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J Cancer Res Clin Oncol (2004) 130: 259–265
DOI 10.1007/s00432-004-0543-1

ORIGINAL PAPER

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A single nucleotide polymorphism in the *MMP-1* promoter is correlated with histological differentiation of gastric cancer

Received: 1 August 2003 / Accepted: 28 December 2003 / Published online: 18 February 2004

Abstract *Purpose*: Matrix metalloproteinase-1 (MMP-1) plays a key role in cancer invasion and metastasis by degradation of extracellular matrix (ECM) and basement membrane barriers. The 1G/2G single nucleotide polymorphism (SNP) in the *MMP-1* promoter at position −1607 bp has been reported to affect the transcriptional activity. In the light of these findings, we investigated whether this SNP in the *MMP-1* promoter is associated with the development, differentiation, and progression of gastric cancer. *Methods*: The 215 gastric cancer patients and 166 controls were used in this study. The SNP of the *MMP-1* promoter was analyzed by PCR-RFLP and sequencing. The genotype frequency was compared between cases and controls, and the association with clinicopathological parameters among cases was studied. *Results*: The frequency of 1G/2G genotypes in gastric cancer patients was similar to those in controls ($p = 0.57$). The degree of tumor invasion, the presence of lymph node metastasis, and clinical stage showed no significant association with the SNP. On the other hand, we found a significant association with histological differentiation and gender among gastric cancer patients ($p < 0.05$, respectively). *Conclusions*: The presence of 2G allele in the *MMP-1* promoter did not enhance the risk of gastric cancer; however, it may be involved in differentiation of gastric cancer.

Keywords SNP · MMP-1 · Gastric cancer

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Introduction

Gastric cancer is one of the most common cancers in many Asian countries including Japan and Korea. The poor prognosis depends on the degree of stomach wall invasion and on metastatic spread to regional lymph nodes. Degradation of extracellular matrix (ECM) and basement membrane barriers by MMPs plays an important role in tumor invasion and metastasis (Forget et al. 1999; Kohn and Liotta 1995; Liotta et al. 1991). A prognostic value of MMPs expression in tumor tissue has been reported (McDonnel and Matrisian 1991). Overexpression of MMP-1 has been demonstrated in a variety of cancers (Hewitt et al. 1991; Murray et al. 1998a; Templeton et al. 1990), and the expression of MMP-1 is associated with poor prognosis of esophageal cancer patients (Murray et al. 1998b). In colorectal cancer, the expression of MMP-1 correlated with pathological factors such as Dukes' stage, differentiation, lymphatic or vascular invasion, and tumor depth (Baker and Leaper 2003).

Genetic polymorphism of insertion of a guanine (G) nucleotide at −1607 bp in the *MMP-1* gene promoter sequence, which generates the sequence 5'-GGA-3', has been identified. This sequence generates a new binding site for ETS transcription factor, influencing its transcriptional activity (Rutter et al. 1998). Moreover, the presence of 2G allele in the *MMP-1* promoter has been reported to associate with the development and

progression of carcinomas of the ovary, endometrium, and colorectum (Ghilardi et al. 2001; Kanamori et al. 1999; Nishioka et al. 2000). The frequency of ovarian cancer patients carrying 2G alleles was significantly higher than that in non-cancer individuals (Kanamori et al. 1999); hence, the presence of 2G allele is thought to be a risk factor of endometrial cancer (Nishioka et al. 2000). Similarly, the frequency of 2G allele was higher in colorectal patients than that in controls (Ghilardi et al. 2001). The levels of *MMP-1* expression in ovarian cancer tissues among the patients carrying 2G alleles were significantly elevated, compared with those homozygously carrying 1G alleles (Kanamori et al. 1999; Nishioka et al. 2000).

In gastric cancer, *MMP-1*expression has been associated with both peritoneal and lymph node metastasis (Inoue et al. 1999); however, there is no report on the association between the *MMP-1* promoter polymorphism and the development of gastric cancer. In this study we investigated whether the 1G/2G polymorphism in the *MMP-1* promoter is associated with the development of gastric cancer. Moreover, we examined the relationship between the 1G/2G polymorphism and the clinicopathological factors among gastric cancer patients.

Materials and methods

Samples

A total of 381 peripheral blood samples from 166 healthy control subjects and 215 gastric cancer patients were employed in this study. Controls were randomly selected from those visited Hiroshima University Hospital for regular healthy check or symptoms such as appetite loss or epigastralgia. They were proved to be free from malignancy by medical examination with gastrointestinal scope and biopsy. Gastric cancer patients underwent surgical operation or endoscopic mucosal resection (EMR) at Hiroshima University Hospital. All patients and controls gave informed consent prior to enrollment in the study. The human genome research ethics screening committee of Hiroshima University School of Medicine approved this study. Gastric cancer patients were 153 males and 62 females (median age 67.7 ± 11.4 years). Histology of gastric cancer was classified according to the criteria of Lauren (1965). There were 122 patients who had an intestinal type of gastric cancer, and 93 patients who had a diffuse type. Intestinal type and diffuse type correspond to well-differentiated type and poorly differentiated type, respectively, in the histological classification of the Japanese Gastric Cancer Association (Japanese Gastric Cancer Association 1998). Alternative histological classification of the 215 gastric carcinomas was those with either intestinal or diffuse type components (pure type) or with coexistence of both types of components (mixed type; Stelzner and Emmrich 1997). Gastric cancer patients were grouped according to TNM classification (Sobin and Wittekind 2002), on the basis of the postoperative histopathological evaluation. Moreover, they were assigned to two subgroups according to the presence (N+) or absence (N–) of detectable lymph node metastasis at the time of diagnosis.

