test. Tumor staging was performed according to TNM stage grouping.[27] In addition, methylation status of these genes was determined in 10 samples of normal gastric mucosa obtained endoscopically from 10 healthy, young individuals (age range, 22–35 yrs; average age, 26.4 yrs) with no clinical symptoms and no microscopic mucosal changes. Because written informed consent was not obtained, for strict privacy protection, all samples were disidentified before analyzing DNA methylation status. This procedure is in accordance with Ethical Guidelines for Human Genome/Gene Research enacted by the Japanese Government.

Genomic DNA Extraction and Methylatign Analysis

To examine DNA methylation patterns of the *RARβ*, *CRBP1*, and *TIG1* genes, we extracted genomic DNAs with a genomic DNA purification kit (Promega, Madison, WI) and performed methylation-specific polymerase chain reaction (PCR) (MSP) analysis.[28] In brief, 2 μg of genomic DNA were denatured by treatment with 2 M NaOH and modified with 3 M sodium bisulfate for 16 hours. DNA samples were purified with Wizard DNA purification resin (Promega), treated with 3 M NaOH, precipitated with ethanol, and resuspended in 25 μL water. Two-microliter aliquots were used as templates for PCR reactions. Primer sequences and annealing temperatures for MSP were as described previously (Table 1).[19,22,29]

Reverse Transcriptase-PCR

mRNA expression of *RARβ*, *CRBP1*, and *TIG1* was analyzed by reverse transcriptase (RT)-PCR. Total RNA was extracted with an RNeasy Mini Kit (QIAGEN, Tokyo, Japan), and 1 μg of total RNA was converted to cyclic DNA (cDNA) with a first-strand cDNA synthesis kit (Amersham Pharmacia Biotech, Uppsala, Sweden). Primer sequences and annealing temperatures were as described previously (Table 1).[19,23] RT-PCR products were subjected to 1.5% agarose gel electrophoresis, stained with ethidium bromide, and examined under ultraviolet light. *ACTB*-specific PCR products were amplified from the same RNA samples and served as internal controls.

TABLE 1
Primer Sequences for Methylation-Specific PCR, RT PCR, and Quantitative RT-PCR

Primer sequence	Annealing temperature
MSP (*RARβ*, methylated)	64 °C
F: 5′-GGT TAG TAG TTC GGG TAG GGT TTA TC-3′	
R: 5′-CCG AAT CCT ACC CCG ACG-3′	
MSP (*RARβ*, unmethylated)	55 °C
F: 5′-TTA GTA GTT TGG GTA GGG TTT ATT-3′	
R: 5′-CCA AAT CCT ACC CCA ACA-3′	
MSP (*CRBP1*, methylated)	70 °C
F: 5′-TTG GGA ATT TAG TTG TCG TCG TTT C-3′	
R: 5′-AAA CAA CGA CTA CCG ATA CTA CGC G-3′	
MSP (*CRBP1*, unmethylated)	67 °C
F: 5′-GTG TTG GGA ATT TAG TTG TTG TTG TTTT-3′	
R: 5′-ACT ACC AAA ACA ACA ACT ACC AAT ACT ACA-3′	
MSP (*TIG1*, methylated)	56 °C
F: 5′-GCG GGG TTC GGG GAT TTC-3′	
R: 5′-GTA CGC GAA CAA ACA AAC G-3′	
MSP (*TIG1*, unmethylated)	55 °C
F: 5′-GTG GGG TTT GGG GAT TTT GAT-3′	
R: 5′-ATA CAC AAA CAA ACA AAC ACA-3′	
RT-PCR (*CRBP1*)	53 °C
F: 5′-TTG TTG CCA AAC TGG CTC CA-3′	
R: 5′-ACA CTG GAG CTT GTC TCC GT-3′	
RT-PCR (*TIG1*)	68 °C
F: 5′-GAA AAA CCC CTT GGA AAT AGT CAG C-3′	
R: 5′-AGT GTG ACA CCT GTG TTG TCA TTT CC-3′	
RT-PCR (*ACTB*)	55 °C
F: 5′-CTGTCTGGCGGCACCACCAT-3′	
R: 5′-GCAACTAAGTCATAGTCCGC-3′	
Quantitative RT-PCR (*RARβ*)	60 °C
F: 5′-ACC ACT GGA CCA TGT AAC TCT AGT GT-3′	
R: 5′-GGC ATC AAG AAG GGC TGG A-3′	
Quantitative RT-PCR (*CRBP1*)	60 °C
F: 5′-CAA CAG TGA GCT GGG ACG G-3′	
R: 5′-GCC ACG CCC CTC CTT C-3′	
Quantitative RT-PCR (*TIG1*)	60 °C
F: 5′-GGC CGC GCG TGG AT-3′	
R: 5′-GGT TGT AGC GCT CTG TGC TG-3′	
Quantitative RT-PCR (*ACTB*)	60 °C
F: 5′-TCA CCG AGC GCG GCT-3′	
R: 5′-TAA TGT CAC GCA CGA TTT CCC-3′	

