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Int. J. Cancer: **110,** 212–218 (2004)

Publication of the International Union Against Cancer

FREQUENT EPIGENETIC INACTIVATION OF *RIZ1* BY PROMOTER HYPERMETHYLATION IN HUMAN GASTRIC CARCINOMA

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The retinoblastoma protein-interacting zinc finger gene, *RIZ1* (GenBank accession number U17838), is involved in chromatin-mediated gene expression and is also a target for frameshift mutation in microsatellite-unstable cancers. Methylation of the *RIZ1* promoter CpG island has been shown to be a common mechanism in inactivating the *RIZ1* gene in human liver and breast cancers. We investigated levels of *RIZ1* mRNA in 45 gastric carcinoma tissues by quantitative RT-PCR and in gastric carcinoma cell lines by RT-PCR. In addition, we examined CpG island methylator phenotype (CIMP) status, *p53* mutation status, and the correlation between promoter methylation status and *RIZ1* mRNA expression. CIMP status was investigated by examining the methylation status of *MINT1*, *MINT2*, *MINT12*, *MINT25* and *MINT31*. *p53* mutation status was examined by PCR-single strand conformation polymorphism and promoter methylation status was examined by methylation-specific PCR. Promoter hypermethylation of the *RIZ1* gene was found in 31 (69%) of 45 gastric carcinoma tissues and in 3 (21%) of 14 corresponding non-neoplastic mucosae, the incidence being significantly different ($p = 0.002$). None of the 12 normal gastric tissues from young non-cancer individuals showed hypermethylation. Promoter hypermethylation was associated with reduced *RIZ1* expression in gastric carcinoma tissues ($p = 0.029$). Promoter hypermethylation of the *RIZ1* gene was significantly associated with CIMP ($p = 0.002$). Mutation status of the *p53* gene was not associated with methylation status or *RIZ1* expression in gastric carcinoma. In gastric carcinoma cell lines MKN-28 and KATO-III, the *RIZ1* promoter was hypermethylated and *RIZ1* transcription was inactive. Treatment of these cells with demethylating agent 5-aza-2′-deoxycytidine restored *RIZ1* transcription. Our results suggest that transcriptional inactivation of the *RIZ1* gene by promoter hypermethylation may participate in stomach carcinogenesis.

Key words: *DNA methylation; gastric carcinoma; RIZ1; CIMP; p53*

Epigenetic mechanisms, including DNA methylation and alteration of chromatin structure, are important ways to silence many genes, especially defective tumor suppressor genes, involved in human cancers.[1,2] Recent studies have shown that promoter hypermethylation is a crucial mechanism in transcriptional silencing of tumor suppressor genes in gastric cancer.[3–13] We also showed that DNA methylation occurs for *MGMT*,[14] *p16^{INK4a}*, *RAR-beta*, *CDH1*,[15] *TSP1*,[16] *HLTF*,[17] and *cyclin D2*[18] in gastric carcinomas.

The retinoblastoma protein-interacting zinc finger gene *RIZ* was isolated with a functional screen for Rb-binding protein.[19] Domain analysis suggests that *RIZ1* is a putative methyltransferase. The PR (PRDI-BF1 and RIZ)/SET (Suvar3-9, Enhancer of zeste, Trithorax) domain is involved in chromatin-mediated gene expression[19,20] and plays an important role in human cancers as evidenced by genetic mutations of several family members.[21] The *RIZ* gene produces 2 mRNA and protein products through alternative promoters. *RIZ1* contains the PR domain, but *RIZ2* lacks this domain.[22] The *RIZ* gene is located on human chromosome 1p36, a region frequently deleted in many human cancers, including gastric cancer.[23,24] Expression of *RIZ1* but not *RIZ2* is frequently silenced in many human cancers, including carcinomas of the breast, colon and liver.[25–27] The *RIZ* gene is also a target for frameshift mutations in microsatellite-unstable cancers of the colon, stomach, endometrium and pancreas.[27–29] Missense mutations of *RIZ1* are common in human diffuse large B cell lymphoma but not in other tumors, including gastric carcinoma.[30] *RIZ1* is considered to be a tumor suppressor gene because *RIZ1* can induce G_2-M arrest and apoptosis in breast cancer, liver cancer and microsatellite-unstable colon cancers.[25–27,31] Moreover, a knockout study showed that *RIZ1* is a tumor susceptibility gene in mice.[30] *RIZ1* and *p53* deficiencies are likely to cooperate in tumor formation in mice and are expected to occur in human cancers as well.[30] Many sporadic human cancers carry both *p53* mutation and silenced *RIZ1* gene.[25,30] Recently, methylation of the *RIZ1* promoter CpG island has been shown to be a common mechanism in inactivating the *RIZ1* gene in human liver and breast cancers.[32] Although frameshift mutations of *RIZ* have been found in some microsatellite-unstable gastric cancers,[28,29] little is known about correlation between *RIZ1* expression, and *RIZ1* methylation status, clinicopathological features and *p53* mutation status in gastric carcinoma.

Gastric carcinomas frequently have CpG island methylator phenotype.[33] Another common phenotype after the CpG island methylator phenotype (CIMP) is CIMP in bracelets. These gastric carcinomas, designated for the CIMP-positive, show methylation at more than 3–5 loci (*methylated in tumors* [*MINT*]*1*, *MINT2*, *MINT12*, *MINT25* and *MINT31*). CIMP-positive gastric carcinomas are frequently associated with promoter methylation of *p16^{INK4a}*[33] and *hMLH1*,[34] suggesting that CIMP is an important pathway involved in stomach carcinogenesis. Association between promoter hypermethylation of *RIZ1* and CIMP was found in colon carcinoma,[35] although it remains unclear in gastric carcinoma.

We investigated promoter methylation status and expression levels of the *RIZ1* gene in primary gastric carcinoma tissues as well as corresponding non-neoplastic mucosa and gastric carcinoma cell lines. We also examined promoter methylation status of the *RIZ1* gene in normal gastric mucosa obtained endoscopically from young healthy individuals to investigate whether methylation of the *RIZ1* gene is associated with aging.[1,2,36,37] To determine whether transcriptional silencing of the *RIZ1* gene is caused by promoter hypermethylation, we compared the methylation status

Grant sponsor: Ministry of Education, Culture, Science, Sports, and Technology of Japan; Grant sponsor: Ministry of Health, Labor, and Welfare of Japan.

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Received 17 September 2003; Revised 5 November 2003; Accepted 24 November 2003

DOI 10.1002/ijc.20090
Published online 19 February 2004 in Wiley InterScience (www.interscience.wiley.com).

with mRNA expression levels of the *RIZ1* gene in 8 gastric carcinoma cell lines and 45 gastric carcinoma tissues. We compared methylation status and the expression levels of the *RIZ1* gene to clinicopathological features. We also examined the relation of *RIZ1* methylation status with CIMP status and *p53* mutation status.

MATERIAL AND METHODS

Cell lines

Eight cell lines derived from human gastric carcinomas were used. The TMK-1 cell line was established in our laboratory from a poorly differentiated adenocarcinoma.[38] Five gastric carcinoma cell lines of the MKN series (MKN-1, adenosquamous cell carcinoma; MKN-7; MKN-28; MKN-74, well differentiated adenocarcinoma; and MKN-45, poorly differentiated adenocarcinoma) were kindly provided by Dr. T. Suzuki. KATO-III and HSC-39 cell lines, which were established from signet ring cell carcinomas, were kindly provided by Dr. M. Sekiguchi and Dr. K. Yanagihara, respectively. All cell lines were maintained in RPMI 1640 (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 10% FBS (Whittaker, Walkersville, MA) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

Drug treatment

Cells were treated with a final concentration of 1 μM 5-aza-2′-deoxycytidine (Aza-dC, Sigma Chemical Co., Tokyo, Japan) for 5 days or with 300 nM of Trichostatin A (TSA, Sigma Chemical Co.) for 1 day before cells were harvested for RNA extraction.

Tissue samples

Forty-five gastric carcinoma tissue specimens from 45 patients and 14 corresponding non-neoplastic mucosae were analyzed for promoter methylation of *RIZ1*. Total RNA was available for 45 pairs of gastric carcinomas and corresponding non-neoplastic mucosae to study expression of *RIZ1*. Tumors and corresponding non-neoplastic mucosae were surgically removed, immediately frozen in liquid nitrogen, and stored at −80°C until use. We confirmed microscopically that the tumor tissue specimens consisted mainly of carcinoma tissue and that non-neoplastic mucosa did not exhibit any tumor cell invasion or show significant inflammatory involvement. Histological classification was carried out according to the Lauren classification system.[39] Diffuse-type gastric carcinomas were further classified into diffuse-adherent and diffuse-scattered subtypes.[40] In addition, gastric carcinomas were classified into 2 types: carcinoma with either intestinal or diffuse-type components (pure type) and carcinoma with coexistence of both types of components (mixed type).[41] Tumor staging was carried out according to the TNM stage grouping.[42] In addition, we examined methylation status of the *RIZ1* gene in 12 samples of normal gastric mucosae obtained endoscopically from 12 healthy young individuals (age range = 22–35 years; average = 25.4 years) with no clinical symptoms and no microscopic changes.

Bisulfite PCR and methylation-specific PCR

To examine the DNA methylation patterns of the promoters, genomic DNA was treated with sodium bisulfite as described previously.[43] A total of 2 μg of genomic DNA was denatured by treatment with 2 M NaOH and modified with 3 M sodium bisulfite for 16 hr. DNA samples were purified with Wizard DNA purification resin (Promega, Madison, WI), treated with 3 M NaOH, precipitated with ethanol, and resuspended in 25 μl water. Two-microliter aliquots were used as templates for PCR reactions. For analysis of DNA methylation of the *RIZ1* promoter (Fig. 1*a*), MSP was carried out with primers for *RIZ1* promoters as described previously.[32] For analysis of DNA methylation of *MINT1*, *MINT2*, *MINT12*, *MINT25*, and *MINT31*, we carried out bisulfite-PCR and then restriction digestion as described previously.[33] PCR products (15 μg) were loaded onto 8% nondenaturing polyacrylamide gels, stained with ethidium bromide and visualized under UV light. We considered cases with methylation at more than 3 of 5 loci (*MINT1*, *MINT2*, *MINT12*, *MINT25* and *MINT31*) to be positive for CIMP.[33] The presence or absence of CIMP was determined previously in 39 of 45 gastric carcinoma samples.[34]

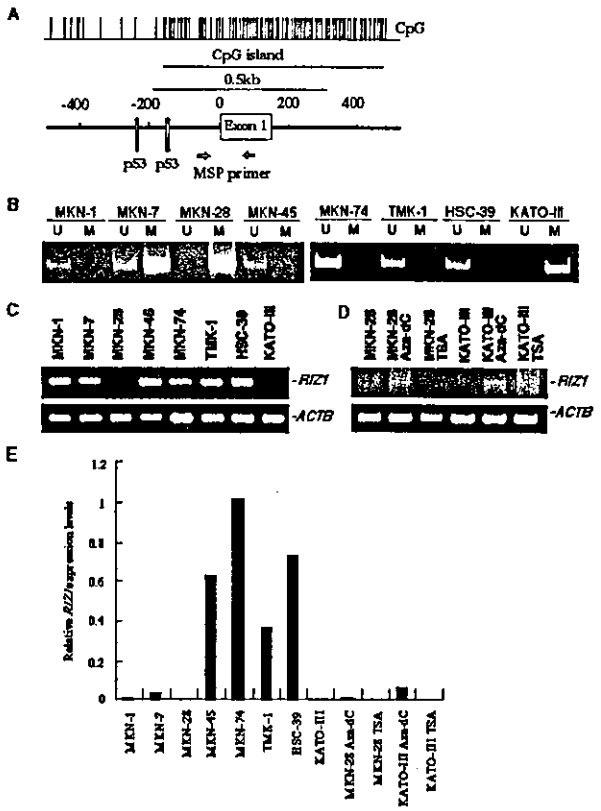

FIGURE 1 – Promoter methylation status and *RIZ1* mRNA expression in gastric carcinoma cell lines. (*a*) Map of the *RIZ1* promoter, depicting the CpG island, MSP primer, and *p53* consensus binding sites. Vertical lines show each CpG site. (*b*) MSP of *RIZ1* in gastric carcinoma cell lines. Primer sets were either unmethylated (U) or methylated (M). The methylated allele was detected in MKN-7, MKN-28, and KATO-III cell lines. (*c*) RT-PCR of gastric carcinoma cell lines. Expression of *RIZ1* was abolished in the MKN-28 and KATO-III cell lines. (*d*) Treatment of MKN-28 and KATO-III cells with the demethylating agent Aza-dC restored *RIZ1* expression. Treatment of these cells with TSA, a histone deacetylase inhibitor, was unable to reactivate *RIZ1* gene expression. (*e*) Quantitative real-time PCR of gastric carcinoma cell lines. The units are arbitrary, and *RIZ1* mRNA expression was calculated by standardization with 1 μg of total RNA from the MKN-74 cell line, which was assigned a *RIZ1* mRNA expression value of 1.0. As shown by RT-PCR analysis, expression of *RIZ1* was abolished in the MKN-28 and KATO-III cell lines. Treatment of MKN-28 and KATO-III cells with Aza-dC, the demethylating agent, restored *RIZ1* expression. Treatment of these cells with TSA, a histone deacetylase inhibitor, was unable to reactivate *RIZ1* gene expression. *RIZ1* expression in gastric carcinoma cell lines harboring *p53* abnormalities tended to be lower than that in gastric carcinoma cell lines harboring wild-type *p53*.