Eight human gastric carcinoma cell lines (MKN-1, MKN-7, MKN-28, MKN-45, MKN-74, TMK-1, HSC-39, and KATO-III) were used. The TMK-1 cell lines was established in our laboratory from poorly differentiated adenocarcinoma (Ochiai et al. 1985). 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 T. Suzuki (Fukushima Medical University, Fukushima, Japan). KATO-III and HSC-39 cell lines, which were established from signet ring cell carcinoma, were kindly provided by M Sekiguchi (University of Tokyo, Tokyo, Japan) and by K. Yanagihara (National Cancer Center, Tokyo, Japan), respectively, (Yokozaki 2000). All of these cell lines were routinely maintained in RPMI-1640 (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (Whittaker, Walkersville, Md.), penicillin (0.1 mg/ml), and streptomycin (0.1 mg/ml) under conditions of 5% CO_2 in air at 37°C.

DNA extraction and PCR-RFLP analysis

The genomic DNA purification kit (Promega, Madison, Wis.) and QIAamp 96 DNA Blood kit (QIAGEN, Valencia, USA) were used for DNA extraction. The PCR-restriction fragment length polymorphism (RFLP) assay was used to determine the *MMP-1* genotypes. The PCR primers used for amplifying MMP-1 polymorphism were: forward primer 5'-TGACTTTTAAAACA TAGTCTATGTTCA-3'; reverse primer 5'-TCTTGGATTGATT TGAGATAAGTCATAGC-3'. The reverse primer was specially designed to introduce a recognition site of restriction enzyme *Alu*I (AGCT) by replacing a T with a G at the second position close to the 3' end of the primer (Zhu et al. 2001). The 1G alleles have this recognition site, whereas the 2G alleles destroy the recognition site by inserting a guanine. The target sequence was amplified in a 25-µl reaction volume containing 10–20 ng of genomic DNA, 0.2 µM dNTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM MgCl2, 0.3 µM of each primer, and 0.75 units of Ampli Taq Gold (Perkin-Elmer, Norwalk, Conn.). The PCR amplification was carried out with 40 cycles of denaturation at 94°C for 30 s, annealing at 58°C (depending on the primer) for 30 s, and extension at 72°C for 30 s after the initial activation step of 94°C for 10 min. The 269-bp fragment was then digested with *Alu*I (TaKaRa Biomedicals, Shiga, Japan) overnight at 37°C. After overnight digestion, 269-bp (2G allele), 241-bp, and 28-bp (1G allele) fragments were loaded on an ethidium bromide stained 2.5% NuSieve 3:1 agarose (FMC Bioproducts, Rockland, Md.) gel for 60 min at 100 V. Heterozygotes displayed a combination of both alleles (269, 241, and 28 bp).

Sequencing analysis of PCR products

The PCR products were purified and cloned into the pCR2.1 vector (Invitrogen, Carlsbad, Calif.). Plasmid DNA was extracted from individual clones by alkaline lysis plasmid minipreparation. The inserted PCR fragments obtained from each sample were sequenced with both M13 reverse and M13 forward primer using the PRISM AmpliTaq DNA polymerase FS Ready Reaction Dye Terminator Sequencing kit (Perkin-Elmer ABI, Foster City, Calif.). Reamplified DNA fragments were purified with Centri-Sep Columns (Princeton, Adelphia, N.J.) and were sequenced by ABI PRISM 310 genetic analyzer (Perkin-Elmer ABI, Foster City, Calif.).

Statistical analysis

Statistical analysis was performed with the use of Fisher's exact test. A value of $p < 0.05$ was considered significant. Odds ratios (OR) and 95% confidence intervals (95% CI) were used for estimating the risk of association with genotypes. Odds ratios for the genotypes were calculated by the logistic regression model, adjusting for age and gender. The logistic regression analysis was performed for the association between the genotypes and clinicopathological factors (SPSS software, ver 11.0).

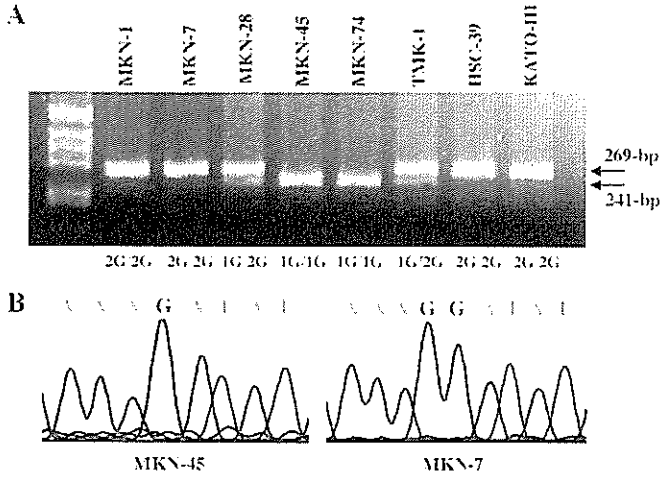

Fig. 1 A Representative PCR-RFLP analysis to confirm the variants in eight human gastric cancer cell lines. Ethidium bromide-stained 2.5% NuSieve 3:1 agarose gel used for genotyping. The target products (269-bp) in the *MMP-1* gene promoter was PCR amplified and digested with *AluI*, which cleaved the 1G allele at the polymorphic site, generating two fragments (241-bp and 28-bp, respectively), but did not cut the 2G allele. **B** Representative electropherogram of each type of polymorphism. The genotype of the SNP was proved to be 1G/1G in MKN-45 and 2G/2G in MKN-7

Results

We first examined the 1G/2G polymorphism in the *MMP-1* gene promoter by PCR-RFLP in eight gastric cancer cell lines, followed by sequencing: three patterns of PCR-RFLP were detected (Fig. 1A). MKN-1, MKN-7, HSC-39, and KATO-III cells had a single 269-bp DNA band; MKN-45 and MKN-74 cells showed a single 241-bp DNA band. On the other hand, MKN-28 and TMK-1 cells had heterozygous bands. Subsequent sequencing confirmed the 1G/2G genotyping of MKN-45 and MKN-7 cells (Fig. 1B). MKN-45 and MKN-7 cells were identified with 1G/1G and 2G/2G genotypes, respectively; MKN-45 and MKN-74 cells carried 1G/1G genotype; MKN-1, MKN-7, HSC-39, and KATO-III cells carried 2G/2G genotype; MKN28 and TMK-1 cells carried heterozygous 1G/2G genotype. Eight human gastric cancer cell lines used in this study had been derived from Japanese gastric cancer patients and established in Japan. There were no particularly prominent genotypes among these cell lines.

