), *p16^{INK4a}* (P=0.018), *CDH1* (P=0.024), and *RAR-β* (P=0.044). Our overall results suggest that in some intestinal- and diffuse-adherent-type gastric carcinomas, DNA hypermethylation affects non-specific gene promoters concordantly, at least in part, whereas in diffuse-scattered-type gastric carcinoma, DNA hypermethylation affects specific genes such as *CDH1* and *RAR-β*. (Cancer Sci 2003; 94: 901–905)

Alterations in DNA methylation patterns, such as hypermethylation of CpG islands, are common changes in human cancers. Hypermethylation of CpG islands in promoters is associated with silencing of various tumor suppressor genes.[1–3] Recent studies of gastric carcinomas identified a CpG island methylator phenotype (CIMP).[4] Cases showing methylation at more than three of the five loci (*methylated in tumors* (*MINT*) *1*, *2*, *12*, *25*, and *31*) were designated as CIMP-positive, and such CIMP-positive gastric carcinomas are frequently associated with *p16^{INK4a}* gene methylation. Although several reports have been published about methylation of various genes in gastric carcinoma,[5–15] in most of these studies, the methylation status was investigated for just a single gene and no reports have described an association between DNA methylation pattern and CIMP status or histological classification. Kang *et al.*[16] studied DNA methylation of multiple genes and reported that Epstein-Barr virus (EBV)-positive gastric carcinoma frequently shows aberrant methylation; however, they did not investigate whether any association exists between DNA hypermethylation and CIMP status or histological classification. Therefore, we investigated methylation of multiple genes in primary gastric carcinoma specimens, and we found an association between DNA hypermethylation and both histological classification and CIMP status.

Materials and Methods

Samples and DNA extraction. One hundred and three gastric carcinoma specimens from 103 patients were studied. Tumor tissues were removed surgically, frozen immediately in liquid nitrogen, and stored at −80°C until use. We confirmed microscopically that the tumor specimens consisted mainly (>80%) of carcinoma tissue. Genomic DNAs were extracted with a genomic DNA purification kit (Promega, Madison, WI). Histological classification (intestinal-type and diffuse-type) was made according to the Lauren classification system.[17] Diffuse-type gastric carcinomas were further classified into diffuse-adherent and diffuse-scattered subtypes.[18] The pathologists assigning histological classifications (Yasui W, Nakayama H) were blinded with respect to promoter methylation status of all specimens. The presence or absence of hypermethylation of CpG islands overlapping exon 1 of *MGMT* gene had been determined previously in 50 of the 103 gastric carcinoma samples.[19] The status of hypermethylation of CpG islands in the promoter of *CDH1* gene, CpG islands overlapping exon 1 of *p16^{INK4a}* gene, and a non-CpG island in exon 1 of the *RAR-β* gene was determined previously in 45 of the 103 gastric carcinoma samples.[20] Because written informed consent was not available, for strict privacy protection, all samples were disidentified before analyzing DNA methylation status. This procedure is in accordance with Ethical Guidelines for Human Genome/Gene Research enacted by the Japanese Government.

Bisulfite polymerase chain reaction (PCR) and methylation-specific PCR (MSP). To examine the DNA methylation patterns, we treated genomic DNA with sodium bisulfite, as described previously.[21] In brief, 2 μg of genomic DNA was denatured by treatment with NaOH and modified with 3 *M* sodium bisulfite for 16 h. DNA samples were purified with Wizard DNA purification resin (Promega), treated with NaOH, precipitated with ethanol, and resuspended in 25 μl of water. Two-microliter aliquots were used as templates for PCR reactions. For analysis of DNA methylation of the *MGMT*, *p16^{INK4a}*, *CDH1*, and *RAR-β*

The abbreviations used are: PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR; hMLH1, human Mut L homologue 1; MGMT, O^6-methylguanine-DNA methyltransferase; CDH1, cadherin 1; RAR-β, retinoic acid receptor-β; MINT, methylated in tumors; CIMP, CpG island methylator phenotype; MSP, methylation-specific PCR.

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genes, we performed MSP. MSP primers for *MGMT*, *p16*^{INK4a}, *CDH1*, and *RAR-β* genes were as described previously.[21-23] For analysis of DNA methylation of *hMLH1*, *MINT1*, *MINT2*, *MINT12*, *MINT25*, and *MINT31*, we performed bisulfite-PCR followed by restriction digestion as previously described.[4,24] Fig. 1 shows CpG maps of *hMLH1*, *MGMT*, *p16*^{INK4a}, *CDH1*, and *RAR-β* genes analyzed, along with the location of the regions amplified.[21-24] PCR products (15 μg) were loaded onto 8% non-denaturing polyacrylamide gels, stained with ethidium bromide, and visualized under UV light. We considered cases with methylation at more than three of five loci (*MINT 1*, *2*, *12*, *25*, and *31*) to be positive for the CIMP.[4]

Statistical analysis. Statistical significance of differences was assessed with Fisher's exact test by using InStat 2.01 software for Macintosh. Two-sided tests were used to determine significance, and *P* values less than 0.05 were regarded as statistically significant.

Results

DNA methylation pattern of gastric carcinoma. Representative data for bisulfite-PCR followed by restriction digestion of the *hMLH1* gene and MSPs of the *MGMT*, *p16*^{INK4a}, *CDH1*, and *RAR-β* genes are shown in Fig. 2A. Among the 103 gastric carcinomas, DNA hypermethylation was detected at the following frequencies: 14 (14%) for *hMLH1*, 26 (25%) for *MGMT*, 26 (25%) for *p16*^{INK4a}, 54 (52%) for *CDH1*, and 53 (52%) for *RAR-β*. Overall results are shown in Fig. 3.

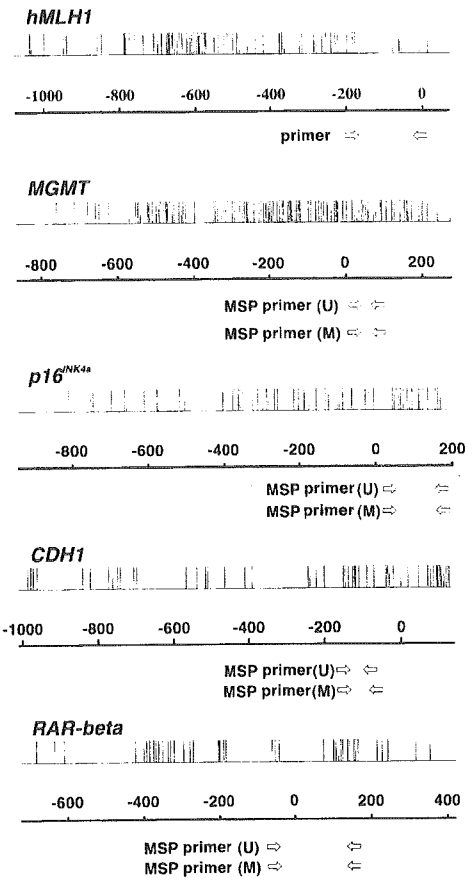

Fig. 1. Maps of the genes analyzed. Shown are CpG maps of the sequences around exon 1 of the five genes analyzed in this study. For each gene, the CpG density is indicated by short vertical bars. Arrows represent PCR primer (U, unmethylated; M, methylated). The numbering in this scheme corresponds to position relative to known transcription start sites.