RT-PCR

RIZ1 expression in gastric carcinoma cell lines was analyzed by RT-PCR. Total RNA was extracted by RNeasy Mini Kit (QIAGEN, Tokyo, Japan), and 1 μg of total RNA was converted to cDNA with a first strand cDNA synthesis kit (Amersham Pharmacia Biotech, Uppsala, Sweden). To investigate *RIZ1* mRNA expression, RT-PCR was carried out with primers 5′-TGG CTG CGA TAT GTG AAT TG-3′ (sense) and 5′-CCT CTG AGC AGT CTT CAA GAG T-3′ (antisense). PCR conditions were 35 cycles at 94°C for 2 min, 60°C for 2 min and 72°C for 3 min. The amplification products were then analyzed by 1.5% agarose gel

electrophoresis with ethidium bromide and examined under UV light. *ACTB* -specific PCR products from the same RNA samples were amplified and these products served as internal controls.

Quantitative RT-PCR analysis

Total RNA was isolated with an RNeasy Mini Kit (QIAGEN, Tokyo, Japan) and 1 μg of total RNA was converted to cDNA with a first strand cDNA synthesis kit (Amersham Pharmacia Biotech, Uppsala, Sweden). PCRs were carried out with the SYBR Green PCR Core Reagents kit (Applied Biosystems, Tokyo, Japan). Real-time detection of the emission intensity of SYBR Green bound to double-stranded DNAs was carried out with the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Initial template concentration was derived from the cycle number at which the fluorescent signal crossed a threshold in the exponential phase of the PCR reaction. Relative gene expression was determined by the threshold cycles for the *RIZ1* gene and the *ACTB* gene (internal control). Reference samples (gastric carcinoma cell line MKN-74) were included on each assay plate to verify plate-to-plate consistency. Plates were normalized to each other with these reference samples. PCR amplification was carried out with a 96-well optical tray and caps in a final reaction mixture volume of 25 μl, according to the manufacturer's instructions. *RIZ1* primer sequences were 5′-ATT GAT GCC ACT GAT CCA GAG A-3′ (sense) and 5′-GCT CTG TTG ATT TCC AGT GGG A-3′ (antisense). *ACTB* primer sequences were 5′-TCA CCG AGC GCG GCT-3′ (sense) and 5′-TAA TGT CAC GCA CGA TTT CCC-3′ (antisense).

p53 *mutation analysis*

Exons 5–8 of the *p53* gene were examined for mutation by PCR single-strand conformation polymorphism (SSCP) analysis. Genomic DNA was PCR-amplified with 10 sets of primers. Primers for exon 5a were 5′-TGC CCT GAC TTT CAA CTC TGT-3′ (sense) and 5′-CAT GTG CTG TGA CTG CTT GTA-3′ (antisense). Primers for exon 5b were 5′-CTG TGC AGC TGT GGG TTG ATT-3′ (sense) and 5′-GCA ACC AGC CCT GTC GTC TCT-3′ (antisense). Primers for exon 6 were 5′-CAC TGA TTG CTC TTA GGT-3′ (sense) and 5′-AGT TGC AAA CCA GAC CTC-3′ (antisense). Primers for exon 7 were 5′-TAG GTT GGC TCT GAC TGT ACC-3′ (sense) and 5′-TGA CCT GGA GTC TTC CAG TGT-3′ (antisense). Primers for exon 8 were 5′-AGT GGT AAT CTA CTG GGA CGG-3′ (sense) and 5′-ACC TCG CTT AGT GCT CCC TG-3′ (antisense). PCR conditions for exon 5a and exon 6 were 35 cycles at 95°C for 30 sec, 58°C for 30 sec and 72°C for 30 sec. PCR conditions for exon 5b were 45 cycles at 95°C for 1 min and 62°C for 1 min. PCR conditions for exons 7 and 8 were 35 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec. PCR products were diluted 10-fold with formamide dye solution, denatured at 85°C for 10 min, and electrophoresed on 6% polyacrylamide gels. Gels were stained and visualized with a Silver Staining II kit (WAKO, Osaka, Japan). The presence or absence of *p53* mutation was determined previously in 30 of 45 gastric carcinoma samples.[16]

Statistical methods

Statistical analyses were carried out with Fisher's exact test and the Mann-Whitney *U* -test; *p* -values <0.05 were regarded as statistically significant.

RESULTS

Promoter methylation status and expression of RIZ1 *in gastric carcinoma cell lines*

To study whether promoter hypermethylation of *RIZ1* induces transcriptional inactivation, we carried out an *in vitro* analysis using 8 gastric carcinoma cell lines. As shown in Figure 1*b*, MSP showed promoter hypermethylation of *RIZ1* in MKN-7, MKN-28 and KATO-III cells and no promoter hypermethylation of *RIZ1* in MKN-1, MKN-45, MKN-74, TMK-1 and HSC-39 cells. In MKN-7 cells, *RIZ1* promoter was considered to be partially methylated, as described previously[32] because both methylated and unmethylated PCR products were detected. To investigate the relation between promoter methylation status and *RIZ1* expression levels, we carried out RT-PCR and quantitative RT-PCR analyses. Transcriptional inactivation was observed in MKN-28 and KATO-III cells with promoter hypermethylation (Fig. 1*c*,*e*). The other 6 cell lines expressed *RIZ1* as detected by RT-PCR analysis (Fig. 1*c*,*e*). To investigate whether methylation is responsible for transcriptional inactivation of *RIZ1*, we treated MKN-28 and KATO-III cells with Aza-dC, a demethylating agent, and carried out RT-PCR analysis. *RIZ1* mRNA expression appeared in these cells after 5 days of Aza-dC treatment (Fig. 1*d*,*e*). Treatment of these cells over 1 day with 300 nM TSA, a histone deacetylase inhibitor, was unable to reactivate *RIZ1* gene expression (Fig. 1*d*,*e*).

mRNA expression levels of RIZ1 *in gastric carcinoma*

We measured the levels of *RIZ1* mRNA expression using quantitative RT-PCR in 45 pairs of gastric carcinoma tissues and their corresponding non-neoplastic mucosae. The overall results of quantitative RT-PCR analysis are summarized in Table I. No association was found by Mann-Whitney *U* -test between mRNA levels of *RIZ1* in the tumor tissues and age ($p = 0.795$), gender ($p = 0.785$), T grade (depth of invasion, $p = 0.315$), N grade (lymph node metastasis, $p = 0.409$) or tumor stage ($p = 0.223$) (data not shown). No association was found between mRNA levels of *RIZ1* in the tumor tissues and histological classification (data not shown).

RIZ1 *promoter methylation status and mRNA expression levels in gastric carcinoma*

Methylation status of the *RIZ1* promoter was examined in a total of 45 gastric carcinoma tissue specimens from 45 patients and 14 corresponding non-neoplastic mucosae. Promoter hypermethylation of the *RIZ1* gene was found in 31 (69%) of 45 gastric carcinoma tissues and in 3 (21%) of 14 corresponding non-neoplastic mucosae. The incidence of hypermethylation between the carcinomas and the non-neoplastic mucosae were significantly different ($p = 0.002$, Fisher's exact test). Representative results of MSP for *RIZ1* are shown in Figure 2*a*,*b* and the overall results of MSP in the tumors are summarized in Tables I and II. No association was detected by Fisher's exact test between methylation status of *RIZ1* and age ($p = 0.143$), gender ($p = 0.492$), T grade ($p = 0.885$), N grade ($p = 0.497$), tumor stage ($p = 0.478$) or pure and mixed types of histological classification ($p = 0.520$) (Table II). When we further classified pure type gastric carcinomas into diffuse-scattered type and intestinal and diffuse-adherent type, however, methylation of the *RIZ1* gene was found more frequently in intestinal and diffuse-adherent type than in diffuse-scattered type ($p = 0.047$, Fisher's exact test) (Table II). To determine whether transcriptional silencing of the *RIZ1* gene is caused by promoter hypermethylation, we compared the methylation status with mRNA expression of the *RIZ1* gene. As shown in Tables I and III, levels of *RIZ1* mRNA in tumor tissues with *RIZ1* hypermethylation (0.13 ± 0.04, mean ± SE) were significantly lower than those in tumor tissues without promoter hypermethylation (0.27 ± 0.10; $p = 0.029$, Mann-Whitney *U* -test) and those in corresponding non-neoplastic mucosae (0.50 ± 0.14; $p = 0.0009$, Mann-Whitney *U* -test). Levels of *RIZ1* mRNA in tumor tissues without *RIZ1* hypermethylation did not differ significantly from those in corresponding non-neoplastic mucosa ($p = 0.557$, Mann-Whitney *U* -test).

Methylation status of RIZ1 *in normal gastric mucosa from non-cancerous healthy individuals*

We examined methylation status of the *RIZ1* gene in a total of 12 normal gastric mucosae obtained endoscopically from non-cancerous individuals (age = 22–35 years; average, 25.4 years). Hypermethylation of the *RIZ1* gene was not found in any of these samples (Fig. 2*c*).

TABLE I - MRNA EXPRESSION LEVELS AND METHYLATION STATUS OF *RIZ1* IN GASTRIC CARCINOMAS

Methylation status in tumor tissue	*RIZ1* mRNA expression level in tumor tissue[1]	*RIZ1* mRNA expression level in non-neoplastic mucosa[1]	CIMP status	*p53* mutation status	Gender	Age	Stage[2]	T grade[3]	N grade[4]	Histology[5]
Unmethylated	1.365	0.053	Negative	Wild-type	F	76	IA	1	0	Intestinal
	0.570	1.173	Negative	Wild-type	M	41	IB	2b	0	Diffuse
	0.122	0.097	Negative	Wild-type	M	79	II	2b	1	Intestinal
	0.162	0.403	Negative	Wild-type	F	75	II	2b	1	Diffuse
	0.084	0.045	Negative	Wild-type	M	51	II	3	0	Intestinal
	0.036	0.077	Negative	Wild-type	F	66	IIIA	3	1	Diffuse
	0.079	0.013	Negative	Wild-type	M	50	IIIB	3	3	Diffuse
	0.096	5.426	Negative	Wild-type	M	34	IV	4	2	Intestinal
	0.747	0.271	Negative	Wild-type	M	66	IV	3	3	Intestinal
	0.093	0.097	Negative	Wild-type	F	74	IV	4	2	Diffuse
	0.183	1.890	Negative	Mutant-type	F	74	IB	2a	0	Diffuse
	0.128	0.086	Negative	Mutant-type	F	86	IB	2b	0	Intestinal
	0.032	0.036	Negative	Mutant-type	M	64	IV	3	3	Diffuse
	0.127	2.694	Positive	Mutant-type	M	59	IV	3	2	Intestinal
Methylated	0.002	0.019	Negative	Wild-type	M	75	IB	2b	0	Intestinal
	0.046	2.231	Negative	Wild-type	M	74	IB	2a	0	Intestinal
	0.008	0.025	Negative	Wild-type	M	73	IB	2a	0	Intestinal
	0.050	0.266	Negative	Wild-type	M	62	II	2b	1	Diffuse
	0.051	0.078	Negative	Wild-type	F	67	II	3	0	Diffuse
	0.147	0.075	Negative	Wild-type	F	64	IIIA	3	1	Diffuse
	0.064	0.102	Negative	Wild-type	M	55	IV	2b	3	Diffuse
	0.012	0.238	Negative	Wild-type	F	65	IV	3	3	Diffuse
	0.061	0.203	Negative	Mutant-type	M	85	IB	2b	0	Intestinal
	0.012	0.153	Negative	Mutant-type	M	72	II	3	0	Intestinal
	0.158	0.659	Negative	Mutant-type	F	46	IIIA	2b	2	Diffuse
	0.068	0.503	Negative	Mutant-type	M	57	IIIA	2b	2	Intestinal
	0.066	0.038	Negative	Mutant-type	F	75	IIIA	3	1	Intestinal
	1.246	0.139	Negative	Mutant-type	M	70	IIIB	3	2	Intestinal
	0.055	0.156	Positive	Wild-type	F	67	IB	2b	0	Intestinal
	0.002	0.602	Positive	Wild-type	M	57	II	2b	1	Diffuse
	0.032	0.082	Positive	Wild-type	F	81	II	2b	1	Diffuse
	0.659	0.152	Positive	Wild-type	M	62	IIIA	3	1	Intestinal
	0.176	0.158	Positive	Wild-type	M	69	IIIA	4	1	Diffuse
	0.004	0.011	Positive	Wild-type	M	81	IIIA	2b	2	Diffuse
	0.099	0.056	Positive	Wild-type	M	85	IIIA	3	1	Diffuse
	0.093	1.514	Positive	Wild-type	M	58	IIIB	3	2	Intestinal
	0.097	0.405	Positive	Wild-type	F	61	IIIB	3	2	Intestinal
	0.131	0.262	Positive	Wild-type	M	70	IIIB	3	2	Diffuse
	0.029	0.370	Positive	Wild-type	M	69	IV	3	3	Intestinal
	0.081	0.153	Positive	Wild-type	M	69	IV	4	2	Diffuse
	0.238	0.535	Positive	Wild-type	M	72	IV	3	3	Intestinal
	0.064	0.114	Positive	Mutant-type	M	61	IA	1	0	Intestinal
	0.100	0.135	Positive	Mutant-type	F	73	II	2b	1	Intestinal
	0.010	0.594	Positive	Mutant-type	F	67	IIIB	3	2	Diffuse
	0.275	0.143	Positive	Mutant-type	M	75	IV	4	3	Intestinal

[1]The units are arbitrary, and we calculated the *RIZ1* mRNA expression in tumor tissues and corresponding non-neoplastic mucosa by standardization with 1 μg of the MKN-74 gastric carcinoma cells, taken as 1.0. – [2]Stage was classified according to the criteria of the UICC TNM stage Grouping 6th edition, 2002, Stomach. – [3]T grade was classified according to the criteria of the UICC TNM stage Grouping 6th edition, 2002, Stomach. – [4]N grade was classified according to the criteria of the UICC TNM stage Grouping 6th edition, 2002, Stomach. – [5]Histology was classified according to the criteria of Lauren.