We next determined the 1G/2G genotyping among gastric cancer patients and controls; typical PCR-RFLP patterns are shown in Fig. 2 (i.e., 1G/1G or 2G/2G genotype with a single 241-bp or 269-bp band, respectively; 1G/2G genotype with 241-bp and 269-bp bands). The 1G/2G genotype distribution in gastric cancer patients and controls is shown in Table 1. The genotype distribution among controls was in good agreement with Hardy-Weinberg equilibrium. The allelic frequency in controls in our study was similar to the allele frequency reported in healthy Japanese subjects (Kanamori et al. 1999). The frequency of those carrying at least one 2G allele (1G/2G and 2G/2G) was almost equal between patients with gastric cancers (87.9%) and controls (89.7%; $p=0.57$, OR = 0.83, 95% CI = 0.43–1.59). In addition, we found that the frequency of genotypes (1G/1G vs 1G/2G + 2G/2G) did not differ by gender, age, and status of *H. pylori* infection.

Finally, we analyzed the association between the 1G/2G genotyping and clinicopathological factors among gastric cancer patients (Table 2). We found a significant difference in genotype distribution (1G/1G vs 1G/2G + 2G/2G) by histological classification (intestinal type vs diffuse type, $p=0.03$, OR = 2.84, 95% CI = 1.09–7.39). The patients carrying at least one 2G allele were more frequent in diffuse type than those in intestinal type. Furthermore, the patients carrying at least one 2G allele were more frequent in mixed type than those in pure type, with an OR of 3.81, although this was not statistically significant due to a small number of the mixed type. We also found a significant difference by gender (male vs female, p = 0.04, OR = 3.48, 95% CI = 1.00–12.04). On the other hand, depth of tumor invasion, the presence of lymph node metastasis, and TNM classification showed no significant correlation with genotyping. In general, intestinal and diffuse types of gastric carcinomas are more frequent in males and females, respectively, implying a possible interaction between histological typing and gender. The logistic

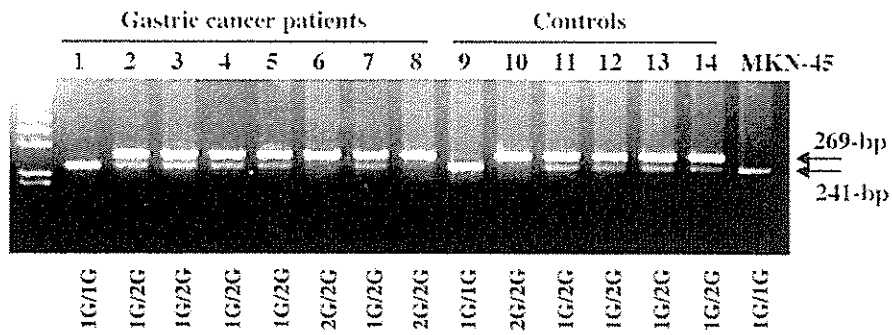

Fig. 2 Representative 8 cases of gastric cancer patients and 5 cases of controls are shown. The target products (*269-bp*) in the *MMP-1* gene promoter were PCR amplified and digested with *AluI*. MKN-45 cells were used as positive controls for digestion with *AluI*. *Numbers above the panel* are case numbers. Genotypes are shown below each case. Case numbers 1, 3, 6, and 7 are intestinal type, and the others are diffuse type

Table 1 *MMP-1* genotype distribution of the study subjects. *.CI* confidence interval

Genotype	Controls (%)[a] ($n=166$)	Patients (%) ($n=215$)	P value[b]	Crude OR[c] (95% CI)
Overall				
1G/1G	17 (10.3)	26 (12.1)		
1G/2G	61 (36.7)	88 (40.9)		
2G/2G	88 (53.0)	101 (47.0)	0.57	0.83 (0.43–1.59)
Gender				
Male				
1G/1G	9 (9.5)	23 (15.0)		
1G/2G	41 (43.2)	60 (39.2)		
2G/2G	45 (47.3)	70 (45.8)	0.20	0.59 (0.26–1.34)
Female				
1G/1G	8 (11.3)	3 (4.8)		
1G/2G	20 (28.1)	28 (45.2)		
2G/2G	43 (60.6)	31 (50.0)	0.18	2.49 (0.63–9.86)
Age (years)				
<65				
1G/1G	14 (11.1)	11 (11.7)		
1G/2G	46 (36.5)	39 (41.5)		
2G/2G	66 (52.4)	44 (46.8)	0.89	0.94 (0.41–2.18)
≥65				
1G/1G	3 (7.5)	15 (12.4)		
1G/2G	15 (37.5)	49 (40.5)		
2G/2G	22 (55.0)	57 (47.1)	0.39	0.57 (0.16–2.09)
H. pylori infection				
Negative	$n=47$	$n=44$		
1G/1G	4 (8.5)	7 (15.9)		
1G/2G	16 (34.0)	14 (31.8)		
2G/2G	27 (57.5)	23 (52.3)	0.28	0.49 (0.13–1.81)
Positive	$n=90$	$n=49$		
1G/1G	12 (13.4)	6 (12.2)		
1G/2G	31 (34.4)	20 (40.8)		
2G/2G	47 (52.2)	23 (47.0)	0.86	1.10 (0.39–3.15)