When we compared hypermethylation of the five genes, we found concordant hypermethylation of the *MGMT* and *p16*^{INK4a} genes (P=0.035), the *p16*^{INK4a} and *RAR-β* genes (P=0.043), and the *CDH1* and *RAR-β* genes (P<0.001). However, the cases showing *hMLH1* gene hypermethylation did not coincide with those showing *MGMT* gene hypermethylation (P=0.019; Table 1).

Association between CIMP and DNA hypermethylation. We then examined the methylation status of *MINT1*, *2*, *12*, *25*, and *31*. Representative data are shown in Fig. 2B, and overall results are shown in Fig. 3. Of the 103 gastric carcinomas, CpG island hypermethylation of the *MINT* loci was detected at the following frequencies: 47 (46%) for *MINT1*, 36 (35%) for *MINT2*, 47 (46%) for *MINT12*, 77 (75%) for *MINT25*, and 22 (21%) for *MINT31*. In total, 42 (41%) of the 103 gastric carcinomas were positive for the CIMP. When we compared the DNA methylation status of the five genes with the CIMP status, we found a significant association between the presence of the CIMP and both *hMLH1* gene hypermethylation (P=0.018) and *p16*^{INK4a} gene hypermethylation (P<0.001; Table 1). In contrast, we found no association between the presence of the CIMP and hy-

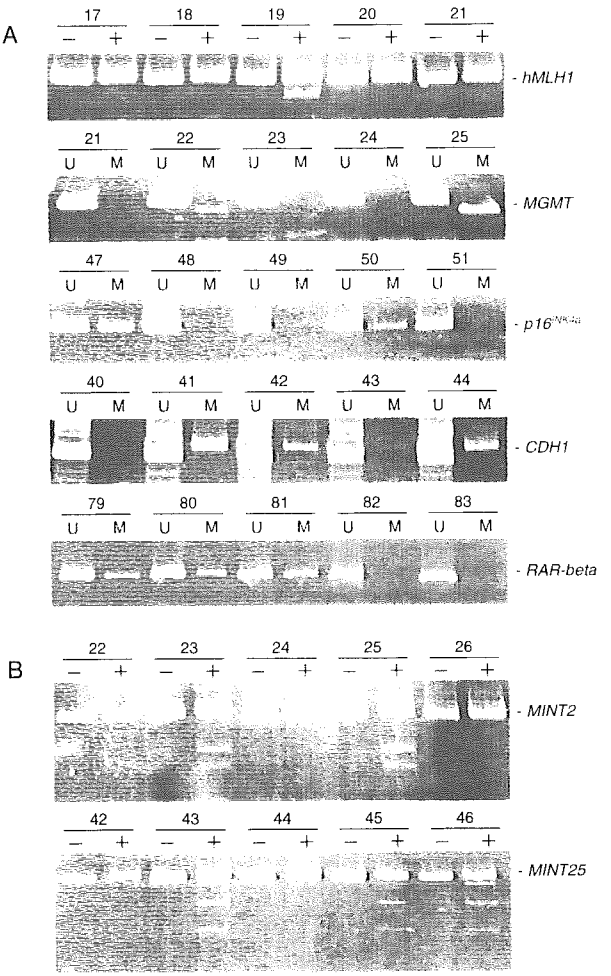

Fig. 2. (A) Bisulfite-PCR followed by restriction digestion of the *hMLH1* gene and MSPs of the *MGMT*, *p16*^{INK4a}, *CDH1*, and *RAR-β* genes. The methylated allele was detected in case 19 (*hMLH1*), cases 22 and 25 (*MGMT*), cases 47, 50, and 51 (*p16*^{INK4a}), cases 41, 42, and 44 (*CDH1*), and cases 79, 80, and 81 (*RAR-β*). (B) Bisulfite-PCR followed by restriction digestion of the *MINT2* and *MINT25*. Methylated allele was detected in cases 23 and 25 (*MINT2*) and cases 43, 45, and 46 (*MINT25*). U, unmethylated; M, methylated; +, after digestion of restriction enzyme; −, before digestion of restriction enzyme.

permethylation of the *MGMT* (P=0.356), *CDH1* (P=0.160), or *RAR-β* (P=0.229) genes.

Association between histological classification and DNA methylation. When we classified gastric carcinoma cases into diffuse-scattered type (37/103) and intestinal and diffuse-adherent types (66/103), the CIMP and hypermethylation of the $p16^{INK4a}$ gene were found more frequently in the intestinal and diffuse-adherent types than in the diffuse-scattered type (P=0.013 and 0.017, respectively). In contrast, hypermethylation of *CDH1* and *RAR-β* genes was more common in the diffuse-scattered type than in the other types (P=0.008 and 0.007, respectively).

Because the CIMP was detected more commonly in the intestinal and diffuse-adherent types than in the diffuse-scattered type, we compared the DNA methylation status of the five genes with the CIMP status in intestinal- and diffuse-adherent-type gastric carcinomas. As shown in Table 2, we found a significant association between the presence of CIMP and hypermethylation of several genes: *CDH1* (P=0.024), *RAR-β* (P=0.044), *hMLH1* (P=0.006), and $p16^{INK4a}$ (P=0.018). The presence of the CIMP was not associated with *MGMT* gene hypermethylation (P=1.000).