Association of RIZ1 *promoter hypermethylation with CIMP*

We analyzed CIMP by examining the methylation status of *MINT1*, *MINT2*, *MINT12*, *MINT25* and *MINT31* in 45 gastric carcinoma tissues. CpG island hypermethylation of the *MINT* loci was detected in 22 (49%) tissues for *MINT1*, 19 (42%) for *MINT2*, 21 (47%) for *MINT12*, 33 (73%) for *MINT25*, and 12 (27%) for *MINT31*. In total, 18 (40%) tissues were regarded as CIMP-positive (Table I). We then compared the promoter methylation status of the *RIZ1* gene with CIMP status. A significant association was found between the presence of CIMP and *RIZ1* promoter hypermethylation ($p = 0.002$, Fisher's exact test) (Table II).

Correlation between p53 *mutation status, methylation status and mRNA levels of the* RIZ1 *gene in gastric carcinoma*

To examine whether a correlation exists between *p53* mutation status and methylation status or mRNA level of the *RIZ1* gene, we examined *p53* mutation status of 45 gastric carcinoma tissues by PCR-SSCP analysis (Fig. 2*d*). In gastric carcinoma tissues, mutations in *p53* were observed in 14 (31%) tissues (Fig. 2*d*). No correlation was found between *p53* mutation status and methylation status of the *RIZ1* gene ($p = 0.805$, Fisher's exact test) (Tables I, II). Mutation status of *p53* also did not correlate with *RIZ1* mRNA levels in gastric carcinoma tissues (*RIZ1* mRNA expression level: wild-type *p53*, 0.18 ± 0.05 (mean ± SE); mutant *p53*, 0.18 ± 0.08; $p = 0.607$, Mann-Whitney U - test) (Tables I, III). *p53* is reported to be mutant in MKN-1, MKN-7, MKN-28, TMK-1 and HSC-39, wild-type in MKN-45 and MKN-74, and completely deleted in KATO-III.[44] Methylation of the *RIZ1* gene and abnormalities in *p53* coexisted in MKN-7, MKN-28 and KATO-III. Moreover, *RIZ1* mRNA levels in *p53* wild-type cell lines were higher than those in *p53* mutant cell lines except TMK-1 and HSC-39 (Fig. 1*e*).

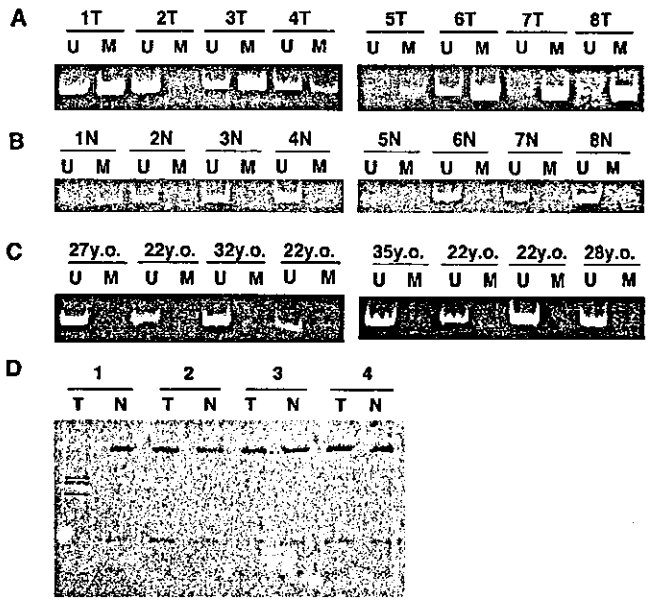

FIGURE 2 – MSP analysis of the *RIZ1* promoter and *p53* mutation analysis of gastric tissues. U, unmethylated PCR product; M, methylated PCR product. (*a*) Methylation status of *RIZ1* in gastric carcinoma tissues. The methylated allele was detected in 1T, 3T, 4T, 5T, 6T, 7T and 8T. (*b*) Methylation status of *RIZ1* in corresponding non-neoplastic mucosae. The methylated allele was detected in 1N. (*c*) Methylation status of *RIZ1* in normal gastric mucosae obtained from young healthy individuals (age = 22–35 years; average = 25.4 years). Promoter methylation of the *RIZ1* gene was not detected in any sample. (*d*) Mutation analysis of *p53* by SSCP in gastric carcinoma tissues. Mutation of *p53* exon 8 was observed in 1T.

TABLE II – ASSOCIATION BETWEEN *RIZ1* METHYLATION STATUS AND CLINICOPATHOLOGICAL FEATURES, CIMP STATUS AND P53 MUTATION STATUS IN GASTRIC CARCINOMAS

Feature	*RIZ1* methylation status		*p*-value[1]
	Positive	Negative	
Gender			
Male	21	8	0.492
Female	10	6	
Age			
<60	5	5	0.143
>61	26	9	
T grade			
1, 2	14	6	0.885
3, 4	17	8	
N			
N0	8	5	0.497
N1, N2, N3	23	9	
Stage			
I, II	12	7	0.478
III, IV	19	7	
Histology			
Intestinal	17	7	0.763
Diffuse	14	7	
Histology			
Mixed type	7	2	0.520
Pure type	24	12	
Intestinal & Diffuse-adherent type	21	7	0.047
Diffuse-scattered type	3	5	
CIMP			
Positive	17	1	0.002
Negative	14	13	
p53 mutation			
Positive	10	4	0.805
Negative	21	10	

[1]Fisher's exact test.

TABLE III – ASSOCIATION BETWEEN *RIZ1* MRNA EXPRESSION AND METHYLATION STATUS AND *P53* MUTATION STATUS IN GASTRIC CARCINOMAS

	RIZ1 expression levels[1]
Non-neoplastic mucosa (n = 45)	$0.50 \pm 0.14^{2,3}$
Tumor tissue without promoter hypermethylation (n = 14)	$0.27 \pm 0.10^{2,4}$
Tumor tissue with promoter hypermethylation (n = 31)	$0.13 \pm 0.04^{3,4}$
Tumor tissues with wild-type *p53* (n = 31)	0.18 ± 0.05^{5}
Tumor tissue with mutant-type *p53* (n = 14)	0.18 ± 0.08^{5}

[1]Mean ± SE.–[2]$p = 0.557$.–[3]$p = 0.0009$.–[4]$p = 0.029$.–[5]$p = 0.607$.

DISCUSSION

DNA methylation and alteration of chromatin structure are important ways of transcriptionally silencing many genes. In our present study, promoter hypermethylation of the *RIZ1* gene was detected in 3 cell lines (MKN-7, MKN-28 and KATO-III), which expressed undetectable levels of the *RIZ1* gene product except MKN-7 with partially methylated *RIZ1* promoter. Moreover, treatment of *RIZ1* mRNA-negative cells (MKN-28, KATO-III) with Aza-dC led to a reactivation of *RIZ1* expression. These results suggest that hypermethylation of the *RIZ1* promoter region plays an important role in transcriptional silencing of *RIZ1* in gastric carcinomas. In support of this conclusion, we found that the *RIZ1* gene was frequently targeted for methylation and silencing in gastric carcinoma tissues and that promoter hypermethylation of the *RIZ1* gene was associated with reduced expression. We found several tumor samples, however, with *RIZ1* gene hypermethylation that did not have low *RIZ1* gene expression. This result may be related to the extreme sensitivity of MSP, which can theoretically detect as little as 0.1% methylated cells.[43] Alternatively, a tumor may exhibit heterogeneity in *RIZ1* methylation. In this case, partial methylation of the *RIZ1* promoter region is likely to reduce the level of transcriptional repression. In contrast, several samples show low levels of *RIZ1* gene expression in the absence of *RIZ1* promoter methylation. Alternative inactivating pathways, such as hemizygous deletion and alteration of transcription factors, may account for low level of *RIZ1* gene expression.

The incidence of *RIZ1* gene promoter methylation was significantly different between gastric tumors and non-neoplastic mucosae; the *RIZ1* gene promoter was hypermethylated in 31 (69%) of 45 gastric carcinoma samples and in 3 (21%) of 14 corresponding non-neoplastic mucosa samples. This result suggests that methylation of the *RIZ1* gene promoter may contribute to gastric carcinogenesis. Methylation of the *RIZ1* gene also has been reported in non-malignant liver tissues.[32] Epigenetic changes including DNA methylation occur in premalignant and histologically normal gastric epithelium.[36,45,46] Furthermore, recent evidence suggests that methylation of certain genes such as E-cadherin and $p16^{INK4a}$ is associated with aging.[1,2,36,37] We confirmed that normal gastric mucosa from young healthy individuals was not hypermethylated in the *RIZ1* promoter region. Therefore, the aging mechanism can partly explain detection of methylation in non-neoplastic mucosae. Age-related methylation of the *RIZ1* gene may also explain the possible link between aging and increased risk for gastric cancer.

In addition to classifying tumors with the Lauren system, recent studies show the importance of histologically distinguishing between gastric tumors that are pure type or mixed type because patients with mixed type gastric tumors tend to have worse outcomes than those with pure types.[41] In our present study, methylation status of the *RIZ1* gene was not associated with histological classification by pure or mixed type. In the pure type of gastric carcinoma, however, methylation of the *RIZ1* gene was found more frequently in intestinal and diffuse-adherent types of carcinomas than in the diffuse-scattered type of carcinoma. Methylation of the *RIZ1* gene may primarily contribute to the intestinal and

diffuse-adherent types of gastric carcinomas. We also reported that methylation of $p16^{INK4a}$ and CIMP occurs more frequently in intestinal and diffuse-adherent types of gastric carcinomas than in the diffuse-scattered type of gastric carcinoma[34] and that methylation of *RAR-beta* and *CDH1* preferentially occurs in the diffuse-scattered type of gastric carcinoma.[15] Our findings suggest that promoter methylation of these genes must play a role in the morphogenesis of gastric carcinomas.

In our present study, a significant association was detected between the presence of CIMP and hypermethylation of the *RIZ1* promoter in gastric carcinomas. This result is consistent with that for colon cancer,[35] indicating that methylation of the *RIZ1* gene is not a random event in gastric carcinogenesis. CIMP is considered to lead to cancer formation and progression through the silencing of multiple tumor suppressor genes.[33] We suggest that the *RIZ1* gene is a likely target gene associated with CIMP. Because CIMP is associated with inactivation of $p16^{INK4a}$[33] and *hMLH1* ,[34] unbridled cell cycle progression and genetic instability is likely to be involved in carcinogenesis of CIMP-positive carcinomas. Genetic instability may cause mutations of target genes such as *Bax*, *TGF-beta receptor type II*, and *RIZ1*, and loss of function of these genes is believed to contribute to carcinogenesis.[28,29,47] Microsatellite instability has been found in only 9–12% of gastric carcinomas,[33,48] and frameshift mutation of RIZ has been found in 36–48 % of microsatellite-unstable gastric carcinomas[28,29] but not in microsatellite-stable gastric carcinomas.[28] Although we did not examine frameshift mutations of *RIZ*, this mutation must be infrequent (5% or less) in gastric carcinoma. Taken together, these findings suggest that hypermethylation is the major mechanism of inactivation of the *RIZ1* gene in gastric carcinomas, especially in CIMP-positive carcinomas.

Abnormalities in *p53* and methylation-mediated silencing of the *RIZ1* gene coexist in MKN-28 and KATO-III, as described previously in other carcinoma cell lines.[25,30] No correlation was observed between *p53* mutation status and *RIZ1* methylation status in gastric carcinoma tissues. Molecular mechanisms underlying *RIZ1* and *p53* cooperation in tumor formation remain to be clarified.[30] Tumor suppressor p53 is a sequence-specific DNA-binding protein, and its biological effects are mediated by transactivation of various target genes.[49] A potential *p53* binding site exists within the promoter of *RIZ1* (Fig. 1*a*). Therefore, it is possible that *p53* directly binds to this potential binding site and activates *RIZ1* expression. In our present study, *RIZ1* mRNA levels were relatively high in MKN-45 and MKN-74, which both harbor wild-type *p53*, and relatively low in MKN-1, MKN-7 and MKN-28, all of which harbor mutant-type *p53* , and in KATO-III, with completely deleted *p53*. *RIZ1* mRNA levels in TMK-1 and HSC-39, both harboring mutant-type *p53* , were also relatively high. Moreover, there was no correlation between *p53* mutation status and *RIZ1* expression in gastric carcinoma tissues. Although *MGMT* and *TSP1* are regulated by promoter hypermethylation and *p53* , *p53* mutation status was not associated with methylation status or expression of these genes in gastric carcinoma.[14,16] Therefore, *RIZ1* is a likely *p53* -target gene, and further investigation is needed.

In conclusion, our results suggest that transcriptional inactivation of *RIZ1* by promoter hypermethylation associated with CIMP participates in the development of gastric carcinoma, especially of the intestinal and diffuse-adherent types.

ACKNOWLEDGEMENTS

We thank M. Takatani for excellent technical assistance and advice. This work was carried out with the kind cooperation of the Research Center for Molecular Medicine, Faculty of Medicine, Hiroshima University.