[a]The observed genotype distribution of controls was in agreement with Hardy-Weinberg equilibrium
[b]Correlation was analyzed by Fisher's test. The p values <0.05 were regarded as statistically significant. The p values of 1G/2G+2G/2G genotypes relative to 1G/1G genotype
[c]Odds ratio of 2G/2G+1G/2G genotypes relative to 1G/1G genotype

Table 2 Correlation between the genotype of SNP in the *MMP-1* promoter and clinicopathological characteristics of gastric cancer patients

	Genotype (%)			OR (95%CI)[a]	
	1G/1G	1G/2G	2G/2G	Crude	Adjusted[b]
Age (years)					
<65 ($n=94$)	11 (11.7)	39 (41.5)	44 (46.8)	0.94	
≥65 ($n=121$)	15 (12.4)	49 (40.5)	57 (47.1)	(0.41–2.15)	
Gender					
Male ($n=153$)	23 (15.0)	60 (39.2)	70 (45.8)	3.48	
Female ($n=62$)	3 (4.8)	28 (45.2)	31 (50.0)	(1.00–12.04)	
Histological classification 1[c]					
Intestinal ($n=121$)	20 (16.5)	49 (40.5)	52 (43.0)	2.84	3.56
Diffuse ($n=94$)	6 (6.4)	39 (41.5)	49 (52.1)	(1.09–7.39)	(1.15–11.11)
Histological classification 2[d]					
Mixed type ($n=26$)	1 (3.8)	12 (46.4)	13 (50.0)	3.81	4.73
Pure type ($n=189$)	25 (13.2)	76 (40.2)	88 (46.6)	(0.49–29.38)	(0.58–38.53)
Depth					
m, sm ($n=85$)	10 (11.8)	29 (34.1)	46 (54.1)	0.97	1.02
mp ~ ($n=130$)	16 (12.3)	59 (45.4)	55 (42.3)	(0.42–2.25)	(0.24–4.35)
Lymph node metastasis					
N (–; $n=126$)	15 (11.9)	46 (36.5)	65 (51.6)	0.98	1.14
N (+; $n=89$)	11 (12.4)	42 (47.2)	36 (40.4)	(0.43–2.25)	(0.16–8.13)
TNM classification[e]					
Stages I, II ($n=145$)	20 (13.8)	55 (37.9)	70 (48.3)	1.71	3.04
Stages III, IV ($n=70$)	6 (8.6)	33 (47.1)	31 (44.3)	(0.65–4.46)	(0.83–11.16)

Correlation was analyzed by Fisher's test. The P values <0.05 were regarded as statistically significant
[a]Odds ratio of 2G/2G+1G/2G genotypes relative to 1G/1G genotype
[b]The ORs were adjusted for age and gender
[c]Histology of gastric cancer was classified according to the criteria of the Lauren
[d]Pure-type gastric cancer with either intestinal or diffuse type components; mixed-type gastric cancer with coexistence of both types of components
[e]Tumor staging was classified according to the criterion of the UICC TNM stage grouping, 6th edition, 2002, stomach

regression analysis then revealed that histological classification remained significant ($p=0.03$, OR = 3.56, 95% CI = 1.15–11.11) even after adjustment for age and gender.

Discussion

Studies of ovarian cancer and colorectal cancer have shown that the frequency of patients carrying at least one 2G allele in the *MMP-1* promoter was significantly higher than in control subjects (Ghilardi et al. 2001; Kanamori et al. 1999); thus, the presence of 2G allele is considered to be one of the risk factors for the development of these cancers. Furthermore, pancreatic cancers frequently showed a positive staining for *MMP-1* protein in immuno-histochemical analysis, whereas the *MMP-1* expression in fetal and normal pancreatic tissues was very faint (Ito et al. 1999). In this study, we examined whether the risk of gastric cancer is associated with the 1G/2G polymorphism in the *MMP-1* promoter region. The allelic frequency in the patients with gastric cancer was similar to that in controls. It seems that the presence of 2G allele did not enhance the susceptibility for the development of gastric cancer.

However, we found a significant association between the 1G/2G polymorphism and the histological classification. The frequency of those carrying at least one 2G allele was significantly higher in the diffuse type (poorly differentiated type) of gastric cancer than that in the intestinal type (well-differentiated type) with an OR of 3.56. The *MMP-1* promoter with 2G allele has displayed significantly increased transcriptional activity than that with 1G allele in melanoma cell lines and normal fibroblasts through the ETS binding site (Rutter et al. 1998). Diffuse type of gastric cancer is usually characterized by an abundant deposition of collagen fibers, possibly requiring higher levels of *MMP-1*expression for degradation of ECM. It has beensuggested that carcinogenesis was a multicellular and multistage process in which the destruction of the microenvironment was required for conversion of normal tissue to tumor (Park et al. 2000). Although MMPs are not oncogenic or mutagenic, they alter the microenvironment and may affect the process of carcinogenesis and its histology. This polymorphism may have a more profound impact on histology and differentiation of gastric cancer.

Recent studies showed the significance of a modified histological classification, pure type or mixed type. Patients with mixed-type gastric cancer revealed poorer prognosis than those with pure type did (Stelzner and Emmrich 1997). In this study, the patients carrying at least one 2G allele were more frequent in mixed type than those in pure type, with an OR of 3.81, although this was not statistically significant due to a small number of the mixed type. Take together; these findings suggest that the presence of 2G allele in the *MMP-1* promoter may contribute to the morphogenesis of gastric carcinomas.