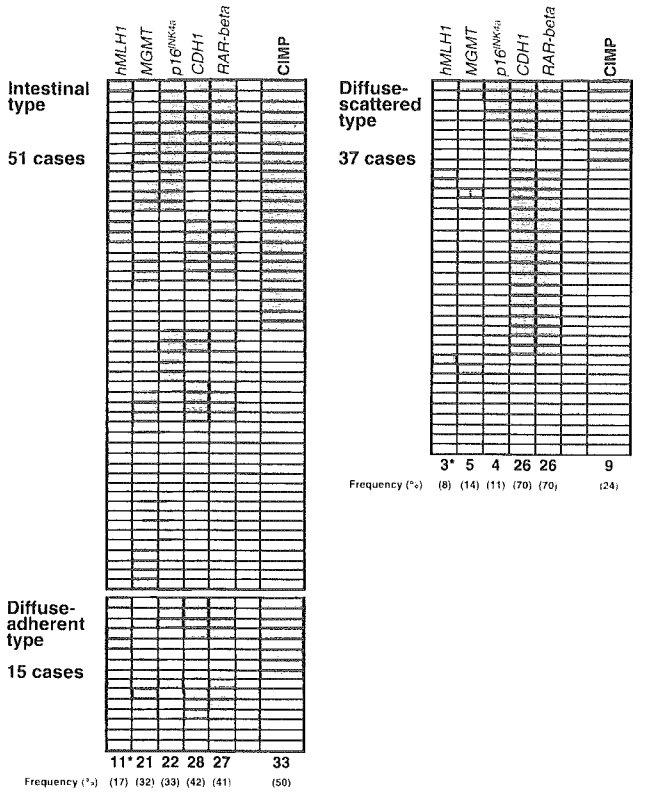

Fig. 3. Summary of methylation of *hMLH1*, *MGMT*, $p16^{INK4a}$, *CDH1*, and *RAR-β* in primary gastric carcinoma. White boxes represent samples that are not methylated or CIMP-negative and gray boxes represent samples that are methylated or CIMP-positive. *, the number of cases with promoter hypermethylation.

Discussion

We found that the CIMP and DNA hypermethylation of $p16^{INK4a}$ occurred preferentially in intestinal- and diffuse-adher-

Table 1. Correlation of promoter methylation status in gastric carcinomas

		MGMT		$p16^{INK4a}$		*CDH1*		*RAR-β*		CIMP	
		U	M	U	M	U	M	U	M	−	+
hMLH1	U	63	26	68	21	44	45	45	44	57	32
	M	14	0	9	5	5	9	5	9	4	10
P value[1]		0.019		NS[2]		NS		NS		0.018	
MGMT	U			62	15	39	38	40	37	48	29
	M			15	11	10	16	10	16	13	13
P value				0.035		NS		NS		NS	
$p16^{INK4a}$	U					40	37	42	35	55	22
	M					9	17	8	18	6	20
P value						NS		0.043		<0.001	
CDH1	U							48	1	33	16
	M							2	52	28	26
P value								<0.001		NS	
RAR-β	U									33	17
	M									28	25
P value										NS	

1) Fisher's exact test.
2) NS, not significant.
U, unmethylated; M, methylated; +, CIMP-positive; −, CIMP-negative.

Table 2. Correlation of promoter methylation status and CIMP in intestinal- and diffuse-adherent-type gastric carcinoma

		CIMP		*P* value[1]
		Positive	Negative	
hMLH1 methylation status	Methylated	10	1	0.006
	Unmethylated	23	32	
MGMT methylation status	Methylated	11	10	1.000
	Unmethylated	22	23	
$p16^{INK4a}$ methylation status	Methylated	16	6	0.018
	Unmethylated	17	27	
CDH1 methylation status	Methylated	19	9	0.024
	Unmethylated	14	24	
RAR-β methylation status	Methylated	18	9	0.044
	Unmethylated	15	24	

1) Fisher's exact test.

ent-type gastric carcinomas rather than diffuse-scattered-type gastric carcinomas. DNA hypermethylation of *CDH1* and *RAR-β* occurred preferentially in diffuse-scattered-type gastric carcinomas rather than the other types. These tendencies were reported previously for *CDH1*[25] and for *p16*^{INK4a} and *RAR-β*.[20] Considering that gastric carcinoma with the CIMP occurred preferentially in intestinal- and diffuse-adherent-type gastric carcinomas, gastric carcinomas with CIMP may not always involve hypermethylation of the *CDH1* and *RAR-β* genes. Though hypermethylation of the *CDH1* and *RAR-β* genes was observed more frequently in diffuse-scattered-type gastric carcinomas, among the combined group of 66 intestinal- and diffuse-adherent-type gastric carcinomas, 27 showed *CDH1* hypermethylation and 28 showed *RAR-β* hypermethylation. In this combined group, we found a significant association between the presence of the CIMP and *CDH1* or *RAR-β* gene hypermethylation. Suzuki *et al.*[26] studied 61 primary gastric cancers and reported that cases with hypermethylation of the *hMLH1* gene also showed hypermethylation of the *CDH1* and *p16*^{INK4a} genes. This phenomenon of hypermethylation of multiple genes is similar to that observed in the present study. Our present data show that in some intestinal- and diffuse-adherent-type gastric carcinomas, DNA hypermethylation affects non-specific gene concordantly, at least in part. However, no information is available about the concordant hypermethylation pattern in gastric carcinomas except for *hMLH1*, *p16*^{INK4a}, and *CDH1*. We should examine concordant hypermethylation of other genes. On the other hand, in diffuse-scattered-type gastric carcinomas, DNA hypermethylation affects specific genes such as *CDH1* and *RAR-β*. Previously, the PML-RAR fusion protein was reported to induce *RAR-β* promoter hypermethylation and silencing by recruiting DNA methyltransferases to target promoters.[27] Considering our present findings of concordant hypermethylation of *CDH1* and *RAR-β*, we believe that an oncogenic transcription factor may induce specific gene hypermethylation in diffuse-scattered-type gastric carcinomas. In intestinal- and diffuse-adherent-type gastric carcinomas, aging may induce DNA hypermethylation of non-specific genes and be associated with the CIMP. In fact, age-related hypermethylation of the *hMLH1* gene has been reported,[28] and diffuse-scattered-type gastric carcinoma tends to affect relatively young individuals.