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Int. J. Cancer: 112, 1003–1009 (2004)

Publication of the International Union Against Cancer

EPIGENETIC INACTIVATION OF *SOCS-1* BY CpG ISLAND HYPERMETHYLATION IN HUMAN GASTRIC CARCINOMA

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***Suppressor of cytokine signaling (SOCS)-1* inhibits signaling of the Janus kinase (JAK)/signal transducers and activators of transcription (STAT) pathway by several cytokines and has tumor suppressor activity. Methylation of the *SOCS-1* CpG island has been shown to inactivate the *SOCS-1* gene in certain human cancers. In our study, we investigated methylation status of the *SOCS-1* gene by methylation-specific PCR in 75 gastric carcinoma (GC) tissues, 25 corresponding nonneoplastic mucosae and 10 normal gastric mucosae from healthy young individuals. We also performed bisulfite sequencing of DNAs from 2 GC tissues. In addition, *SOCS-1* mRNA levels were examined in 50 GCs by quantitative RT-PCR. Hypermethylation of the *SOCS-1* gene was detected in 33 (44%) of 75 GC tissues and in 3 (12%) of 25 corresponding nonneoplastic mucosae; the incidence was significantly different ($p = 0.004$). None of the 10 normal gastric tissues from healthy individuals showed hypermethylation. Methylation of the *SOCS-1* gene was associated with lymph node metastasis, advanced tumor stage and reduced expression of *SOCS-1* in GC tissues ($p = 0.009$, 0.034 and 0.002, respectively). Reduced expression of *SOCS-1* in GC tissues was associated with lymph node metastasis and advanced tumor stage ($p = 0.013$ and 0.002, respectively). Our results suggest that transcriptional inactivation of the *SOCS-1* gene by hypermethylation may be involved in development, progression and metastasis of GC.**

Key words: *DNA methylation; gastric carcinoma; SOCS-1; JAK/STAT*

A variety of genetic and epigenetic alterations are associated with gastric cancer (GC).[1] Epigenetic mechanisms, such as DNA methylation of CpG islands, are common changes in human cancers. Hypermethylation of CpG islands is associated with silencing of many genes, especially defective tumor suppressor genes (TSGs), and has been proposed as a means of inactivating TSGs in cancer.[2,3] Identification of genes subject to DNA hypermethylation may provide insights into gastric carcinogenesis. Moreover, methylated genes may serve as targets for cancer diagnosis and therapies. Recent studies have shown that DNA hypermethylation is a crucial mechanism in transcriptional silencing of TSGs in GC.[4–13] We found that *MGMT, p16^{INK4a}, RAR-beta, CDH1, TSP1, HLTF, RUNX3* and *RIZ1* are methylated in GC.[14–19] Methylation of TSGs occurs in the early stages of carcinogensis and tends to accumulate along the multiple pathways of gastric carcinogenesis.[20] Therefore, methylation of most TSGs is thought to be involved in gastric carcinogenesis; however, methylation of only a limited number of genes is associated with progression of GC.[9,21–23]

Cytokines are secreted proteins that regulate cellular proliferation and differentiation. The stimuli of these mediators leads mainly to the transcriptional activation of cytokine-induced genes through the Janus kinase (JAK)/signal transducers and activators of transcription (STAT) pathway.[24] Recent studies have indicated that the JAK/STAT pathway is involved in development of several types of tumors.[25–27] The suppressor of cytokine signaling (SOCS) proteins are negative regulators of several cytokine pathways, particularly the JAK/STAT pathway. These proteins are characterized by a central src homology (SH2) domain and a conserved carboxyl-terminal domain called the SOCS box. The SOCS family comprises at least 9 proteins, SOCS-1 to SOCS-8 and CIS (cytokine-inducible SH2-containing protein).[28–30] Through 3 independent approaches, SOCS-1 protein was identified as a protein involved in a negative feedback loop for cytokine signaling.[31–33] SOCS-1 interacts directly with active JAKs by binding to their activation loop in a phosphorylation manner. Blockade of JAK activation results in downregulation of the JAK/STAT pathway.[32] SOCS-1 suppresses cellular responses to various cytokines, including interleukin (IL)-6, IL-4, leukemia inhibitory factor, oncostatin M, interferon-gamma, thrombopoietin and growth hormone.[30] Recently, it was reported that the *SOCS-1* gene is silenced by CpG island methylation in human hepatocellular carcinoma,[34] hepatoblastoma,[35] multiple myeloma,[36] acute myeloid lymphoma[37] and pancreatic ductal neoplasm.[38] Inactivation of *SOCS-1* by methylation results in constitutive activation of the JAK/STAT pathway and activation of target genes.[34] *SOCS-1* is considered a TSG because restoration of *SOCS-1* expression can suppress growth rate and anchorage-independent growth of cells with methylation-silenced *SOCS-1* and constitutively activated JAK2.[34] Moreover, ectopic expression of *SOCS-1* abolishes proliferation mediated by a constitutively active form of the KIT receptor *TEL-JAK2* and *v-ABL*, and it reduces metastasis of *BCR-ABL* transformed cells.[39] However, little is known about the correlation between *SOCS-1* expression, *SOCS-1* methylation status and clinicopathologic features of GC.

In our study, we investigated methylation status and expression of the *SOCS-1* gene in primary gastric carcinoma tissues and in corresponding nonneoplastic mucosa. To determine whether transcriptional inactivation of the *SOCS-1* gene is caused by hypermethylation, we compared methylation status with levels of *SOCS-1* mRNA in 50 gastric carcinomas. We also analyzed the relation between methylation status, expression of *SOCS-1* and clinicopathologic features of GC.

MATERIAL AND METHODS

Tissue samples

Seventy-five gastric tumor tissue specimens from 75 patients were analyzed for methylation of the *SOCS-1* gene. Twenty-five corresponding nonneoplastic mucosae were also analyzed. Total

Grant sponsor: Ministry of Education, Culture, Science, Sports, and Technology of Japan; Grant sponsor: Ministry of Health, Labor, and Welfare of Japan.

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Received 14 December 2003; Accepted after revision 26 May 2004

DOI 10.1002/ijc.20521
Published online 18 August 2004 in Wiley InterScience (www.interscience.wiley.com).

RNA was available for 50 tumor tissues and corresponding nonneoplastic mucosae to study expression of *SOCS-1*. Tumors and corresponding nonneoplastic 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 of carcinoma tissue and that nonneoplastic mucosae did not exhibit any tumor cell invasion or significant inflammatory involvement. Histologic classification was performed according to the Lauren classification system.[40] Diffuse-type gastric carcinomas were further classified into diffuse-adherent and diffuse-scattered subtypes.[41] Tumor staging was performed according to the TNM staging system.[42] In addition, we examined methylation status of the *SOCS-1* gene in 10 samples of normal gastric mucosae obtained endoscopically from 10 young healthy individuals (age range 22–35 years; average 26.4 years) with no clinical symptoms and no microscopic changes.

Methylation analysis

To examine DNA methylation patterns, genomic DNA was treated with sodium bisulfite, as described previously.[43] In brief, 2 μg of genomic DNA was denatured by treatment with 2 M NaOH and modified with 3 M sodium bisulfite for 16 hr. DNA samples were purified with Wizard DNA Purification Resin (Promega, Madison, WI), treated with 3 M NaOH, precipitated with ethanol and resuspended in 25 μl water. Two-microliter aliquots were used as templates for PCR. The *SOCS-1* gene lies within a CpG island spanning 2.5 kb and consists of 2 exons. Exon 1 of the *SOCS-1* gene is untranslated (Fig. 1). Methylation-specific PCR (MSP) was performed with 2 primer sets, MSP3′ and MSP5′, which amplify the CpG island, as described previously.[34,35] These primers are located 5′ (MSP5′) and 3′ (MSP3′) of the translation start site of the *SOCS-1* gene (Fig. 1). We further analyzed methylation status of the *SOCS-1* gene in 2 GC tissues by bisulfite sequencing with 3 sets of primers (Fig. 1). Region 1 and region 2 are located 5′ of the translation start site; region 3 is located 3′ of the translation start site and was studied previously (Fig. 1).[34] Primers for region 1 were 5′-GAG GAG GGA GGG GAG TTT AGG GTA GTT-3′ (sense) and 5′-TTC AAC CTC AAT AAA CAC AAC TAA AAA A-3′ (antisense). Primers for region 2 were 5′-TTT TTT AGT TGT GTT TAT TGA GGT TGA A-3′ (sense) and 5′-CCA CCT AAT TAT ATA CTA CCA TCC TAC AA-3′ (antisense). Primers for region 3 were 5′-TGT AGG ATG GTA GTA TAT AAT TAG GTG GT-3′ (sense) and 5′-TAA TAC TCC AAC AAC TCT AAA AAA CAA TC-3′ (antisense).[34] PCR products were purified and cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA). Cloned PCR fragments were sequenced with the M13 forward primer and the PRISM AmpliTaq DNA polymerase FS Ready Reaction Dye Terminator Sequencing Kit (Applied Biosystems, Tokyo, Japan). Reamplified DNA fragments were purified with Centri-Sep Columns (Applied Biosystems) and sequenced with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). In addition, we confirmed the specificity of MSP5′ and the absence of methylation in region 1 by restriction endonuclease digestion. Lack of methylation of products from the MSP5′ PCR was confirmed by digestion with *Bst* UI (New England Biolabs, Beverly, MA), and lack of methylation of region 1 was confirmed by digestion with *Bst* UI and *Taq* I (New England Biolabs). These enzymes digest DNA only if the CG site(s) in the recognition sequence are methylated prior to bisulfite treatment and thus are not converted to TG.

Quantitative RT-PCR

Total RNA was isolated with an RNeasy Mini Kit (Qiagen, Tokyo, Japan), and 1 μg of total RNA was converted to cDNA with a First Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, Uppsala, Sweden). 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 performed using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Initial template concentration was determined from the cycle number at which the fluorescent signal crossed the threshold in the exponential phase of the PCR reaction. Relative gene expression was determined by the threshold cycles for the *SOCS-1* gene and the *ACTB* gene, which was amplified as an internal control. Reference samples (GC cell line MKN-74) were included on each assay plate to verify plate-to-plate consistency. Plates were normalized to each other with these reference samples. PCR amplification was performed in 96-well optical trays with caps in a final reaction volume of 25 μl according to the manufacturer's instructions. *SOCS-1* primer sequences were 5′-ATC CCC CTC AAC CCC GT-3′ (sense) and 5′-TGC CGG TCA AAT CTG GAA G-3′ (antisense). *ACTB* primer sequences were 5′-TCA CCG AGC GCG GCT-3′ (sense) and 5′-TAA TGT CAC GCA CGA TTT CCC-3′ (antisense). Reduced expression of *SOCS-1* was defined as a ratio of expression of *SOCS-1* mRNA in tumor tissues to expression of *SOCS-1* mRNA in corresponding nonneoplastic tissues (T/N ratio) of < 0.5.

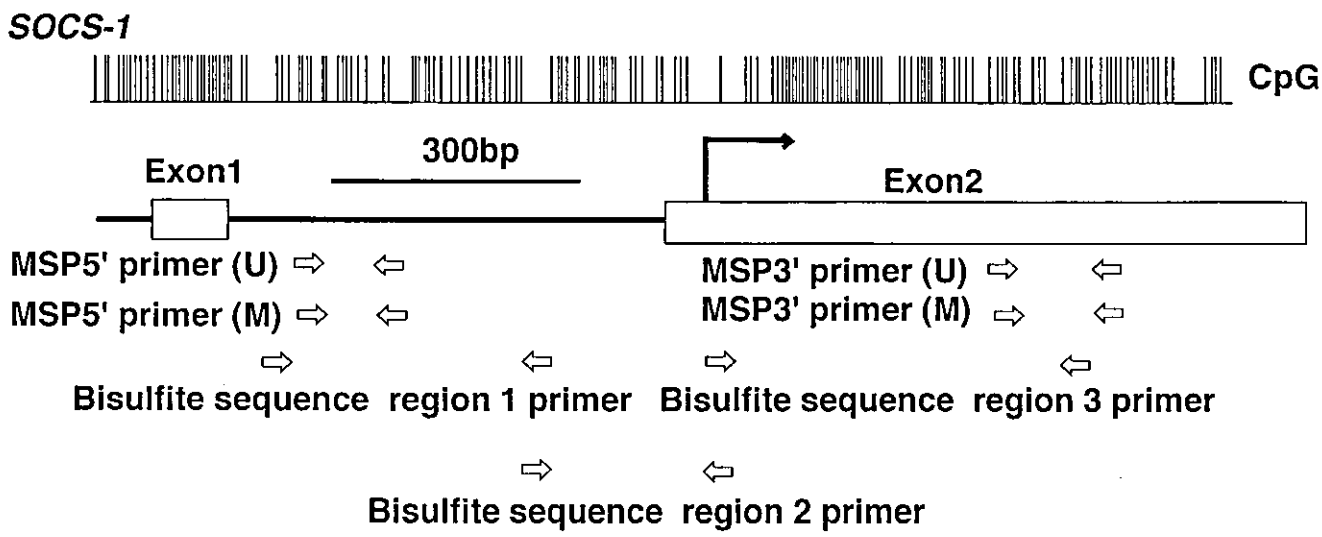

FIGURE 1 – Schematic of the *SOCS-1* gene. CpG density is represented by vertical lines with each line corresponding to a single CpG site. A high density of these sites is indicative of a CpG island. *SOCS-1* lies within a 2.5-kb CpG island. Arrow indicates translation start site. *SOCS-1* contains 2 exons. Exon 1 is untranslated. Open arrows indicate locations of the primers used for MSP and bisulfite sequencing.

Statistical methods

Statistical analyses were performed using Fisher's exact test and the Mann-Whitney U test. p-values < 0.05 were regarded as statistically significant.