Several MMPs, including MMP-1, have been reported to play an important role in cancer invasion through their overexpression, which is associated with metastasis and unfavorable prognosis in esophageal cancer, ovarian cancer, cutaneous malignant melanoma, and colorectal cancer (Ghilardi et al. 2001; Kanamori et al. 1999; Murray et al. 1998b; Ye et al. 2001).Kanamori et al. (1999) observed a high expression level of the *MMP-1* in tumors carrying 2G allele than 1G homozygotes in ovarian cancers; however, in this study, 1G/2G polymorphism in the *MMP-1* promoter was not associated with invasion, lymph node metastasis, and TNM classification in gastric cancer patients. Possible explanations may be the following:

1. The degree of tumor invasion in gastric carcinoma might be determined not only by the presence of 2G allele in the *MMP-1* promoter but also the response to growth factors and cytokines. In fact, cytokines, such as interleukin-1 (IL-1), influence the expression levels of *MMP-1*(Singer et al. 1997). Especially, IL-1A acts as a growth stimulator for gastric carcinoma (Ito et al. 1993), and it correlates with liver metastasis of gastric carcinoma (Furuya et al. 1999; Tomimatsu et al. 2001). Although the presence of 2G allele in the *MMP-1* promoter displays a significantly higher transcriptional activity than the *MMP-1* promoter with 1G allele (Rutter et al. 1998), the polymorphism may not significantly affect mean expression levels of *MMP-1* in normal stromal cells. The polymorphism may increase the *MMP-1* expression in response to growth factors and cytokines (Wyatt et al. 2002).
2. Some reports revealed that overexpression of *MMP-1* was observed in stromal cells of gastric carcinoma but not in carcinoma cells (Migita et al. 1999; Otani et al. 1999). So, the presence of 2G allele of *MMP-1* promoter may not necessarily contribute to the degree of tumor invasion in gastric carcinoma.
3. In stage-I cases in our study, a large portion of them (99 of 112 cases) have 2G allele, and 37 of 54 cases carrying 2G homozygotes were treated by means of EMR. So, they are in the status of before invading to the submucosa, they have no significant correlation with tumor invasion and metastasis. In fact, our results revealed a tendency of increasing risk of development of gastric cancer with an 8.33 exceeding risk for the patients with the 2G allele (stage II vs stage III + IV;$p=0.05$). It has been reported that MMPs can alter the microenvironment and may influence tumor formation. So, MMPs may contribute to the initial stages of cancer development, and overexpression of MMPs may be associated with elevated risk of tumorigenesis. Moreover, the polymorphism in the *MMP-1* promoter is associated with early tumor stages in lung cancer (Zhu et al. 2001). The polymorphism in the *MMP-1* promoter may be one of the pathways of the increased neoplastic risk

in the stomach. However, we do not have any data concerning pre-cancerous lesions (i.e., patients with gastric adenoma, severe atrophic gastritis with intestinal metaplasia) at present; therefore, we could not verify a relationship between the polymorphism in the *MMP-1* promoter and a pre-cancerous lesion. However, since this is a very important point to elucidate the pathway of increasing risk of tumorigenesis and progression in gastric cancer, we should clarify this issue in the near future.

On the other hand, we found a significant association with gender among gastric cancer patients ($p = 0.037$, OR = 3.48, 95% CI = 1.00–12.04). This finding suggested a gender-specific effect of the *MMP-1* polymorphism. The presence of 2G allele in *MMP-1* promoter enhances the transcriptional activity. Moreover, it has been reported that the activity of MMP-1 might be regulated by sex hormones (Marbaix et al. 1992; Schneikert et al. 1996). The expression of *MMP-1* was negatively regulated by androgen (Schneikert et al. 1996), whereas the secretion and activation of *MMP-1* was inhibited by physiological concentrations of progesterone (Marbaix et al. 1992). Furthermore, being consistent with the above-mentioned information, IL-1A is a key inducer of *MMP-1* in the human endometrium. Ovarian steroids inhibited the release of IL-1A and repress MMP-1 production (Singer et al. 1997); therefore, the presence of 2G allele in the *MMP-1* promoter may affect the risk of gastric cancer in women. However, in our study, almost all women with gastric cancer are elderly and in menopausal status; the mean levels of ovarian steroids may be low among them. So, we did not find a significant association among gastric cancer patients and controls in women.

Conclusion

In conclusion, our studies suggest that the presence of the 2G allele in the *MMP-1* promoter might be associate with histological differentiation of gastric cancer; however, we could not consider lifestyle factors such as cigarette smoking, alcohol consumption, and dietary habits of our patients. These factors may contribute to the development of gastric cancer (Chen et al. 2000). Further investigations are necessary to clarify a role of this *MMP-1* polymorphism with increased number of study subjects and epidemiological data.

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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}* 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]