We found no association between hypermethylation of the *MGMT* gene and histological classification; however, we did note that the cases showing *hMLH1* gene hypermethylation did not coincide with those showing *MGMT* gene hypermethylation. Our current data suggest that an alternative pathway for hypermethylation of *MGMT* may be present. Hypermethylation of *MGMT* characterizes a subset of colorectal cancers with low-level DNA microsatellite instability.[29] Kang *et al.* reported that EBV-positive gastric carcinomas frequently showed aberrant methylation of multiple genes such as *p16*^{INK4a} and *MGMT*, and that the methylation frequencies of *hMLH1* and *CDH1* were not significantly higher in EBV-positive than in EBV-negative gastric carcinoma.[16] So, carcinomas showing concordant hypermethylation of *MGMT* and *p16*^{INK4a} may be associated with EBV. In fact, we found a significant association between hypermethylation of the two genes. A subset of gastric carcinomas, known as lymphoepithelioma-like gastric carcinoma, is known to harbor the EBV genome in a high proportion of cases.[30–32] The methylation status has no relation to the histological features of EBV-positive gastric carcinoma.[16]

Although methylations of the regions of *hMLH1* and *CDH1* genes we analyzed in this study cause silencing,[33,34] it has been reported that the regions of the *MGMT*, *p16*^{INK4a}, and *RAR-β* analyzed in this study do not cause gene silencing.[22–24] Nevertheless, DNA methylation of any regions, whether or not they cause gene silencing, may correlate with reduced gene expression, histology, or the presence of the CIMP. In fact, DNA hypermethylation in the examined regions showed a good correlation with reduced expression of the respective target genes.[7,11,22,23,33–36]

In conclusion, our present results suggest that in some intestinal- and diffuse-adherent-type gastric carcinomas, DNA hypermethylation affects non-specific genes concordantly, at least in part, whereas in diffuse-scattered-type gastric carcinomas, DNA hypermethylation affects specific genes such as *CDH1* and *RAR-β*.

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Correlation of a single nucleotide polymorphism in the E-cadherin gene promoter with tumorigenesis and progression of gastric carcinoma in Japan

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Abstract. A C/A single nucleotide polymorphism (SNP) in the E-cadherin gene promoter has been shown to affect transcriptional efficiency *in vitro*. In this study, we analyzed the C/A SNP in 106 gastric carcinoma cases and 90 cancer-free controls compared with clinicopathological parameters. The genotype of C/C, C/A, and A/A was found in 61 (58%), 34 (32%) and 11 (10%) of 106 gastric carcinoma cases and in 32 (36%), 52 (58%) and 6 (6%) of 90 cancer-free controls, respectively. The genotype of C/C was significantly more frequent in gastric carcinoma cases than in cancer-free controls ($p<0.05$). The E-cadherin protein expression level in non-neoplastic gastric mucosa was higher in the C/C genotype than in the C/A. In gastric carcinoma cases, the genotype of C/C was significantly more frequent in the cases of poorly differentiated adenocarcinoma (31/44; 70%) than in well differentiated type (30/62; 48%). The C/C genotype was also significantly more frequent in the cases with deep invasion (51/74; 69%) and with lymph node metastasis (38/54; 70%) than in those confined superficially (10/32; 31%) and without lymph node metastasis (23/52; 44%), respectively ($p<0.05$). These results suggest that the C/A SNP in the E-cadherin promoter may be a good marker for malignancy of gastric carcinomas.

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Abbreviations: C, cytosine; A, adenine; SNP, single nucleotide polymorphism; SSCP, single-strand conformational polymorphism; PCR, polymerase chain reaction

Key words: SNP, E-cadherin, gastric carcinoma, tumorigenesis, tumor progression

Introduction

Cell-cell adhesions play crucial roles not only in regulating morphogenesis of both normal and neoplastic tissues but also in invasion and metastasis of cancer. Cadherins are a multigene family of transmembrane glycoproteins, which are located on the cell surface and responsible for calcium-dependent intercellular adhesion (1). Almost all epithelial cells express E-cadherin and is considered to be the main cadherin type for intercellular adhesion of epithelial cells. There are many observations suggesting that E-cadherin may function as tumor suppressor (2,3). Inverse correlation between E-cadherin expression and metastasis and/or invasion was observed in various cancers including carcinomas of the ovary, prostate and the head and neck (4,5). Cancer cell lines expressing E-cadherin show non-invasive pattern of growth, whereas cells without E-cadherin expression show invasive growth (6).

In gastric carcinomas, well-differentiated adenocarcinomas maintain strong expression of E-cadherin, while poorly differentiated carcinomas especially scirrhous-type carcinomas display marked reduction of E-cadherin expression (7-10). Somatic mutations in the E-cadherin gene occur preferentially in about 50% of poorly differentiated type gastric cancer (11,12). Germline mutations in the E-cadherin gene were found in some of the familial gastric carcinomas (13,14). Epigenetic silencing of E-cadherin expression through CpG hypermethylation at the promoter region has also been reported in a section of gastric carcinomas (15-17).

Genetic factors contribute to many human diseases, conferring susceptibility or resistance, or influencing interaction with environmental factors. The most common type of human genetic variation is single nucleotide polymorphism (SNP), some of which occur in the promoter resulting in difference in transcriptional efficiency (18). A C/A SNP exists at -160 from the transcriptional start site of the E-cadherin gene promoter and the A allele decreases transcriptional efficiency by 68% compared with the C allele *in vitro* (19). In the present study, we examined the genotype of this C/A SNP in gastric carcinoma cases and cancer-free controls. We also analyzed

the correlation between genotype of the SNP and clinicopathological characteristics of carcinomas. During preparation of this manuscript, Wu *et al* reported that the individuals with A/A genotype in the E-cadherin promoter have a decreased risk of gastric carcinoma in Taiwanese (20). We will discuss the similarity and difference of the results between Taiwanese and Japanese.

Materials and methods

Tissue samples. We analyzed 106 gastric carcinoma cases and 90 cancer-free controls. Tissue samples of non-neoplastic gastric mucosae from 106 gastric carcinoma cases were obtained by surgery, frozen immediately thereafter in liquid nitrogen. We confirmed microscopically that all gastric carcinoma patients had gastric adenocarcinomas and the corresponding non-neoplastic mucosae did not exhibit any tumor-cell invasion or show significant inflammatory involvement. Histology, stroma, depth of tumor invasion were classified according to the criteria of the Japanese Classification of Gastric Carcinoma (1999, 13th edition). Non-neoplastic gastric mucosae of 90 cancer-free controls were obtained by endoscopic mucosal resection and fixed in formalin followed by paraffin-embedding. No gastric tumors were confirmed endoscopically or histopathologically. The mean age of gastric carcinoma cases (86 males and 20 females) and non-gastric carcinomas cases (45 males and 45 females) was 66.8±12.4 years and 63.0±12.0 years, respectively. Coding anonymously protects privacy in all samples. The study was performed under the permission of the human genome research ethics screening committee of Hiroshima University School of Medicine.