RESULTS

SOCS-1 methylation status and mRNA expression levels in GC

Methylation status of the *SOCS-1* gene was examined in 75 gastric carcinoma tissue specimens from 75 patients and 25 corresponding nonneoplastic mucosae. Methylation of regions 3′ and 5′ of the translation start site was examined with MSP3′ and MSP5′ primer pairs, respectively (Fig. 1). With MSP3′, hypermethylation of *SOCS-1* was detected in 33 (44%) of 75 gastric carcinoma tissues and in 3 (12%) of 25 corresponding nonneoplastic mucosae. The incidence of hypermethylation of this site differed significantly ($p = 0.004$, Fisher's exact test) between the carcinoma tissues and the nonneoplastic mucosae. Representative results of analysis with MSP3′ for *SOCS-1* are shown in Figure 2*a* and *b*, and the overall results of MSP analyses of the tumors are summarized in Table I. In the MSP5′ analysis, hypermethylation of the *SOCS-1* gene was not detected in any gastric carcinoma tissues or corresponding nonneoplastic mucosae. Representative results of MSP5′ for *SOCS-1* are shown in Figure 3*a* and *b*. We confirmed the specificity of the MSP5′ primers by restriction analysis with *Bst* UI (Fig. 3*c*). As expected, unmethylated PCR products were not digested. We also examined methylation status of the *SOCS-1* gene by bisulfite sequencing of DNA from 1 gastric carcinoma tissue (1T) with hypermethylation of the MSP3′ but not the MSP5′ region and by bisulfite sequencing of 1 gastric carcinoma tissue (2T) without hypermethylation of either the MSP3′ or MSP5′ region. Dense methylation was detected throughout the 3′ region, including the MSP3′ region, but was not observed in the 5′ region, which included the MSP5′ region and the region 5′ of exon 1, in gastric carcinoma tissue 1T (Fig. 4*a*). Some clones of 1T were not methylated in region 3, which was considered to be caused by contamination of stromal cells or heterogeneity of tumor tissues. In contrast, methylation was not detected in any of the 3 regions examined in gastric carcinoma tissue 2T (Fig 4*a*). We confirmed the absence of methylation of region 1 in tumor tissues by restriction analyses with *Bst* UI and *Taqs* I (Fig. 4*b*). All PCR products from region 1 were not digested. We defined hypermethylation in the MSP3′ analysis as hypermethylation of the *SOCS-1* gene. As shown in Table I, hypermethylation of the *SOCS-1* gene was associated with lymph node metastasis and advanced tumor stage ($p = 0.009$ and 0.034, respectively). No association was detected between methylation status of *SOCS-1* and age, sex, T grade (tumor invasion) or histologic classification. We measured *SOCS-1* mRNA expression by quantitative RT-PCR in 50 tumor tissues (T) and their corresponding nonneoplastic mucosae (N). Reduced expression of *SOCS-1* in tumor tissues was defined as a T/N ratio <0.5. Reduced expression of *SOCS-1* in tumor tissues was observed in 25 (50%) of the 50 cases (Table II). To determine whether transcriptional inactivation of the *SOCS-1* gene is caused by hypermethylation, we compared methylation status with expression of *SOCS-1*. As shown in Table II, reduced expression of *SOCS-1* mRNA in tumor tissues was associated with hypermethylation of the *SOCS-1* gene ($p = 0.002$, Fisher's exact test). We then compared expression of the *SOCS-1* gene to clinicopathologic features. Reduced expression of *SOCS-1* in the tumor tissues was also associated with lymph node metastasis and advanced tumor stage ($p = 0.013$ and 0.002, respectively, Fisher's exact test) (Table II). However, no association was found between reduced expression of *SOCS-1* in the tumor tissues and age, sex, T grade or histologic classification (Table II).

Methylation status of SOCS-1 in normal gastric mucosae from noncancerous healthy individuals

We examined methylation status of the *SOCS-1* gene in 10 normal gastric mucosae obtained endoscopically from healthy individuals (age 22–35 years; average 26.4 years) by MSP3′

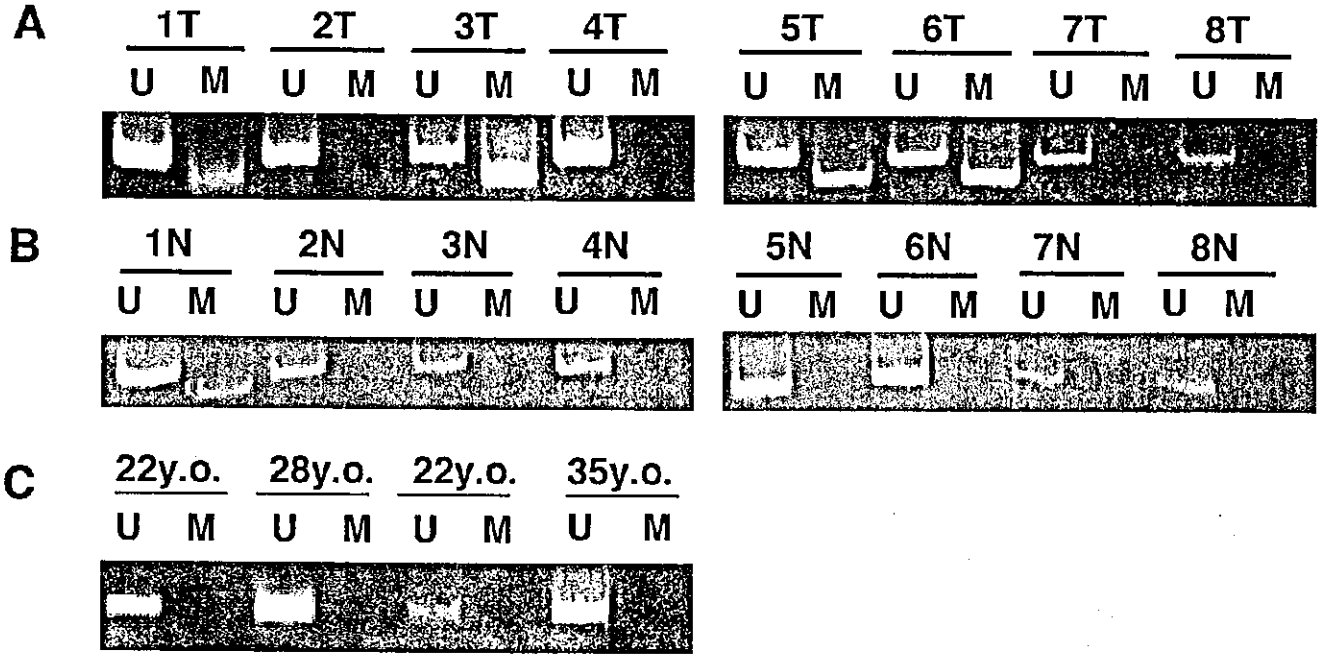

FIGURE 2 – MSP3′ analysis of gastric tissues. U, unmethylated PCR product; M, methylated PCR product. *(a)* Methylation status of *SOCS-1* in gastric carcinoma tissues. Methylated allele was detected in samples 1T, 3T, 5T and 6T. *(b)* Methylation status of *SOCS-1* in corresponding nonneoplastic mucosae. Methylated allele was detected only in specimen 1N. *(c)* Methylation of *SOCS-1* in normal gastric mucosae obtained from healthy young subjects. Methylation of the *SOCS-1* gene was not detected.

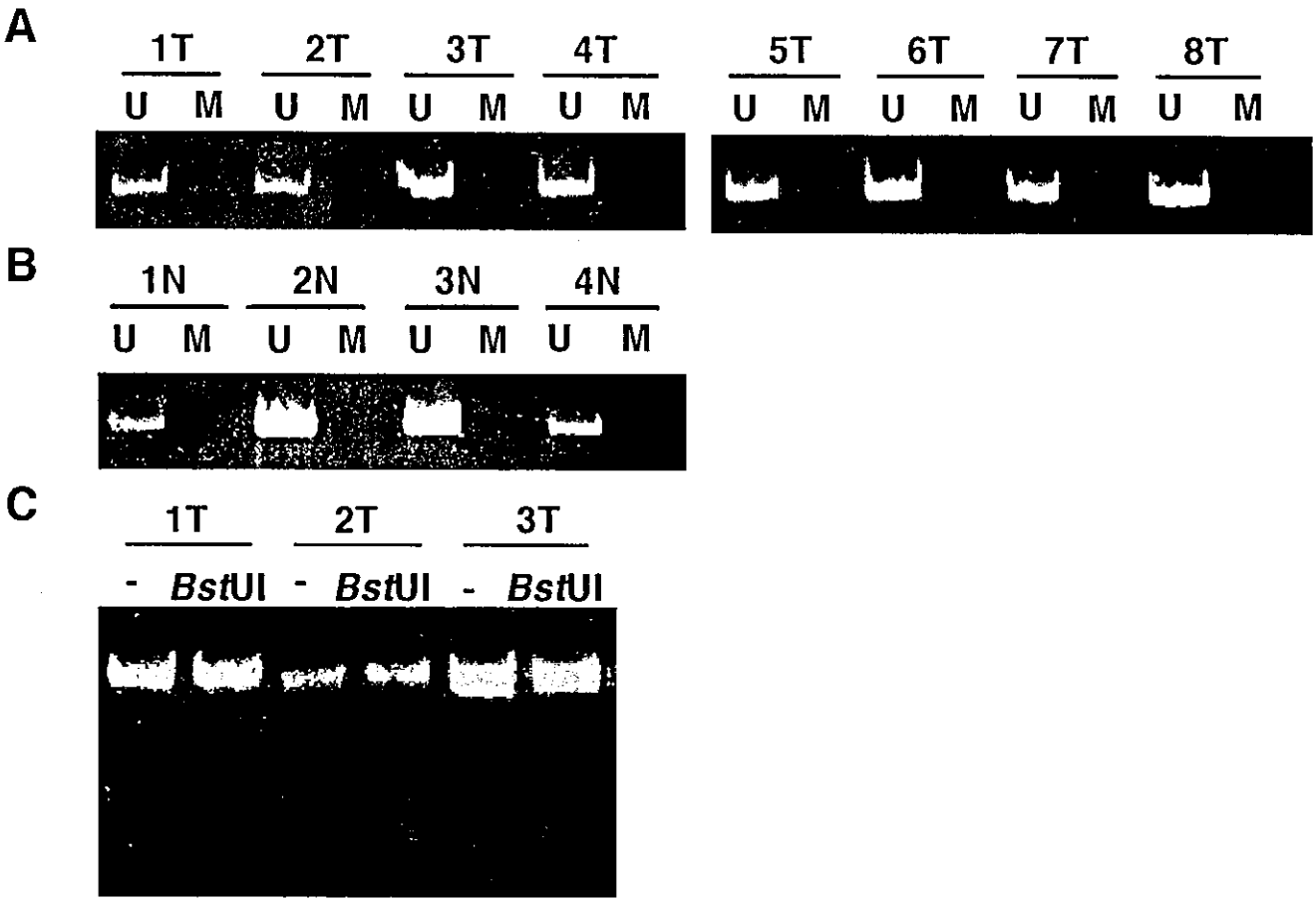

FIGURE 3 – MSP5′ analysis of gastric tissues. U, unmethylated PCR product; M, methylated PCR product. *(a)* MSP5′ analysis of gastric carcinoma tissues. None of the 40 gastric carcinoma tissues show hypermethylation of this region. *(b)* MSP5′ analysis of corresponding neoplastic mucosae. Hypermethylation of this region was not detected. *(c)* Restriction analysis of unmethylated PCR products by *Bst* UI to confirm the specificity of MSP5′ analysis in tumor tissues. Unmethylated PCR products were not digested. -, undigested.

analysis. Hypermethylation of the *SOCS-1* gene was not detected in any of these samples (Fig. 2*c*), whereas 12% of nonneoplastic mucosae from GC patients exhibited hypermethylation as described above.

DISCUSSION

In our study, we found that *SOCS-1* is a target for DNA methylation and silencing in GC and that DNA hypermethylation of the *SOCS-1* gene is associated with reduced *SOCS-1* mRNA expression. These results suggest that hypermethylation of the *SOCS-1* gene plays an important role in transcriptional inactivation of *SOCS-1* in GC. The incidence of *SOCS-1* methylation differed significantly between gastric tumor tissues and nonneoplastic mucosae; the *SOCS-1* gene was hypermethylated in 33 (44%) of 75 tumor samples and in 3 (12%) of 25 corresponding nonneoplastic mucosa samples, indicating that methylation of the *SOCS-1* gene may contribute to development of GC. Epigenetic changes such as DNA methylation occur in premalignant and histologically normal gastric epithelia.[44,45] Recent evidence suggests that methylation of certain genes, such as E-cadherin and $p16^{INK4a}$, is associated with aging.[1,2,39,47] We also found that normal gastric mucosae from young healthy subjects are not hypermethylated at the *SOCS-1* gene. Therefore, the aging mechanism may explain, at least in part, detection of methylation in nonneoplastic mucosae. Age-related methylation of the *SOCS-1* gene may also contribute to gastric carcinogenesis with respect to a possible link between aging and increased risk for gastric cancer.

