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 minipreparation. 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.

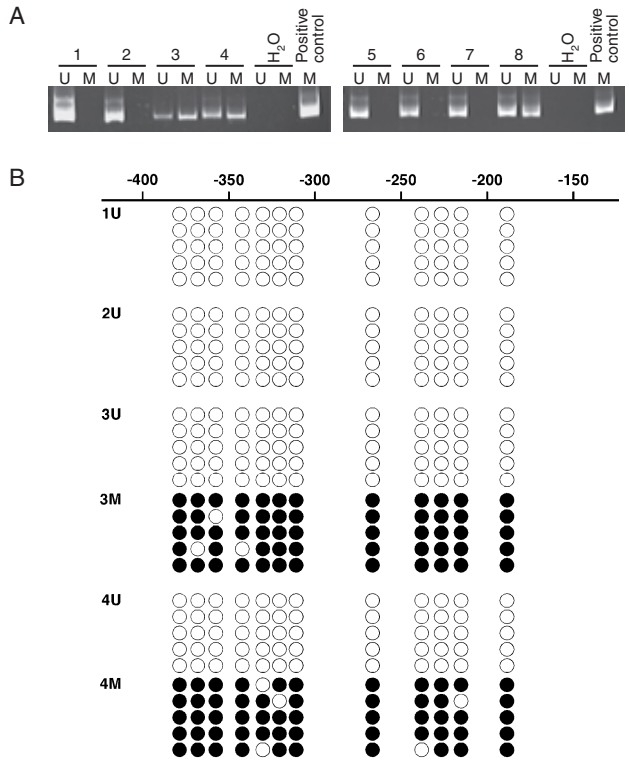

Fig. 1. Methylation status of *HLTF* gene in gastric carcinoma tissues. (A) MSP of *HLTF*. Unmethylated (U), methylated (M). The methylated allele was detected in specimens 3, 4, and 8. (B) Sequencing analysis of 5 cloned MSP products of *HLTF*. Except for primer complementary sequences, *HLTF*-MSP products 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).

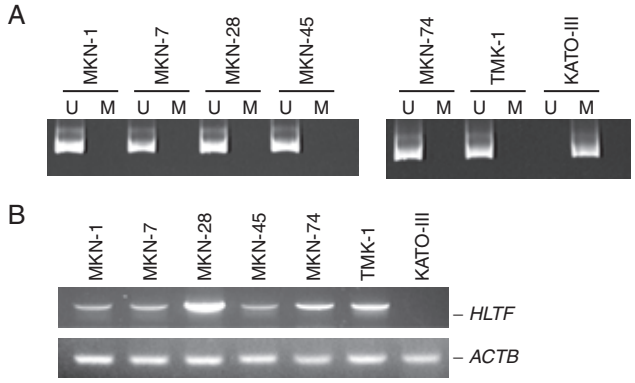

Fig. 2. DNA methylation status and mRNA expression levels of *HLTF* in gastric carcinoma cell lines. (A) MSP of *HLTF*. Unmethylated (U), methylated (M). The methylated allele was detected only in the KATO-III cell line. (B) RT-PCR analysis of gastric carcinoma cell lines. Expression of *HLTF* was absent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

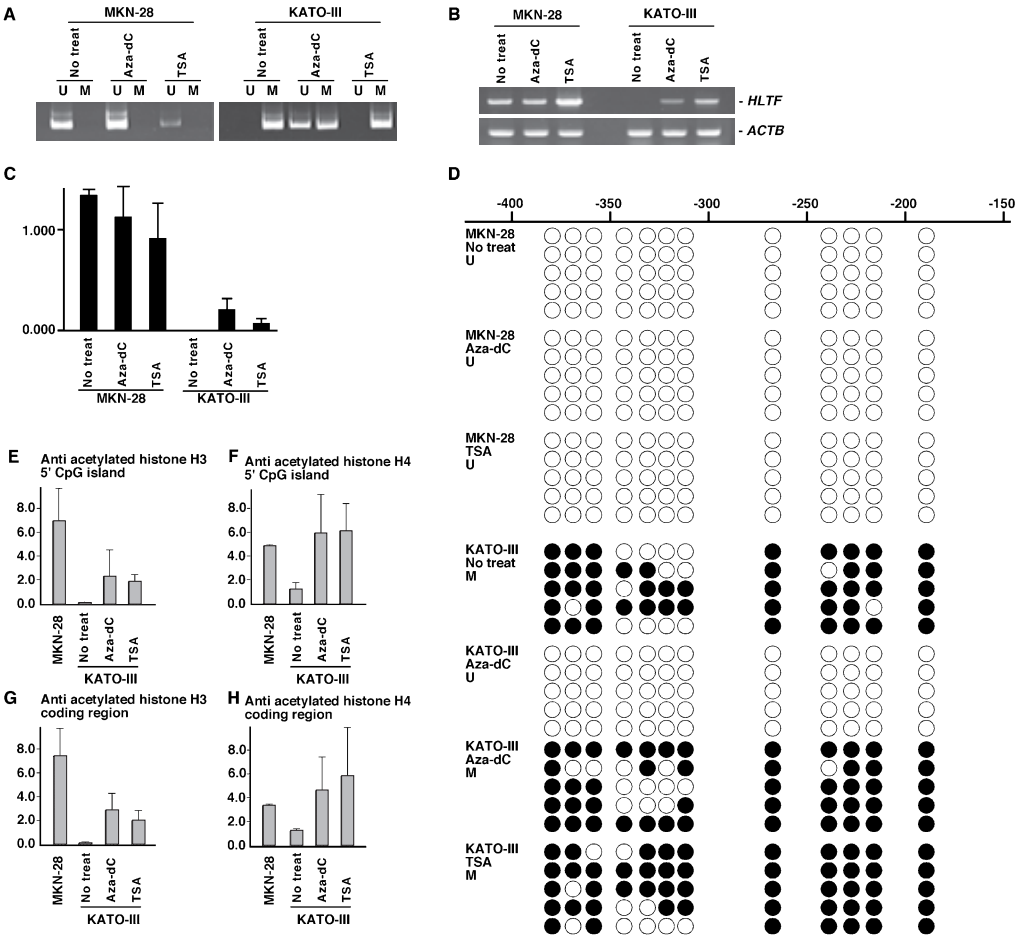

Fig. 3. Effect of Aza-dC and TSA in MKN-28 and KATO-III cells. (A) MSP in Aza-dC-treated and TSA-treated MKN-28 and KATO-III cells. The unmethylated allele was observed only in Aza-dC-treated KATO-III cells. (B) RT-PCR analysis of Aza-dC-treated and TSA-treated MKN-28 and KATO-III cells. Expression of *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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