Cell culture. Eight human gastric carcinoma cell lines (MKN-1, MKN-7, MKN-28, MKN-45, MKN-74, HSC-39, KATO-III and TMK-1) were used (21). The TMK-1 cell line was established in our laboratory from poorly differentiated adenocarcinoma (22). Five gastric carcinoma cell lines of the MKN series (MKN-1, adenosquamous cell carcinoma; MKN-7, MKN-28, and MKN-74, well-differentiated adenocarcinomas; and MKN-45, poorly-differentiated adenocarcinoma), were kindly provided by Dr T. Suzuki (Fukushima Medical University, Fukushima). KATO-III and HSC-39 cell lines, which were established from signet ring cell carcinoma, were kindly provided by Dr M. Sekiguchi (University of Tokyo, Tokyo) and by Dr K. Yanagihara (National Cancer Center, Tokyo), respectively (23). All of these cell lines were routinely maintained as previously described (9).

DNA extraction. Genomic DNA samples were extracted by Wizard Genomic DNA Purification Kit (Promega, Madison, WI) from human gastric carcinoma cell lines, non-neoplastic gastric mucosa freshly frozen. From formalin-fixed paraffin-embedded tissues, genomic DNA samples were extracted with proteinase K as previously described (24).

PCR-single-strand conformational polymorphism (PCR-SSCP) analysis. Genotype of the C/A SNP in the E-cadherin gene promoter region was analyzed by PCR-SSCP. PCR fragments were generated as previously described (24). PCR was performed for 10 min at 94°C followed by 35 cycles at 94°C for 30 sec, 57°C for 30 sec, and 72°C for 1 min, and a final extension step at 72°C for 10 min. The PCR primer sequences used were 5'-GTGGAATCAGAACCGTGCAG-3' (E-Cad/POU) and 5'-CCACCCGGCCTCGCATAGAC-3' (E-Cad/P2L). SSCP analysis was performed as described (23).

DNA sequencing. DNA sequencing analysis confirmed genotype of the C/A SNP in the E-cadherin promoter region. For the sequencing reaction, the PRISM AmpliTaq DNA polymerase FS Ready Reaction Dye Terminator Sequencing kit (Perkin-Elmer ABI) was used and sequenced by ABI PRISM 310 genetic analyzer (Perkin-Elmer ABI, Foster City, CA) as described (25).

Western blot analysis. Tissue extracts from the non-neoplastic gastric mucosa of 22 gastric carcinoma cases were prepared and Western blotting was carried out as described (26). Anti-E-cadherin monoclonal antibody (HECD-1) (Takara, Kyoto, Japan) and peroxidase conjugated anti-mouse IgG was used in the primary and secondary reaction, respectively. The quality and amount of protein samples applied on the gel were confirmed by detection with anti-α-tubulin antibody. We quantified the bands by autoradiographic signal intensities of the bands on Western blots by densitometric scanning, normalized by internal control (α-tubulin) as described (26).

Methylation specific PCR. To determine DNA methylation status in the promoter region of the E-cadherin gene, extracted DNAs from carcinoma tissues of 53 gastric carcinoma cases were treated with sodium bisulfite and methylation specific PCR analysis was carried out as previously described (26,27). Genomic DNA from MKN-28 cell line treated with SssI methyltransferase (New England Biolabs, Inc., Beverly, MA), generating DNA completely methylated at CpG sites, served as the positive control for methylated E-cadherin.

Statistical analysis. Fisher's exact test was used to test whether the distribution of SNP genotype was significantly different between gastric and cancer-free controls. In gastric carcinoma cases, correlation between SNP genotype and clinicopathological characteristics of gastric carcinomas was also examined by Fisher's exact test. A computer program InStat Version 2.01 (GraphPad Software, Inc) performed Fisher's exact test. Odds ratios and their 95% confidence intervals were calculated with adjustment for age as a categorical variable. Mann-Whitney test was used to examine independence between SNP genotype and E-cadherin expression.

Results

We amplified the E-cadherin promoter region containing the C/A SNP at -160 from transcriptional start site by PCR and performed SSCP analysis in gastric carcinoma cell lines. Three patterns of PCR-SSCP bands were detected (Fig. 1A). MKN-1, MKN-45 and MKN-74 had two bands indicating homozygous SNP genotype. MKN-7, MKN-28, HSC-39 and KATO-III showed another homozygous SNP genotype. On the other hand, TMK-1 had three bands all of which corresponded the bands mentioned above, indicating heterozygous SNP

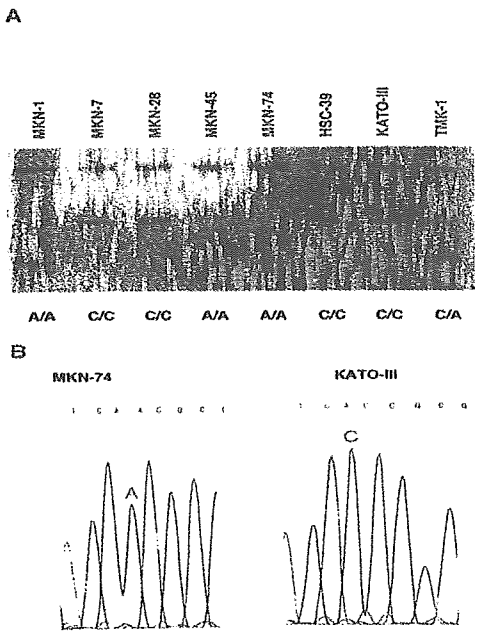

Figure 1. The C/A SNP of the E-cadherin gene promoter in eight human gastric carcinoma cell lines. A, PCR-SSCP analysis of the E-cadherin gene promoter in gastric carcinoma cell lines. The genotype of SNP was decided as indicated, considering together with the results of DNA sequencing of MKN-74 and KATO-III shown in (B). B, DNA sequence analysis of the E-cadherin promoter in MKN-74 and KATO-III. Nucleotide at -160 from the transcriptional initiation site was A/A in MKN-74 and C/C in KATO-III, respectively. This analysis allowed to determine the genotype for the three SSCP patterns in (A).