Although the primers used for MSP3′ amplify exon 2 and not the promoter of the *SOCS-1* gene,[48] methylation status of the MSP3′ region has been associated with *SOCS-1* expression in a variety of tumors. Furthermore, treatment of SOCS-1-negative cell lines harboring hypermethylation with demethylating agent led to a reactivation of *SOCS-1* expression,[34,36,38] indicating that methylation may play an important role in gene silencing. Consistent with previous reports, methylation of the MSP3′ region was detected more frequently in gastric tumor tissues than in corresponding nonneoplastic mucosae and was associated significantly with reduced expression of *SOCS-1*. Bisulfite sequencing revealed that the dense methylation extended from exon 2 to the translation start site. Methylation did not extend into the region 5′ of the translation start site, which included the MSP5′ region, in GC tissues. Similarly, dense methylation was detected in the 3′ region of region 3 in hepatocellular carcinoma cell lines with methylation-mediated silencing of *SOCS-1*.[34] Therefore, methylation of the *SOCS-1* gene appears to occur mainly in exon 2 and does not extend to the 5′ region of exon 1. Methylation of the MSP3′ region may reflect methylation status of the CpG island around the translation start site. Methylation of CpG islands located within the promoter regions of

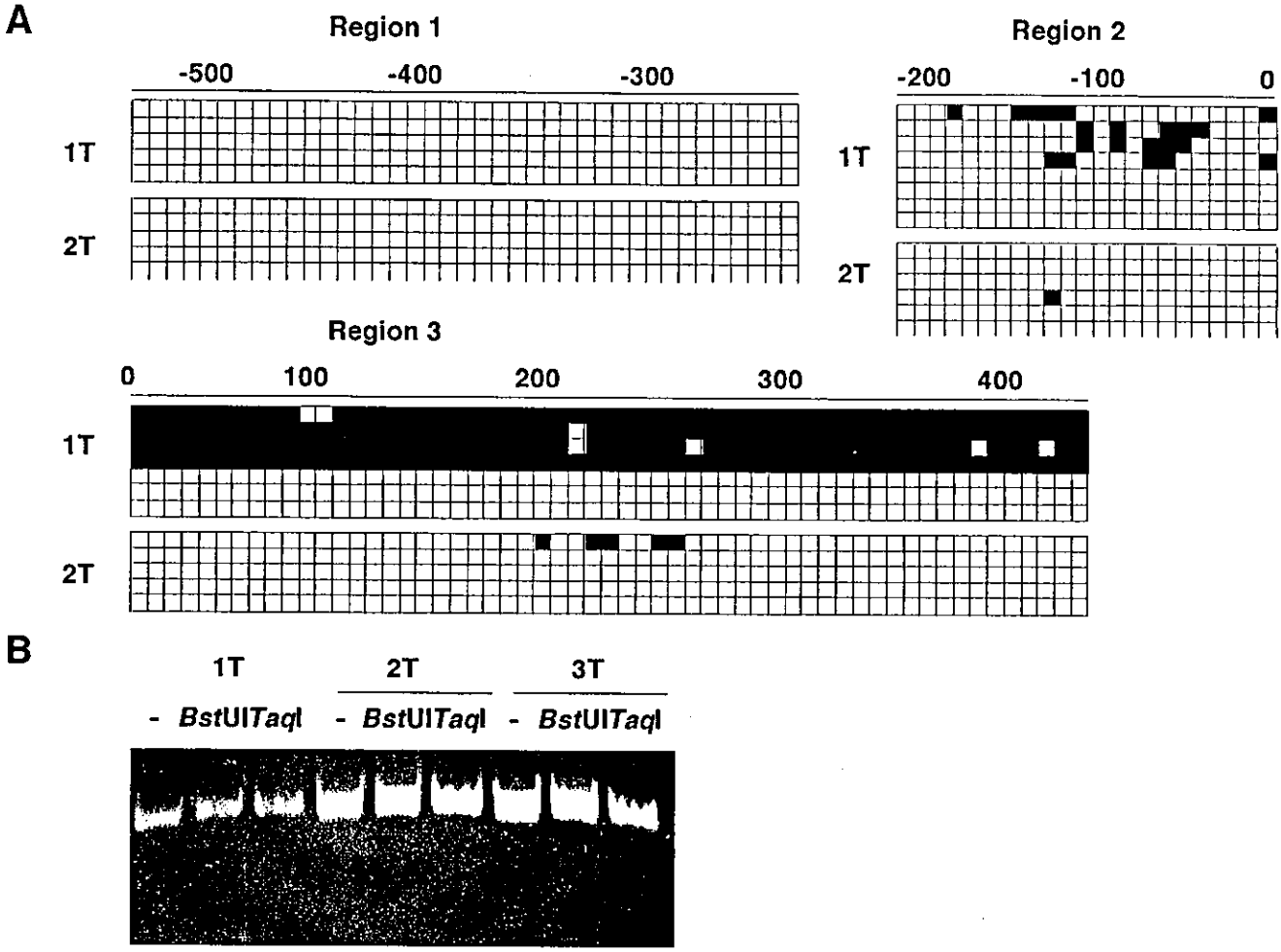

FIGURE 4 – Bisulfite sequencing analysis. *(a)* Bisulfite sequencing analysis of 2 gastric carcinoma tissues. GC tissue (1T) shows methylation of the MSP3′ region, and GC tissue (2T) lacks methylation of the MSP3′ region. Methylation of the *SOCS-1* gene was examined with 3 primer sets. Region 1 and region 2 are located 5′ of the translation start site. Region 3 is located 3′ of the translation start site. We sequenced 5 to 8 clones of PCR products from bisulfite-treated DNA. Filled and open squares represent methylated and unmethylated, respectively. Translation start site is indicated as '0'. *(b)* Restriction analysis of PCR products from region 1 with *Bst* UI and *Taq* I to confirm absence of methylation of region 1 in tumor tissues. PCR products were not digested. -, undigested.

many genes plays an important role in regulation of gene expression. However, the promoter region is not always the best match site that correlates with loss of gene expression. For example, methylation in the first exon is strongly correlated with silencing of genes such as *MGMT* and *p16*^{INK4a}. Methylation of the second exon may also correlate with silencing of genes including *SOCS-1*. The fact that exon 1 is not transcribed may result in loss of SOCS-1 if exon 2 is methylated. However, the mechanism underlying the correlation between hypermethylation of exon 2 and reduced expression of *SOCS-1* remains unclear.

In our study, we observed that methylation of *SOCS-1* and reduced *SOCS-1* expression in tumor tissues were associated with lymph node metastasis and advanced tumor stage, suggesting that loss of SOCS-1 may be involved in lymph node metastasis and tumor progression. Stimulation of the JAK/STAT pathway by cytokines or growth factors results in transactivation of target genes. *SOCS-1* is upregulated by several cytokines and growth factors. This blocks JAK activation, resulting in termination or attenuation of the JAK/STAT pathway. Inactivation of *SOCS-1* by DNA methylation disrupts this negative feedback loop and increases hypersensitivity of GC cells to stimulation by cytokines and growth factors. We previously reported that IL-6 is an essential growth and survival factor in GC,[49] and serum IL-6 levels are correlated with disease status of GC.[50,51] Thus, loss of SOCS-1 may increase responsiveness of GC cells to IL-6 signals, thereby supporting survival and expansion of GC cells. Moreover, *SOCS-1* is considered a candidate tumor suppressor gene because ectopic expression *SOCS-1* suppresses proliferation and anchorage-independent growth of hepatocellular carcinoma cells in which *SOCS-1* expression is silenced by DNA methylation.[34] Furthermore, expression of the *c-fos*, a target gene of the JAK/STAT pathway, is associated with tumor metastasis.[52] Therefore, inactivation of *SOCS-1* by DNA methylation may provide a growth advantage to GC cells through activation of the JAK/STAT pathway, particularly by IL-6 signals, and consequent activation of target genes such as *c-fos*, leading to development, progression and metastasis of GC. Therefore, methylation of the *SOCS-1* gene may be a good molecular marker of tumor progression and metastasis as well as for early detection of GC. Because methylation-mediated inactivation is a potentially reversible phenomenon,[53] induction of expression of *SOCS-1* by

TABLE I – ASSOCIATION BETWEEN *SOCS-1* METHYLATION STATUS AND CLINICOPATHOLOGIC FEATURES AND CIMP STATUS IN GASTRIC CARCINOMAS

	SOCS-1 methylation[1]		*p*-value[2]
	Positive	Negative	
Sex			
Male	24	28	0.572
Female	9	14	
Age			
≤65	9	15	0.437
>66	24	27	
T grade			
1, 2	11	22	0.099
3, 4	22	20	
N stage			
N0	5	18	0.009
N1, 2, 3	28	24	
Stage			
Stage I, II	10	23	0.034
Stage III, IV	23	19	
Histology			
Intestinal	17	22	0.941
Diffuse	16	20	
Histology			
Intestinal and diffuse-adherent	24	27	0.437
Diffuse-scattered	9	15	

[1]Methylation status of the *SOCS-1* gene was examined by MSP3′ analysis.–[2]Fisher's exact test.

TABLE II – ASSOCIATION BETWEEN *SOCS-1* MRNA EXPRESSION AND METHYLATION STATUS AND CLINICOPATHOLOGIC FEATURES IN GASTRIC CARCINOMAS

	SOCS-1 reduced expression		*p* value[1]
	Positive	Negative	
Sex			
Male	19	15	0.225
Female	6	10	
Age			
≤65	8	11	0.382
>66	17	14	
T grade			
1, 2	7	12	0.145
3, 4	18	13	
N grade			
N0	3	11	0.013
N1, N2, N3	22	14	
Stage			
Stage I, II	4	15	0.002
Stage III, IV	21	10	
Histology			
Intestinal	13	13	1.000
Diffuse	12	12	
Histology			
Intestinal and diffuse-adherent	15	18	0.370
Diffuse-scattered	10	7	
SOCS-1 methylation			
Positive	17	6	0.002
Negative	8	19	

[1]Fisher's exact test.

demethylating agents might control tumor formation and progression of GC.

In conclusion, our results suggest that *SOCS-1* is subject to epigenetic silencing by DNA methylation in a considerable proportion of GCs and that epigenetic inactivation of *SOCS-1* by hypermethylation may be involved in development, progression and metastasis of GC. Although additional studies are needed to understand the biologic significance of *SOCS-1* inactivation in gastric tumorigenesis, our findings suggest that *SOCS-1* methylation could be a useful molecular marker for detection and evaluation of progression and metastatic potential of GC. Frequent constitutive activation of the JAK/STAT pathway by hypermethylation of the *SOCS-1* gene may be a molecular target for treatment of GC.

ACKNOWLEDGEMENTS

We thank Mr. M. Takatani for his excellent technical assistance and advice. This work was carried out with the kind cooperation of the Research Center for Molecular Medicine, Faculty of Medicine, Hiroshima University.

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[CANCER RESEARCH 64, 523–529, January 15, 2004]

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

Received 4/30/03; revised 10/21/03; accepted 11/6/03.

Grant support: Grants-in-Aid for Cancer Research from the Ministry of Education, Culture, Science, Sports, and Technology of Japan and from the Ministry of Health, Labor, and Welfare of Japan.

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Green PCR Core Reagent kit (Applied Biosystems, Foster City, CA). Real-time detection of the emission intensity of SYBR Green bound to double-stranded DNAs was by the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). *POT1* cDNA and internal control cDNA (β-actin gene, *ACTB*) were PCR amplified separately. Relative gene expression was determined by the threshold cycles for the *POT1* gene and *ACTB* gene. Reference samples (GC cell line, HSC-39) were included on each assay plate to verify plate-to-plate consistency. Plates were normalized to each other by these reference samples. PCR amplification was performed according to the manufacturer's instructions in 96-well optical trays with caps with a 25-μl final reaction mixture. Quantitative RT-PCRs were performed in triplicate for each sample primer set, and the mean of the three experiments was used as the relative quantification value. *POT1* primer sequences were 5′-TCAGTCTGTTAAACTTCATTGCCC-3′ and 5′-TGCACCATCCTGAAAAATTATATCC-3′. *ACTB* primer sequences were 5′-TCACCGAGCGCGGCT-3′ and 5′-TAATGTCACGCACGATTTCCC-3′.

Telomere Restriction Fragment Length Analysis. High molecular weight genomic DNA was extracted with a DNA Extraction kit (Stratagene Cloning System, La Jolla, CA). Tissue DNA was digested with *Hinf*I, electrophoresed on 0.6% agarose gels, and blotted onto nitrocellulose filters. The filters were hybridized with a telomeric DNA probe and then autoradiographed. We estimated the telomere length as the peak signal using Kodak Digital Science 1D software (Eastman Kodak Company, New Haven, CT).

3′ Telomeric Overhang Assay (In-Gel Hybridization). In-gel hybridization to measure 3′ telomeric overhangs was carried out as described in Wellinger *et al.* (25). DNA samples were digested with *Hinf*I and electrophoresed on 0.6% agarose gels. The gels were dried with a gel dryer for 24–28 min at room temperature. The very thin gels were then hybridized for 16 h at 37°C to end-labeled oligonucleotides in hybridization buffer. $[TTAGGG]_4$ and $[CCCTAA]_4$ oligonucleotide probes were end-labeled with γ-^{32}P-ATP and T4 polynucleotide kinase. After removal of excess hybridization buffer, gels were washed twice with 0.25× SSC for 1.5 h at room temperature, followed by 2-h washes at 30°C. After sequential native gel hybridization, dried gels were alkali-denatured in 0.15 M NaCl, 0.5 M NaOH for 25 min, neutralized in 0.15 M NaCl, 0.5 M Tris-HCl (pH 8.0) for 20 min, and reprobed. Image analysis and quantitation were performed with a Fuji Film BAS 2000 Bio-Imaging Analysis System and NIH Image.

Telomeric Repeat Amplification Protocol Assay. Telomeric repeat amplification protocol assay was done with a TRAPEZE Telomerase Detection kit (Intergen Company, Oxford, United Kingdom). Intensity of the telomeric repeat amplification protocol product bands and of the internal control bands was determined with the use of NIH Image.