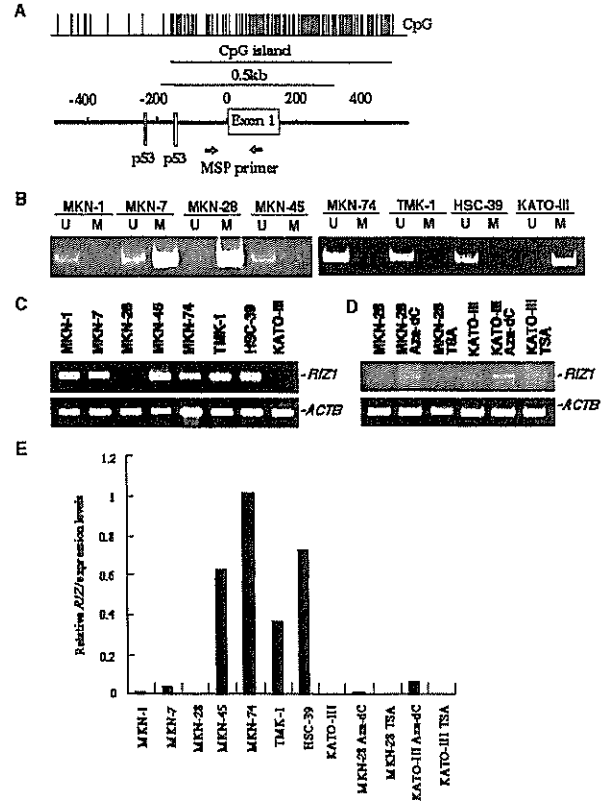

FIGURE 1 – Promoter methylation status and *RIZ1* mRNA expression in gastric carcinoma cell lines. (*a*) Map of the *RIZ1* promoter, depicting the CpG island, MSP primer, and *p53* consensus binding sites. Vertical lines show each CpG site. (*b*) MSP of *RIZ1* in gastric carcinoma cell lines. Primer 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*).

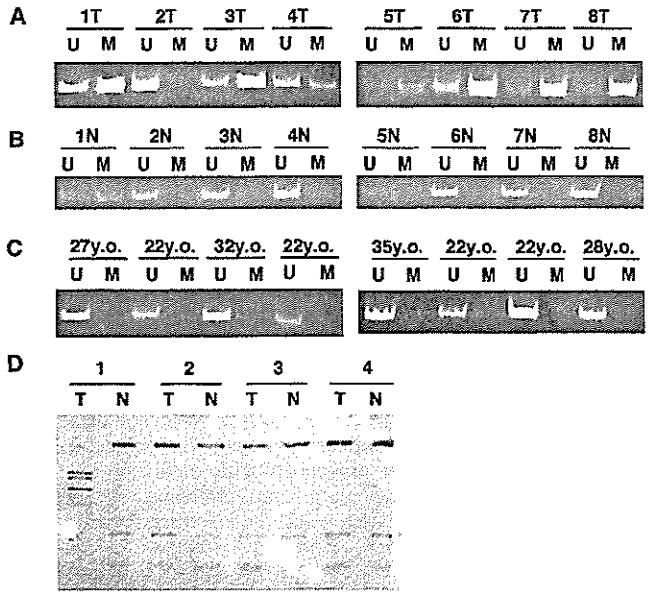

FIGURE 2 – MSP analysis of the *RIZ1* promoter and *p53* mutation analysis of gastric tissues. U, unmethylated PCR product; M, methylated PCR product. (*a*) Methylation status of *RIZ1* in gastric carcinoma tissues. The methylated allele was detected in 1T, 3T, 4T, 5T, 6T, 7T and 8T. (*b*) Methylation 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 ± 0.14[2,3]
Tumor tissue without promoter hypermethylation (n = 14)	0.27 ± 0.10[2,4]
Tumor tissue with promoter hypermethylation (n = 31)	0.13 ± 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.

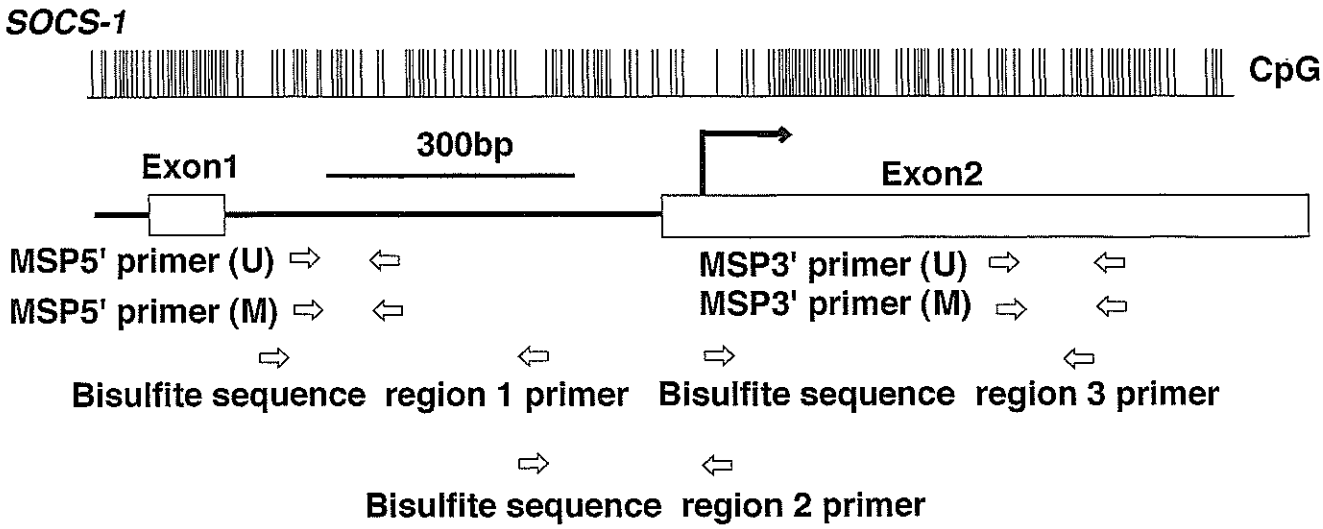

FIGURE 1 – Schematic of the *SOCS-1* gene. CpG density is represented by vertical lines with each line corresponding to a single CpG site. A high density of these sites is indicative of a CpG island. *SOCS-1* lies within a 2.5-kb CpG island. Arrow indicates translation start site. *SOCS-1* contains 2 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 *Taq*s 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′