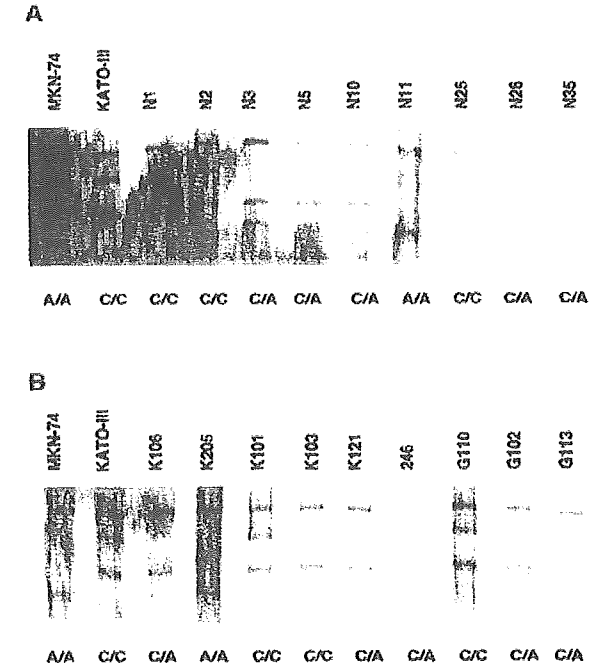

Figure 2. PCR-SSCP analysis of the E-cadherin promoter and predicted C/A SNP genotype in cancer-free controls (A) and gastric carcinoma cases (B). The genotype of SNP shown below each band was decided using MKN-74 and KATO-III as controls. Unrelated band was occasionally detected between the upper two bands (KATO-III, N2, K101, G110) that was not examined further. Numbers above the panel are case numbers.

genotype. We then performed DNA sequencing to reveal genotype of the C/A SNP in MKN-74 and KATO-III. MKN-74 was found to have A/A genotype and KATO-III had C/C genotype by considering together with PCR-SSCP results (Fig. 1B). Therefore, TMK-1 was thought to have heterozygous C/A genotype.

We then examined the C/A SNP in the E-cadherin promoter in non-neoplastic mucosa of gastric carcinoma cases and cancer-free controls by PCR-SSCP analysis. Representative results are shown in Fig. 2. Genotype of the SNP was decided by comparing the band patterns of MKN-74 and KATO-III as controls of A/A and C/C, respectively. Distribution of the SNP genotype among gastric carcinoma cases and cancer-free controls are summarized in Table I. The genotype of C/C, C/A, and A/A was found in 61 (58%), 34 (32%) and 11 (10%) of 106 gastric carcinoma cases and in 32 (36%), 52 (58%) and 6 (6%) of 90 cancer-free controls, respectively. As the number of A/A genotype was very small, we compared C/C with C/A and A/A together. The genotype of C/C was found in 61 (58%), while the genotype of C/A and A/A was found in 45 (42%) of 106 gastric carcinoma cases. On the other hand, the genotype of C/C was found in 32 (36%), while the genotype of C/A and A/A was in 58 (64%) of 90 cancer-free controls. The genotype of C/C was significantly more frequent in gastric carcinoma cases than in cancer-free controls (p=0.003). The estimated odds ratio of C/C to C/A and A/A in gastric carcinoma cases was 2.68 (95% CI, 1.50-4.79) after adjusted for age as a categorical variable (Table I).

We then examined the expression of E-cadherin protein by Western blotting in the 22 gastric carcinoma cases of which protein samples were available. Those having C/C genotype (14 cases) showed significantly higher expression level of E-cadherin protein in non-neoplastic mucosae by approximately 70% than C/A genotype (8 cases) (Mann-Whitney test; U=87, p<0.05). This is consistent with result of the transient transfection experiment showing that the A allele decreases transcriptional efficiency (19). However, the expression of the E-cadherin protein in carcinoma tissue was reduced especially in poorly differentiated type, which was not associated with the SNP genotype (data not shown).

We next evaluated the correlation between genotype of the SNP and clinicopathological characteristics in gastric carcinoma cases. Results are summarized in Table II. The genotype of C/C was significantly more frequent in the cases of gastric carcinoma with poorly differentiated adenocarcinoma, deep invasion and lymph node metastasis. The C/C genotype was found in 31 (70%) of 44 poorly differentiated adenocarcinoma cases, and in 30 (48%) of 62 well differentiated adenocarcinoma cases (p=0.029), respectively. The C/C genotype was more frequent in the cases with invasion beyond muscularis propria (51/74; 69%) than in those confined to submucosa (10/32; 31%) (p=0.001). The genotype of C/C was also found more frequently in the cases with lymph nodes metastasis (38/54; 70%) than in those without metastasis (23/52; 44%) (p=0.012). The estimated odds ratio of C/C to C/A and A/A in poorly differentiated type, invasion beyond muscularis propria and lymph nodes metastasis was 2.31

Table I. Distribution of the genotype of E-cadherin among gastric carcinoma cases and cancer-free controls.

	Total	Genotype		
		C/C (%)	C/A (%)	A/A (%)
Gastric carcinoma cases	106	61	34	11
		(58)	(32)	(10)
Cancer-free controls	90	32	52	6
		(36)	(58)	(6)

	Age[a]	Genotype		p-value[b]	OR (95% CI)[c]
		C/C (%)	C/A and A/A (%)		
Gastric carcinoma cases	66.8±12.4	61	45	0.003	2.68 (1.50-4.79)
(n=106)		(58)	(42)		
Cancer-free controls	63.0±12.0	32	58		
(n=90)		(36)	(64)		

[a]Mean ± standard deviation (years). [b]Fisher's exact test. p-value <0.05 was regarded as statistically significant. [c]Adjusted for age as a categorical variable. CI, confidence interval.

Table II. Correlation between genotype of the E-cadherin promoter and clinicopathological characteristics of gastric carcinoma.

	Genotype		p-value[b]	Odds ratio (95% CI)[c]
	C/C (%)	C/A and A/A (%)		
Histology[a]				
Well	30 (48)	32 (52)		1 (ref.)
Poorly	31 (70)	13 (30)	0.029	2.31 (1.02-5.24)
Stroma[a]				
Non-sci	44 (54)	37 (46)		1 (ref.)
Sci	17 (68)	8 (32)	0.255	1.64 (0.64-4.23)
Depth of invasion[a]				
m, sm	10 (31)	22 (69)		1 (ref.)
mp-si	51 (69)	23 (31)	0.001	4.95 (2.02-12.1)
Lymph node metastasis				
Negative	23 (44)	29 (56)		1 (ref.)
Positive	38 (70)	16 (30)	0.010	2.86 (1.28-6.36)

[a]According to the criteria of the Japanese Classification of Gastric Carcinoma (1999, 13th Edition). Well, well differentiated adenocarcinoma including papillary and tubular adenocarcinoma; poorly, poorly differentiated adenocarcinoma including signet-ring cell carcinoma and mucinous adenocarcinoma; sci, scirrhous gastric carcinoma; m, mucosa propria; sm, submucosa; mp, muscularis propria; si, tumor invades adjacent structures. [b]Correlation was analyzed by Fisher's exact test. p-value <0.05 was regarded as statistically significant. [c]Adjusted for age as a categorical variable. CI, confidence interval.