Telomere Shortening Assay. We harvested MKN-28 and MKN-74 cells maintained at 37°C under 5% CO_2 in RPMI 1640 and 10% fetal bovine serum. Cells were pretreated for 5 and 10 days with medium exchange and addition of AZT (100 μM) each day before being used for experiments.

***POT1* Antisense Oligonucleotides.** The 24-mer phosphorothioate oligonucleotide antisense sequence of the first 24 nucleotides of *POT1* was synthesized and purified by reverse-phase high-performance liquid chromatography (Espec Oligo Service, Tsukuba, Japan). The sequence was 5′-TTGTTGCTGGAACCAAAGACATTG-3′, and for control, complementary (sense) oligonucleotides were synthesized as 5′-CAATGTCTTTGGTTCCAGCAACAA-3′. We harvested MKN-28 cells maintained at 37°C under 5% CO_2 in RPMI 1640 and 10% fetal bovine serum. Cells were pretreated with 2.5 μM antisense or sense oligonucleotides in Lipofectamine (Invitrogen-Life Technologies, Inc., Carlsbad, CA) for 4 and 8 days by medium exchange and addition of antisense or sense oligonucleotides every 2 days before being used for experiments.

Anaphase Bridges. H&E-stained cultures or tissue sections were examined for anaphase bridges under a light microscope at ×100 magnification. The anaphase bridge index (ABI) was determined by dividing the number of anaphases with bridges by the total number of anaphases. Anaphase bridging was defined as reported previously (26). Two investigators independently scored a minimum of 10 anaphases/sample.

Statistical Analysis. Statistical significance was assessed by Fisher's exact test, Mann-Whitney *U* test, Spearman's rank correlation test, or unpaired *t* test. Statview 5.0 Macintosh software was used. All tests were two-sided. A P of <0.05 was regarded as statistically significant.

RESULTS

***POT1* Expression Levels Increase with Tumor Stage.** Expression levels of *POT1* were measured by quantitative RT-PCR in the 51 cases of GC. We calculated the ratio of *POT1* mRNA expression levels in GC tissues relative to levels in nonneoplastic mucosae (T/N ratio). The T/N ratios were significantly higher in stage III/IV cancers than in stage I/II cancers ($P = 0.005$, Mann-Whitney *U* test; Fig. 1*A*). We considered a T/N $>$ 2.0 to represent up-regulation and a T/N $<$ 0.5 to represent down-regulation. Up-regulation of *POT1* was found in 12 (23.5%) of the 51 cases, and down-regulation was found in 18 (35.3%) of the 51 cases. Down-regulation of *POT1* was more frequent in stage I/II tumors (52.4%, 11 of 21) than in stage III/IV tumors (23.3%, 7 of 30; $P = 0.033$, Fisher's exact test; Table 1),

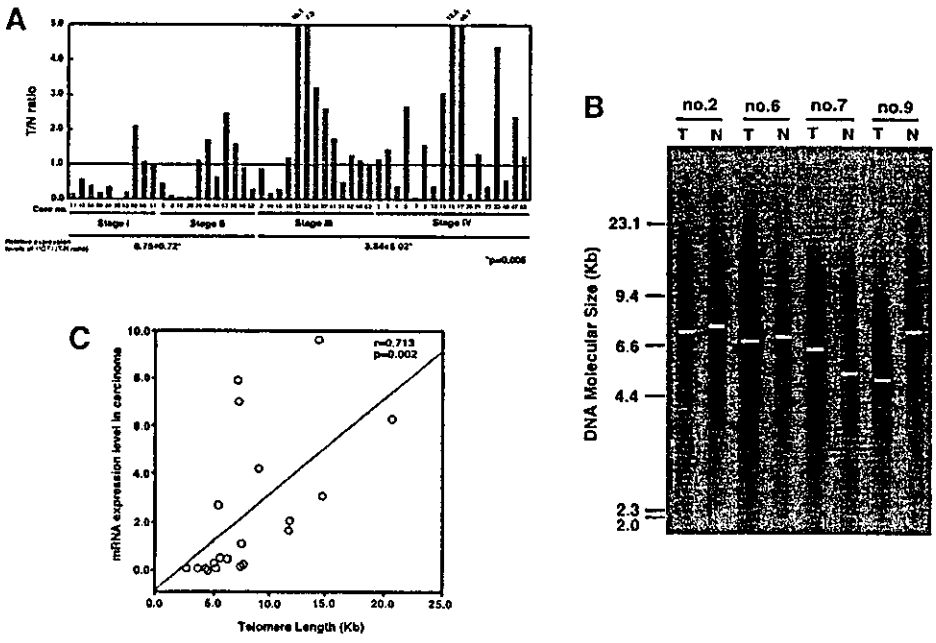

Fig. 1. *POT1* expression levels in gastric cancer (GC) and association with telomere length. *A*, distribution of *POT1* expression in the 51 cases of GC. T/N ratio stage I/II: 0.75 ± 0.72; T/N ratio stage III/IV: 3.84 ± 8.02, $P = 0.005$ by Mann-Whitney *U* test. *B*, representative telomere restriction fragment length analysis. Telomere lengths were determined in tumor tissues (T) and corresponding nonneoplastic mucosae (N). Horizontal *white bars* show the average length. *C*, *POT1* expression levels in tumor tissues correlate positively with telomere length ($r = 0.713$, $P = 0.002$ by Spearman's rank correlation test). T/N ratio = *POT1* mRNA expression levels in GC tissue relative to levels in corresponding nonneoplastic mucosa.

Table 1 *Clinicopathological features of gastric cancers (n = 51) in relation to POT1 expression levels*[a]

	Down-regulation (T/N <0.5)	No change (T/N = 0.5–2.0)	Up-regulation (T/N >2.0)	*P*
Histology[b]				
Intestinal	9 (36.0%)	9 (36.0%)	7 (28.0%)	
Diffuse	9 (34.6%)	12 (46.2%)	5 (19.2%)	n.s.[c]
T-grade[d]				
$T_{1,2}$	12 (50.0%)	9 (37.5%)	3 (12.5%)	
$T_{3,4}$	6 (22.2%)	12 (44.5%)	9 (33.3%)	0.038[e]
N-grade[d]				
N_0	6 (46.2%)	5 (38.4%)	2 (15.4%)	
$N_{1,2,3}$	12 (31.6%)	16 (42.1%)	10 (26.3%)	n.s.
Stage[d]				
I, II	11 (52.4%)	8 (38.1%)	2 (9.50%)	
III, IV	7 (23.3%)	13 (43.4%)	10 (33.3%)	0.033[e] 0.048[f]

[a] T/N ratio = *POT1* mRNA expression levels in GC tissue relative to levels in corresponding nonneoplastic mucosa.
[b] According to the Lauren criteria (23).
[c] n.s., not significant.
[d] According to the criteria of the tumor-node-metastasis stage classification system (24).
[e] By Fisher's exact test, for down-regulation *versus* no change and up-regulation.
[f] By Fisher's exact test, for down-regulation and no change *versus* up-regulation.

whereas up-regulation of *POT1* was more frequent in stage III/IV tumors (33.3%, 10 of 30) than in stage I/II tumors (9.5%, 2 of 21; $P = 0.048$, Fisher's exact test; Table 1). In addition, down-regulation of *POT1* was observed preferentially in low T grade (depth of invasion) cancers ($P = 0.038$, Fisher's exact test; Table 1). No association was found between *POT1* expression level and N grade (degree of lymph node metastasis) or histological type (Table 1).

Positive Correlation between *POT1* Expression Levels and Telomere Length. For association between *POT1* expression levels and telomere length in GC tissues, telomere length was examined by Southern blotting analysis in 20 of the 51 cases (Fig. 1*B*). *POT1* expression levels in GC tissue decreased in accordance with telomere shortening ($r = 0.713$, $P = 0.002$, Spearman's rank correlation test; Fig. 1*C*).

Positive Correlation between *POT1* Expression Levels and 3′ Telomeric Overhang Signals. 3′ overhang signals were examined by in-gel hybridization analysis in the same 20 GC tissues in which telomere lengths were measured (Fig. 2*A*). 3′ overhang signals reduced in accordance with reduced *POT1* expression levels ($r = 0.696$, $P = 0.002$, Spearman's rank correlation test; Fig. 2*B*) and telomere shortening ($r = 0.570$, $P = 0.013$, Spearman's rank correlation test; Fig. 2*C*).

AZT Inhibition of *POT1* Expression in GC Cells. To confirm the positive correlation between *POT1* expression levels and telomere length, we measured *POT1* expression levels in GC cells in which telomeres were shortened by AZT. AZT inhibited telomerase activity and shortened telomeres (Fig. 3, *A* and *B*). Telomerase activity was inhibited with AZT by 68% after 5 days and 82% after 10 days in MKN-28 cells and by 23% after 5 days and 94% after 10 days in MKN-74 cells. AZT reduced telomere length from 3.9 to 3.7 Kb after 5 days and to 3.4 Kb after 10 days in MKN-28 cells and from 3.6 to 3.1 Kb after 5 days and to 2.7 Kb after 10 days in MKN-74 cells. Quantitative RT-PCR analysis showed *POT1* expression to be down-regulated in AZT-treated cells (Fig. 3*C*). Expression of *POT1* in AZT-treated MKN-28 cells was 29% of that in nontreated cells after

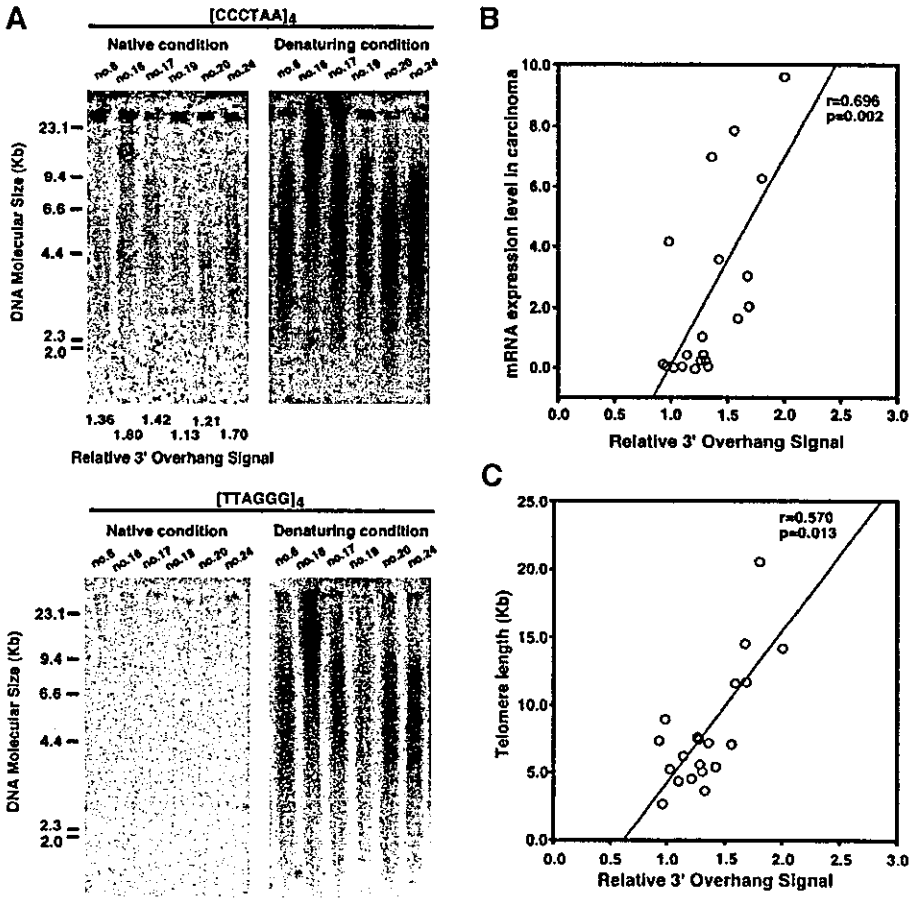

Fig. 2. 3′ Telomeric overhang signals were associated with *POT1* expression levels and telomere lengths. *A*, representative in-gel hybridization assay. The *top left panel* shows native gel probed with $[CCCTAA]_4$, and the *top right panel* shows denatured gel probed with $[CCCTAA]_4$. The *bottom left panel* shows native gel probed with $[TTAGGG]_4$, and the *bottom right panel* shows denatured gel probed with $[TTAGGG]_4$. Signals were expressed relative to the signal in MKN-74 gastric cancer cells. *B*, *POT1* expression levels in tumor tissues correlate positively with 3′ telomeric overhang signals in gastric cancer tissues ($r = 0.696$, $P = 0.002$ by Spearman's rank correlation test). *C*, telomere lengths correlate positively with 3′ telomeric overhang signals in gastric cancer tissues ($r = 0.570$, $P = 0.013$ by Spearman's rank correlation test).