PCR: polymerase chain reaction; RT-PCR: reverse transcriptase-polymerase chain reaction; MSP: methylation-specific polymerase chain reaction; *RARβ*: retinoid acid receptor β; F: forward; R: reverse; *CRBP1*: cellular retinol-binding protein 1; *TIG1*: tazarotine-induced gene 1; RT-PCR: reverse transcriptase-polymerase chain reaction.

Quantitative RT-PCR Analysis

Total RNA was extracted with an RNeasy Mini Kit, and 1 μg of total RNA was converted to cDNA with a first-strand cDNA synthesis kit (Amersham Pharmacia Biotech). PCR was performed with the SYBR Green PCR Core Reagents kit (Applied Biosystems, Foster City, CA). Real-time detection of the emission intensity of SYBR Green bound to double-stranded DNA was performed with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) as described previously.[30] Primer sequences are listed in Table 1. We calculated the ratio of target gene mRNA expression levels between GC samples (T) and samples of corresponding nonneoplastic mucosa (N), with a T/N ratio < 0.5 representing reduced expression. *ACTB*-specific PCR products were amplified from the same RNA samples and served as internal controls.

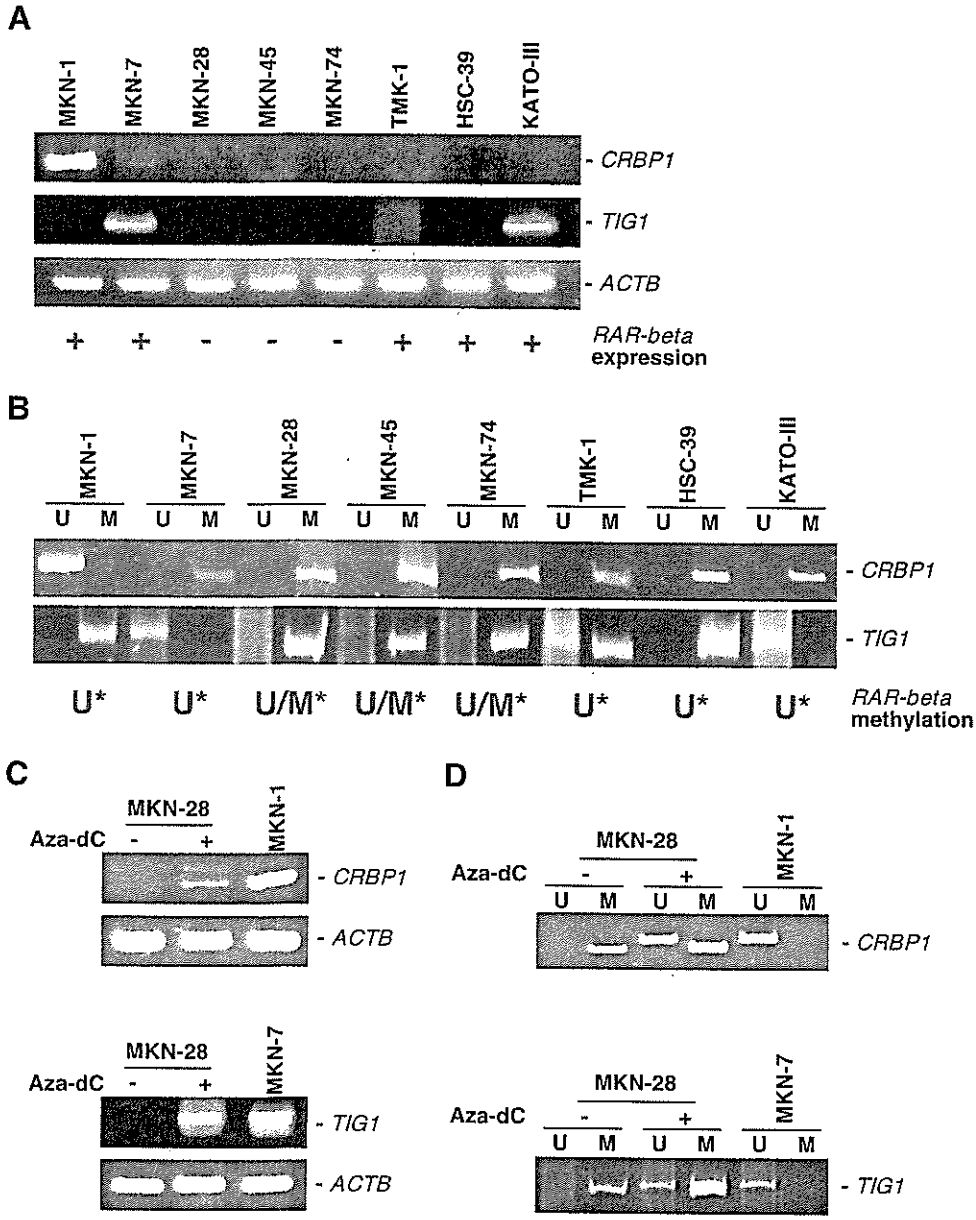

FIGURE 1. mRNA expression and DNA methylation status in gastric carcinoma (GC) cell lines. (A) Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of GC cell lines. Cellular retinol-binding protein 1 (*CRBP1*) was expressed only in the MKN-1 cell line. Tazarotene-induced gene 1 (*TIG1*) expression was identified in the MKN-7 and KATO-III cell lines. A plus sign indicates cell lines with retinoid acid receptor β (RARβ) expression and a minus sign indicates cell lines without RARβ expression (see Hayashi et al.[10]). Note that at least one of the three genes was inactivated in all of the GC cell lines tested. (B) Methylation-specific PCR (MSP) of *CRBP1* and *TIG1* in GC cell lines. Primer sets were either unmethylated (U) or methylated (M). The methylated allele for *CRBP1* was detected in the MKN-7, MKN-28, MKN-45, MKN-74, TMK-1, HSC-39, and KATO-III cell lines. The methylated allele for *TIG1* was detected in the MKN-1, MKN-28, MKN-45, MKN-74, TMK-1, and HSC-39 cell lines. U* indicates cell lines with an unmethylated *RAR*β gene, M* indicates cell lines with a methylated *RAR*β gene, and U/M* indicates cell lines with an unmethylated and a methylated *RAR*β gene (see Hayashi et al.[10]). (C) MSP in untreated and Aza-2′-deoxycytidine (Aza-dC)-treated MKN-28 cells. An unmethylated allele of both *CRBP1* and *TIG1* genes was identified in Aza-dC-treated cells. (D) RT-PCR of untreated and Aza-dC-treated MKN-28 cells. mRNA expression of both *CRBP1* and *TIG1* genes was identified in Aza-dC-treated cells.