(1.02-5.24), 4.95 (2.02-12.1) and 2.86 (1.28-6.36), respectively (95% CI). However, the association of C/C genotype with development of poorly differentiated adenocarcinoma, deeply tumor invasion and presence of metastasis seems to be inconsistent with the fact that C allele must increase transcriptional efficiency (19). We also confirmed by immunohistochemical staining that the expression of E-cadherin in non-neoplastic gastric mucosa at infiltrative margins of

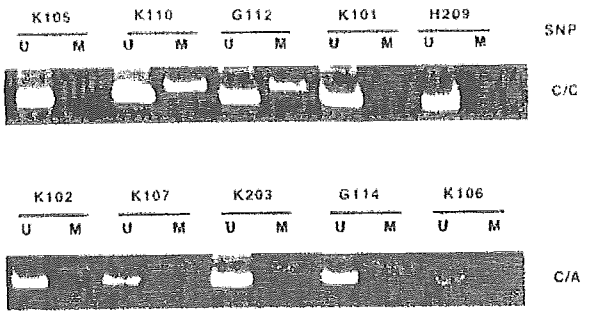

Figure 3. Methylation status in the E-cadherin promoter region in gastric carcinoma cases. Methylation-specific PCR was perfomed as described in Materials and methods. U and M above the panel indicate unmethylated and methylated, respectively.

carcinoma tissue was not specifically reduced in those have the C/C genotype of some cases analyzed in this study (data not shown).

Hypermethylation of CpG islands, one of epigenetic regulations, is associated with the silencing of various tumor suppressor genes (26). This epigenetic regulation may affect the expression of E-cadherin, possibly resulting in the inconsistency. We then analyzed the methylation status of E-cadherin promoter region in gastric carcinoma tissues by methylation specific PCR in part of gastric carcinoma cases used in the present study. Representative results are shown in Fig. 3. The methylation allele was detected in K105, K110, G112, and H209 and in K102, K107, K203, and G114, among C/C and C/A genotype, respectively. The overall results are summarized in Table III. The genotype of C/C was found in 20 (66%) cases and the genotype of C/A and A/A was found in 10 (34%) cases of 30 methylation-positive cases, while 16 (70%) and 7 (30%) of 23 methylation-negative cases. There was no significant correlation between the methylation status of E-cadherin promoter and the SNP genotype.

Discussion

In the present study, we analyzed the C/A SNP at -160 from transcriptional start site of the E-cadherin gene promoter in 106 gastric carcinoma cases and 90 cancer-free controls. We found significant difference in distribution of the SNP genotype between gastric carcinoma cases and cancer-free controls, suggesting that the C/A SNP has a great effect on tumorigenesis of gastric carcinoma. In our present study, C/A and A/A genotype was significantly less frequent in gastric carcinoma cases than in cancer-free controls, but we found no significant difference in the frequency of A/A genotype only between gastric carcinoma cases and cancer-free controls in Japanese (Table I). In Taiwanese, Wu *et al* reported that A/A genotype of this SNP in the E-cadherin promoter was significantly less frequent in gastric carcinoma cases (4/201, 2.0%) than controls (19/196, 9.7%), but there was no significant difference in the frequency of C/A and A/A genotype between gastric carcinoma cases (106/201, 53%) and controls (123/196, 63%) (20). However, the A allele is associated with reduced risk of gastric carcinoma commonly in the two race. Furthermore, we found in this study a significant association of the C/C genotype with the cases with poorly differentiated adenocarcinoma, deep invasion and lymph node metastasis. These observations indicate that the C/C genotype of E-cadherin promoter participates in tumorigenesis, especially of poorly differentiated adenocarcinoma and invasion and metastasis of gastric carcinoma in Japanese. On the other hand, Wu *et al* (20) did not find significant association between the genotype of E-cadherin promoter and cancer location, histology, stage, lymph node metastasis and *H. pylori* infection, suggesting involvement of some other factors, such as racial/ethnical factors, difference of criteria for the histopathological diagnosis of gastric carcinoma, and so on.

The C/C genotype is known to have higher transcriptional efficiency of E-cadherin *in vitro* (19) and the expression of E-cadherin is reduced generally in various cancers including gastric carcinoma (2-12). The association of C/C genotype with tumorigenesis, development of poorly differentiated adenocarcinoma, tumor invasion and metastasis seems to be inconsistent with these facts. We then examined the expression of E-cadherin protein in non-neoplastic gastric mucosa and found that the C/C genotype showed higher expression level of E-cadherin protein than C/A genotype, as expected.

Epigenetic alterations, in addition to multiple genetic abnormalities, are deeply involved in the genesis and progression of human cancers. Methylation status of the CpG island in promoter regions is an important determinant of gene expression. The C/A SNP in the promoter may have certain effect on the CpG island methylation. We therefore studied CpG island methylation of E-cadherin promoter and

Table III. Correlation between genotype and methylation status in the E-cadherin promoter region in gastric carcinoma tissues.

Methylation of E-cadherin promoter[a]	Genotype		p-value[b]	Odds ratio (95% CI)[c]
	C/C (%)	C/A and A/A (%)		
Positive 30	20 (66)	10 (34)		
Negative 23	16 (70)	7 (30)	0.823	0.88 (0.27-2.83)

[a]The methylation status of E-cadherin promoter region was analyzed by methylation specific PCR as described in Materials and methods. [b]Correlation was analyzed by Fisher's exact test. p-value <0.05 was regarded as statistically significant. [c]CI, confidence interval.

compared it with the genotype of the SNP. However, there was no significant correlation between the methylation status of E-cadherin promoter and SNP genotype (Table III). The expression of E-cadherin was consistently reduced in carcinoma tissues with CpG island hypermethylation. Thus, we do not know at present why C/C genotype was associated with tumorigenesis, poorly differentiated type, invasion and metastasis of gastric carcinoma.

There are many lines of evidence suggesting that cell adhesion molecules, growth factors, cell cycle regulators, matrix degradation enzymes have influence on tumorigenesis and progression of gastric carcinomas (28-30). Alterations in the other cadherin-related molecules, such as P-cadherin, α-, ß-catenin have also been found in gastric carcinomas (8). We have recently found that the 2G genotype of SNP in the MMP-1 gene promoter is related to development and progression of gastric cancer (Matsumura and Yasui unpublished data). Those have the C/C genotype of SNP in the E-cadherin promoter analyzed in the present study may have the 2G genotype of SNP in the MMP-1 promoter. Effects of these molecules may cause the discrepancy between the C/A SNP in E-cadherin promoter and clinicopathological characteristics of gastric carcinomas found in the present study. The different distribution of age and sex between gastric carcinoma cases and cancer-free controls may also be one of the reasons for this discrepancy. Although not investigated in this study, the prognosis of those who have the C/C genotype may be better than those with the C/A or A/A genotype. We should resolve this question in the near future by examining the correlation between C/A SNP in E-cadherin and other genetic and epigenetic abnormalities in more detail.