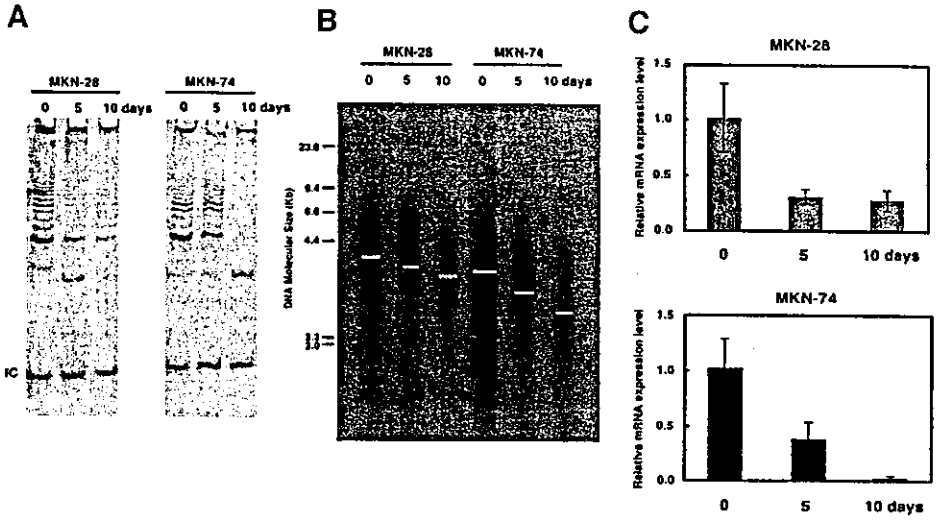

Fig. 3. *POT1* expression levels in relation to telomere shortening. *A*, reduced telomerase activity in MKN-28 and MKN-74 cells is observed after 5 and 10 days' treatment with azidothymidine (AZT). IC = internal control (36 bp). *B*, Telomere shortening is observed in MKN-28 and MKN-74 cells after 5 and 10 days' treatment with AZT. *C*, quantitative reverse-transcription-PCR analysis showed reduced *POT1* expression in MKN-28 and MKN-74 cells treated with AZT. *POT1* expression levels are the mean ± SD and relative to levels in nontreated cells.

5 days and 26% after 10 days. And expression of *POT1* in AZT-treated MKN-74 cells was 36% of that in nontreated cells after 5 days and 1% after 10 days.

Inhibition of *POT1* Induces Telomere Shortening. For additional analysis, we examined MKN-28 cells treated with *POT1* antisense oligonucleotides. Decreased *POT1* expression was confirmed by quantitative RT-PCR in MKN-28 cells after treatment with antisense oligonucleotides (Fig. 4*A*). Expression of *POT1* in antisense oligonucleotide-treated MKN-28 cells was 40% of nontreated cells after 4 days and 34% after 8 days. Treatment with *POT1* antisense oligonucleotides resulted in telomerase inhibition (by 58% after 4 days and 83% after 8 days; Fig. 4*B*) and telomere shortening (from 3.8 to 3.3 Kb after 4 days and to 3.0 Kb after 8 days; Fig. 4*C*). Treatment with sense oligonucleotides did not produce any alterations in *POT1* expression levels, telomerase activity, or telomere length.

Inhibition of *POT1* Reduces 3′ Telomeric Overhang Signals. To test whether *POT1* expression levels are associated with 3′ overhang signals, 3′ telomeric overhang signals were examined by in-gel hybridization in MKN-28 cells treated with *POT1* antisense oligonucleotides. A reduction in 3′ overhang signals was found in antisense

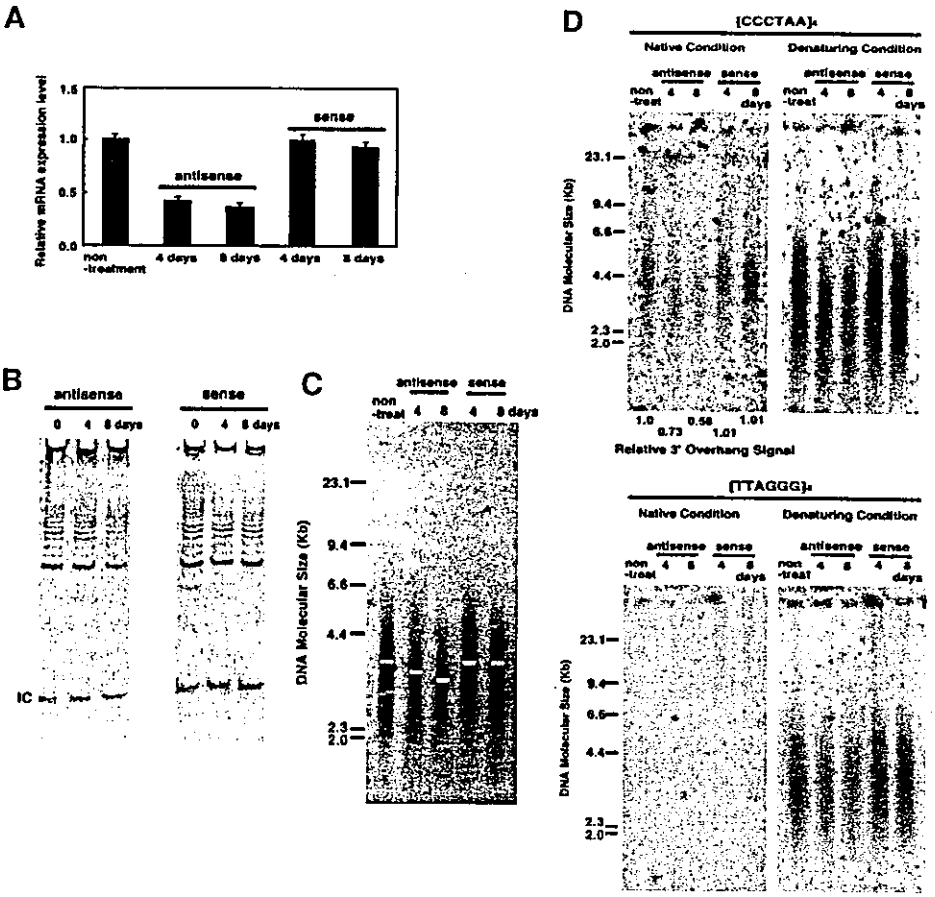

Fig. 4. Telomere shortening, telomerse inhibition and the reduction of 3′ telomeric overhang signals in association with reduced expression of *POT1* in antisense oligonucleotides-treated MKN-28 cells. *A*, reduction of *POT1* expression was confirmed in MKN-28 cells after antisense oligonucleotide treatment for 4 and 8 days. *B*, reduced telomerase activity was detected in antisense oligonucleotides-treated MKN-28 cells, whereas telomerase activity was not changed in sense oligonucleotides-treated MKN-28 cells. *C*, telomere shortening was detected in MKN-28 cells treated with antisense oligonucleotides. *D*, inhibition of Pot1 reduced 3′ telomeric overhang signals. The *top left panel* shows native gel probed with $[CCCTAA]_4$, and the *top right panel* shows denatured gel probed with $[CCCTAA]_4$. The *bottom left panel* shows native gel probed with $[TTAGGG]_4$, and the *bottom right panel* shows denatured gel probed with $[TTAGGG]_4$. Signals were expressed relative to the signal in the nontreated MKN-28 gastric cancer cells and normalized to the amount of DNA (10 μg) loaded based on denatured gels.

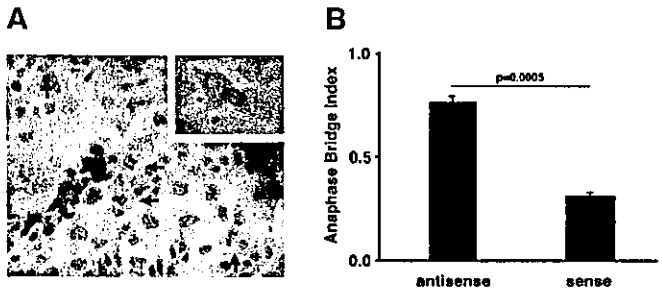

Fig. 5. Inhibition of Pot1 increases the incident of anaphase bridge. *A*, photomicrograph of typical anaphase bridge (*arrows* and *inset*) in MKN-28 treated with *POT1* antisense oligonucleotides H&E-stained section. (magnification, ×400; inset magnification, ×1000). *B*, inhibition of Pot1 by antisense oligonucleotides increases the number of anaphase bridge in MKN-28 gastric cancer cells.

oligonucleotide-treated cells (to 73% after 4 days and to 56% after 8 days; Fig. 4*D*). Signals in sense oligonucleotide-treated cells were almost the same as signals in nontreated cells (101% after both 4 and 8 days).

Inhibition of *POT1* Expression Increases the Frequency of Anaphase Bridges. To examine association between *POT1* expression levels and telomere dysfunction, we determined the ABI in the 51 GC tissues. No significant association was found between *POT1* expression levels and the ABI. Moreover, the ABI did not correlate with telomere length or 3′ telomeric overhang signals (data not shown). We then treated MKN-28 cells with *POT1* antisense and sense oligonucleotides for 8 days and determined the ABI. Treatment with *POT1* antisense increased the ABI in MKN-28 cells to about twice that of sense-treated cells (0.76 for antisense treatment and 0.31 for sense treatment; $P = 0.0005$, unpaired t test; Fig. 5, *A* and *B*).

DISCUSSION

Pot1 is thought to have two functions. One is mediating recruitment of telomerase, and the other is protecting the 3′ telomeric overhang from degradation and DNA repair activities (13, 16–18). We studied *POT1* mRNA expression in GC tissues with respect to both functions. We showed expression levels of *POT1* in the GC tissues relative to levels in nonneoplastic mucosae to be significantly associated with tumor stage and that up-regulation of *POT1* occurs preferentially in late-stage GC. Pot1 is thought to protect telomeres (13). In fact, inhibition of *POT1* by antisense oligonucleotides led to an increase in the frequency of anaphase bridge in MKN-28 cells. Thus, inhibition of Pot1 is associated with telomere dysfunction. Previous studies in a mouse model revealed that severe telomere dysfunction impaired tumor progression (26–31). Severe telomere dysfunction is shown to be reduced in advanced tumors that survive after crisis (26, 32, 33). Thus, up-regulated *POT1* may participate in protection of the telomere ends in late-stage GC. In addition, we found *POT1* mRNA expression levels to be associated with telomere length, as well as 3′ telomeric overhang signals in GC tissues. However, we did not find significant association between telomere length or 3′ telomeric overhang signals and tumor stage (data not shown). High-level *POT1* expression, as well as telomerase activity, may be required for maintenance of telomere function.

Telomere dysfunction appears to occur in cancer precursor lesions and increase the frequency of genetically initiated neoplasms (26, 30, 31, 34–39); therefore, in early-stage GC after neoplasm initiation, down-regulation of *POT1* may occur preferentially. We showed that down-regulation of *POT1* was observed preferentially in low T grade cancers, which might also reflect telomere dysfunction in early-stage GC. Approximately half of our GC patients (21 of 51) showed no changes in the expression level of *POT1*, perhaps because telomere dysfunction is circumvented by chromosomal rearrangement such as in decentric and ring chromosomes in these patients.

The ABI did not correlate with *POT1* expression levels, telomere length, or 3′ telomeric overhang signals. Anaphase bridges are chromatin bridges that are not resolved after anaphase, and they result in breakage-fusion-bridge cycles that produce rapid and widespread changes in the gene (40–46). Anaphase bridges are a hallmark of telomere dysfunction (26) but form not only as a result of defects in telomere structure or length (6, 34) but also as a result of defects in DNA replication (47), recombinations (48), or translocations that introduce a second centromere into the chromosome (46, 49). Therefore, we could not show significant association between ABI and *POT1* expression levels, telomere length, or 3′ telomeric overhang signals.

It was reported recently that the 3′ telomeric overhang is shortened at senescence and that progressive overhang loss occurred in cells that avoided senescence through inactivation of p53 and Rb (50). Evidence indicates that 3′ telomeric overhang shortening is the result of continuous cell division and that it is associated with telomere shortening. We showed telomere length to be associated with 3′ telomeric overhang signals, consistent with previously reported findings (50).

To confirm the association between *POT1* expression levels and telomere length, we measured telomere length in AZT-treated GC cells and showed reduced *POT1* expression as well as telomere shortening. Loayza *et al.* (51) also reported the amount of Pot1 to be correlated with telomere length. Our data support their findings. However, we cannot fully rule out the possibility that the down-regulation of *POT1* may have been because of a direct effect of AZT or other factors. We examined association between *POT1* expression levels and telomere length, 3′ telomeric overhangs, and the frequency of anaphase bridges using GC cells treated with *POT1* antisense oligonucleotides. The inhibition of *POT1* by antisense oligonucleotides was found to shorten the telomere, reduce the 3′ overhang signals, and increase the frequency of anaphase bridges. Colgin *et al.* (52) reported that overexpression of *POT1* led to telomere elongation. In yeast, Pot1-like protein Cdc13 recruits telomerase to the 3′ telomeric overhang (14); thus, inhibition of *POT1* may lead to telomere shortening through inhibition of recruitment of telomerase to the 3′ telomeric overhang. Furthermore, our study showed that inhibition of telomerase activity occurred via *POT1* antisense oligonucleotides. We are unable to explain this phenomenon at the present. However, inhibition of telomerase activity by *POT1* antisense oligonucleotides may participate partly in telomere shortening. We examined the viability of antisense-treated MKN-28 cells (4- and 8-day treatments) by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and observed no change in the viability of treated cells compared with nontreated cells (data not shown). Thus, it seems there is no association between telomerase inhibition and decreased viability of cells. We found inhibition of *POT1* by antisense oligonucleotides to reduce 3′ overhang signals. Therefore, it is possible that *POT1* expression levels may generally depend on the size of the 3′ telomeric overhang. However, it remains a possibility that the reduction of 3′ overhang signals may be because of end-to-end fusion. Indeed, inhibition of *POT1* led to an increase in the frequency of anaphase bridges. In this study, no signal was present at the position of the larger terminal fragments representing the fused telomeres (53). Therefore, at least some of the loss of 3′ overhangs in GC cells must have taken place on unfused chromosome ends, and end-to-end fusion may have little effect on 3′ overhang signals.

Loayza *et al.* (51) observed telomere elongation using Pot1 mutant lacking the oligosaccharide/oligonucleotide-binding (OB) fold required for DNA binding. We are unable to fully explain the discrep-