Statistical Methods

Differences were analyzed statistically with the Fisher exact test and the Mann–Whitney *U* test. *P* values < 0.05 were considered statistically significant.

RESULTS

DNA Methylation Status and Expression of CRBP1 and TIG1 in GC Cell Lines

To determine whether hypermethylation of the *CRBP1* and *TIG1* genes induces transcriptional inactivation, we performed in vitro analysis with 8 GC cell lines. RT-PCR analysis showed that seven of the cell lines (MKN-7, MKN-28, MKN-45, MKN-74, TMK-1, HSC-39, and KATO-III) did not express *CRBP1* mRNA, and six of the cell lines (MKN-1, MKN-28, MKN-45, MKN-74, TMK-1, and HSC-39) did not express *TIG1* mRNA. Figure 1A,B shows that all GC cell lines that lacked *CRBP1* gene expression showed methylation of CpG sites within the *CRBP1* gene, and all GC cell lines that lacked *TIG1* gene expression showed methylation of CpG sites within the *TIG1* gene. Therefore, methylation status is associated with expression of both *CRBP1* and *TIG1*.

Next, we investigated whether methylation status of the *RAR*β, *CRBP1*, and *TIG1* genes was related (Fig. 1A,B). We previously reported on the *RAR*β methylation status in GC cell lines.[10] No clear relation was noted for the methylation status among the three

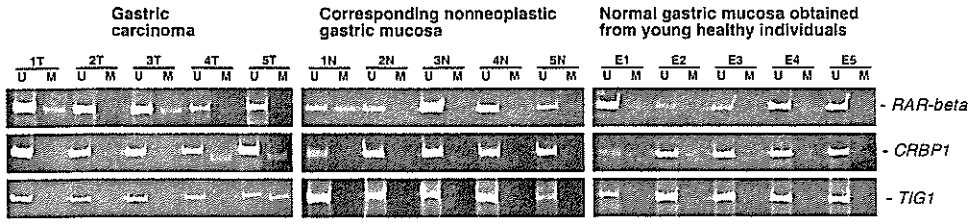

FIGURE 2. Methylation-specific polymerase chain reaction (MSP) analysis of retinoid acid receptor β (*RARβ*), cellular retinol-binding protein 1 (*CRBP1*), and tazarotene-induced gene 1 (*TIG1*) genes in gastric carcinoma, in corresponding nonneoplastic gastric mucosa, and in normal gastric mucosa obtained from young, healthy individuals. U indicates unmethylated polymerase chain reaction (PCR) products and M indicates methylated PCR products. A methylated allele of the *RARβ* gene was detected in Samples 1T and 3T. A methylated allele of the *CRBP1* gene was detected in Samples 4T and 5T. A methylated allele of the *TIG1* gene was detected in Sample 5T. In corresponding nonneoplastic gastric mucosa, a methylated allele of the *RARβ* gene was detected in Sample 1N. Methylated alleles of *CRBP1* and *TIG1* were not detected. In normal gastric mucosa from young, healthy individuals, no methylation was detected.