Our present study suggests that this C/C genotype in the E-cadherin promoter region may be a good marker for malignancy of gastric carcinomas in Japan, although the mechanism how the C/C genotype participates in tumor invasion and metastasis remains unclear.

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Expression of *POT1* is Associated with Tumor Stage and Telomere Length in Gastric Carcinoma

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ABSTRACT

Pot1, a telomere end-binding protein in fission yeast and human, is proposed not only to cap telomeres but also to recruit telomerase to the ends of chromosomes. No study has been performed regarding Pot1 expression status in human cancers. Thus, we examined *POT1* mRNA expression in 51 gastric cancer (GC) tissues and evaluated telomere length and 3′ telomeric overhang signals in 20 of the 51 GC tissues. Quantitative reverse transcription-PCR analysis showed that *POT1* expression levels in the tumor relative to those in nonneoplastic mucosa (T/N ratio) were significantly higher in stage III/IV tumors than in stage I/II tumors ($P = 0.005$). Down-regulation of *POT1* ($T/n < 0.5$) was observed more frequently in stage I/II GC (52.4%, 11 of 21) than in stage III/IV GC (23.3%, 7 of 30; $P = 0.033$), whereas up-regulation of *POT1* ($T/n > 2.0$) was observed more frequently in stage III/IV GC (33.3%, 10 of 30) than in stage I/II GC (9.5%, 2 of 21; $P = 0.048$). *POT1* expression levels showed decreased in accordance with telomere shortening ($r = 0.713$, $P = 0.002$). In-gel hybridization analysis showed that 3′ telomeric overhang signals decreased in accordance with decreases in *POT1* expression levels ($r = 0.696$, $P = 0.002$) and telomere shortening ($r = 0.570$, $P = 0.013$). Reduced *POT1* expression was observed in GC cell lines with telomeres shortened by treatment with azidothymidine. In addition, inhibition of Pot1 by antisense oligonucleotides led to telomere shortening as well as inhibition of telomerase activity in GC cells. Moreover, inhibition of Pot1 decreased 3′ overhang signals and increased the frequency of anaphase bridge ($P = 0.0005$). These data suggest that Pot1 may play an important role in regulation of telomere length and that inhibition of Pot1 may induce telomere dysfunction. Moreover, changes in *POT1* expression levels may be associated with stomach carcinogenesis and GC progression.

INTRODUCTION

Telomeres are distinctive structures consisting of a repetitive DNA sequence (TTAGGG) and associated proteins that cap the ends of linear chromosomes. Telomeres enable cells to distinguish chromosomal ends from double-strand breaks in the genome. Mammalian telomeric DNA is mostly composed of double-stranded 5′-TTAGGG-3′ repeats and terminates with a single-stranded overhang of the G-rich strand (1–3). In human somatic cells, telomeres have 500-3000 TTAGGG repeats, but telomeres shorten gradually with age (4–6). In contrast, telomeres of germ line and cancer cells do not shorten, consistent with the behavior of immortal and unicellular organisms. The telomeres of immortal cells are maintained by telomerase, which is able to extend 3′ telomeric overhangs, or by recombination (7–10). Telomerase activity confers cell immortality through stabilization of the chromosome, and it participates in the development of the majority of human cancers. We have shown that telomerase activity occurs in early-stage gastric cancer (GC; Ref. 11) and that telomerase reverse transcriptase expression is required for telomerase activity in the initiation of carcinogenesis in the stomach (12).

A single-stranded telomeric DNA binding protein, protection of telomeres (Pot1), has been identified in fission yeast and human (13). In fission yeast, most cells lacking Pot1 die because of sequence loss and end-to-end chromosomal fusion, although a few survivors emerge that have circularized all three chromosomes, thereby bypassing the requirement for chromosomal end maintenance. Purified fission yeast and human Pot1 proteins bind specifically to the G-rich strand of their own telomeric DNA but not to the complementary C-rich strand or double-stranded telomeric DNA, consistent with a role in binding to the 3′ telomeric overhang at the ends of telomeres *in vivo*. In *Saccharomyces cerevisiae*, the single-stranded telomeric DNA binding protein Cdc13 not only caps telomeres but also recruits telomerase to the ends of chromosomes (14, 15). Therefore, Pot1 is thought to be involved in this dual task (13, 16–18). A recent study indicated that each Pot1 binds to one telomeric repeat and coats the entire single-stranded overhang of the telomere in *Schizosaccharomyces pombe* (18). However, no study has investigated Pot1 expression status and its association with telomere length in human cancers including GC.

We investigated expression of the *POT1* gene and its relation to telomere length and 3′ telomeric overhang in GC tissues. Moreover, we studied the relation between *POT1* gene expression levels and telomere length in GC cell lines using azidothymidine (AZT) and *POT1* antisense oligonucleotides because AZT causes telomere shortening by inhibiting telomerase activity (19–22). In addition, we investigated alteration of 3′ telomeric overhang signals and the frequency of anaphase bridges in GC cells treated with *POT1* antisense oligonucleotides.

MATERIALS AND METHODS

Samples. Fifty-one pairs of GC tissues and corresponding nonneoplastic mucosae were studied. Specimens were removed surgically, frozen immediately in liquid nitrogen, and stored at −80°C until use. We confirmed microscopically that the carcinoma specimens consisted mainly of carcinoma tissue and that the nonneoplastic mucosae showed no invasion by carcinoma cells or significant inflammatory involvement. Histological classification and tumor staging were done according to the Lauren classification system (23) and tumor-node-metastasis (24) classification systems. From among a total 51 GC cases, we randomly selected 20 cases in which high molecular weight DNA was available to evaluate telomere lengths and 3′ telomeric overhang signals.

GC Cell Lines. Two cell lines derived from human GC were used: MKN-28 and MKN-74 derived from well-differentiated adenocarcinomas and kindly provided by Dr. Toshimitsu Suzuki. Both cell lines were maintained in RPMI 1640 (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 10% fetal bovine serum (Whittaker, Walkersville, MA) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

Quantitative Reverse Transcription (RT)-PCR. Total RNA was extracted with the RNeasy Mini Kit (Qiagen, Valencia, CA). Total RNA (1 μg) was converted to cDNA with the First Strand cDNA Synthesis kit (Amersham Pharmacia Biotech, Uppsala, Sweden). PCRs were performed with the SYBR

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