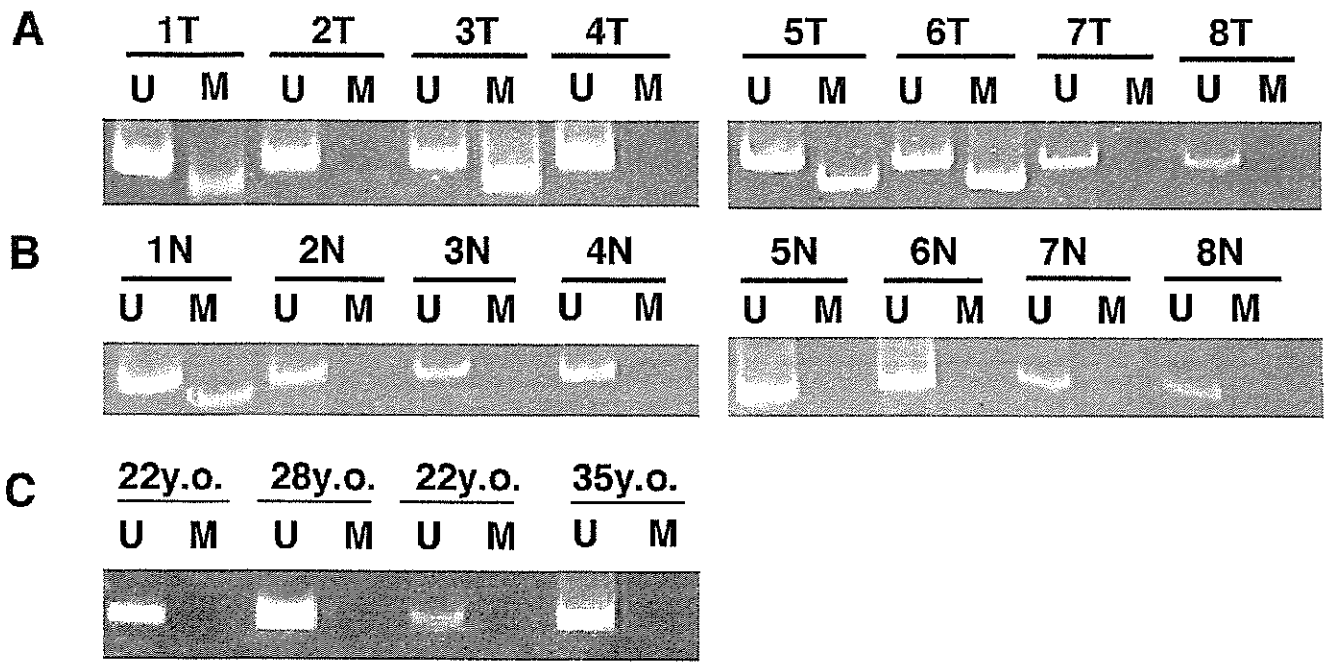

FIGURE 2 – MSP3′ analysis of gastric tissues. U, unmethylated PCR product; M, methylated PCR product. *(a)* Methylation status of *SOCS-1* in gastric carcinoma tissues. Methylated allele was detected in samples 1T, 3T, 5T and 6T. *(b)* Methylation status of *SOCS-1* in corresponding nonneoplastic mucosae. 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.

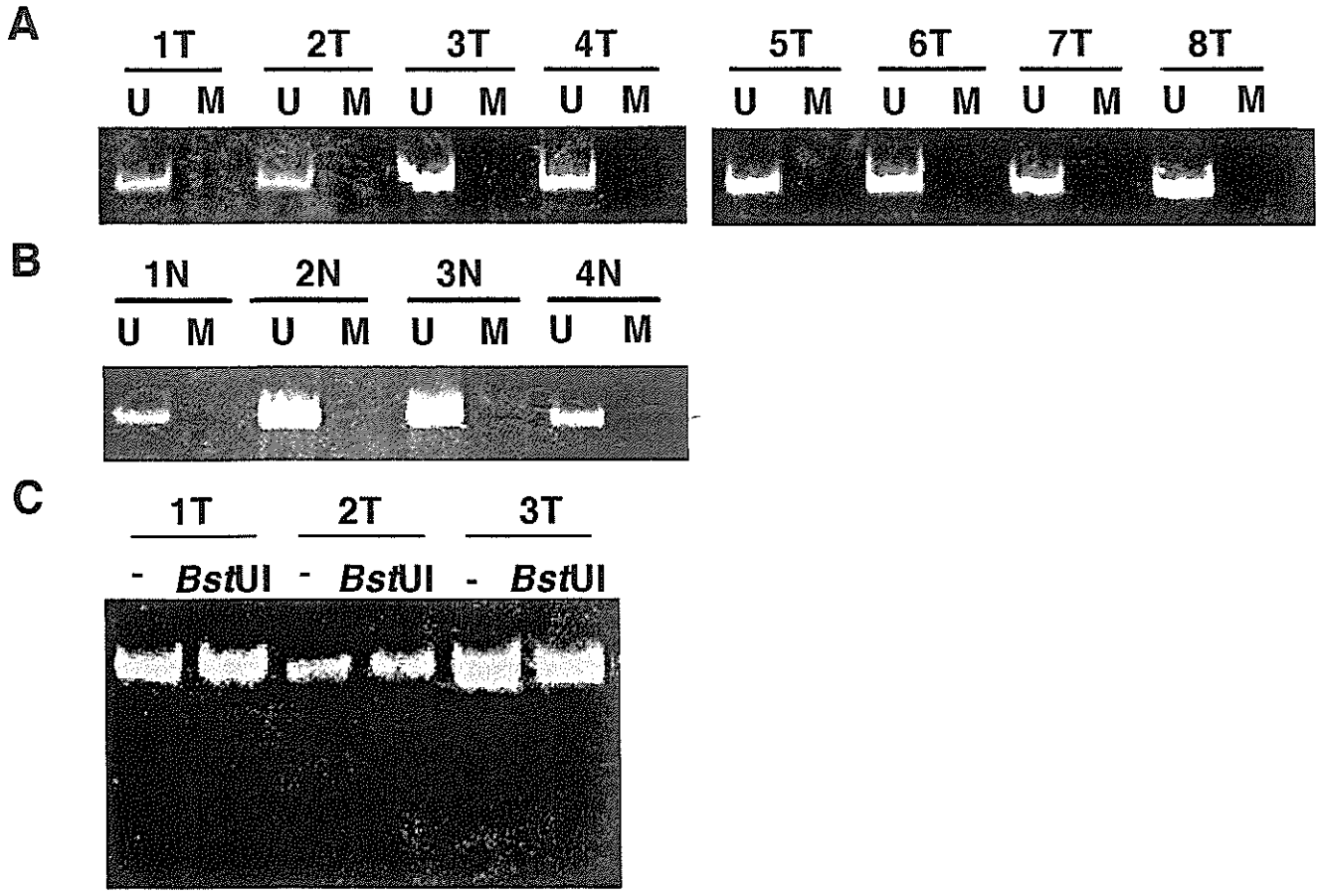

FIGURE 3 – MSP5′ analysis of gastric tissues. U, unmethylated PCR product; M, methylated PCR product. *(a)* MSP5′ analysis of gastric carcinoma tissues. None of the 40 gastric carcinoma tissues show hypermethylation of this region. *(b)* MSP5′ analysis of corresponding neoplastic mucosae. Hypermethylation 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

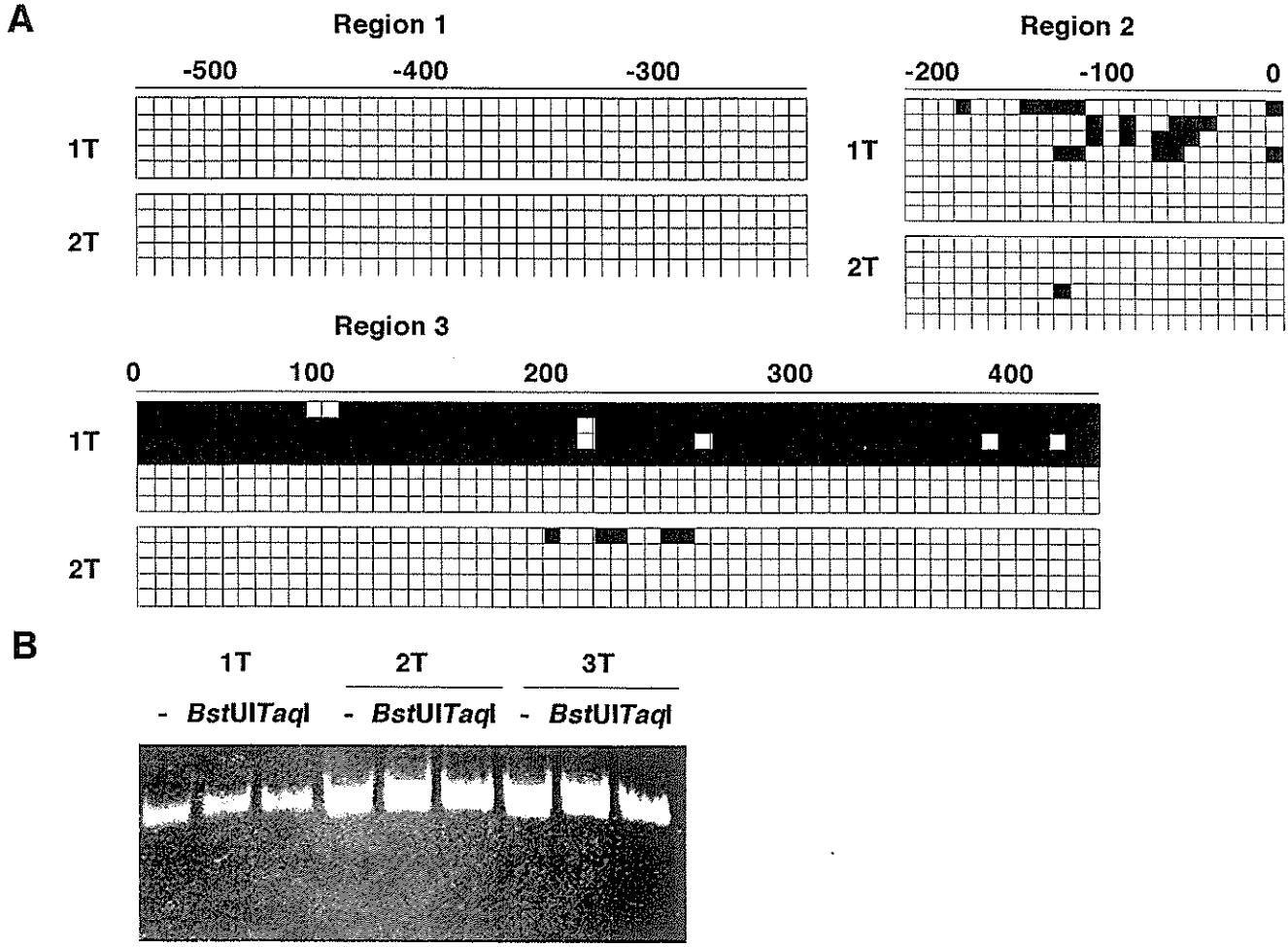

FIGURE 4 – Bisulfite sequencing analysis. *(a)* Bisulfite sequencing analysis of 2 gastric carcinoma tissues. GC tissue (1T) shows methylation of the MSP3′ region, and GC tissue (2T) lacks methylation of the MSP3′ region. Methylation of the *SOCS-1* gene was examined with 3 primer sets. Region 1 and 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