genes; however, our results showed that at least one of the these genes was hypermethylated and inactivated in all of GC cell lines tested.

To confirm whether transcriptional inactivation of *CRBP1* and *TIG1* causes DNA methylation, we treated MKN-28 cells with Aza-dC and then performed RT-PCR (Fig. 1C) and MSP (Fig. 1D) analyses. Treatment with Aza-dC restored *CRBP1* and *TIG1* expression, and an unmethylated allele was detected after Aza-dC treatment.

RARβ, CRBP1, and TIG1 Methylation Status and mRNA Expression Levels in GC Tissues

Representative MSP data for *RARβ*, *CRBP1*, and *TIG1* in GC tissues, in corresponding nonneoplastic mucosa, and in normal gastric mucosa from young, healthy individuals are shown in Figure 2. In the 42 GC samples, CpG island hypermethylation was detected at the following frequencies: 15 of 42 GC samples (36%) were hypermethylated for *RARβ*, 14 of 42 GC samples (33%) were hypermethylated for *CRBP1*, and 4 of 42 samples (10%) were hypermethylated for *TIG1*. The overall results are shown in Table 2. No clear association was found between the 3 genes in terms of methylation status (Table 3). Of the 42 GC samples, at least 1 of the 3 genes was hypermethylated in 24 samples (57%). We analyzed the correlation of methylation status of each gene with clinical characteristics (age, gender, *H. pylori* infection, T classification [depth of tumor invasion], N status [degree of lymph node metastasis], tumor stage, and histologic type) (Table 4). DNA methylation of the *CRBP1* gene was found more frequently in female patients than in male patients. No association was found between the GC samples with hypermethylation of at least one gene and clinical data (data not shown).

In 30 samples of corresponding nonneoplastic mucosa, DNA methylation of *RARβ* was found in 6 of 30 samples (20%), DNA methylation of *CRBP1* was found in 0 of 30 samples (0%), DNA methylation of *TIG1* was found in 1 of 30 samples (3%) (Fig. 2). Only *CRBP1* was methylated significantly more frequently in tumor tissue compared with nonneoplastic tissue (Table 5). Among 30 corresponding nonneoplastic mucosa samples, intestinal metaplasia was found in 22 samples. No correlation was observed between methylation of any of these genes and the presence of intestinal metaplasia (data not shown). There was no correlation of methylation status between tumor and nonneoplastic tissue from the same patient. Methylated alleles were not detected in any of 10 samples of normal gastric mucosa from young, healthy individuals (Fig. 2).

We also used quantitative RT-PCR analysis to determine whether methylation of the *RARβ*, *CRBP1*, and *TIG1* genes affects expression of the respective mRNA. Reduced expression of *RARβ*, *CRBP1*, and *TIG1* was found in 17 of 42 GC samples (40%), 16 of 42 GC samples (38%), and 8 of 42 GC samples (19%) (Table 2). The *RARβ* mRNA expression levels in tumor tissues with DNA methylation of *RARβ* (mean ± standard error [in arbitrary units], 2.40 ± 0.58) were significantly lower compared in the levels in tumor tissues without DNA methylation (8.35 ± 1.56; $P = 0.0087$; Mann–Whitney *U* test) (Table 6). The mRNA expression levels of both *CRBP1* and *TIG1* also were correlated with hypermethylation of the respective genes ($P = 0.0109$ for *CRBP1*; $P = 0.0003$ for *TIG1*; Mann–Whitney *U* test) (Table 6).

DISCUSSION

It is believed that *RARβ* functions as a tumor suppressor. Previous studies have shown that overexpression of *RARβ* induces growth arrest and apoptosis in several carcinoma cell lines.[10,31,32] In the current study, we investigated three genes associated with retinoic

TABLE 2
Summary of DNA Methylation and mRNA Expression of *RARβ*, *CRBP1*, and *TIG1* in Gastric Carcinoma

				TNM classification[a]					DNA methylation			RNA expression		
Patient no.	Gender	Age (yrs)	*H. pylori*	T	N	M	Stage	Histology[b]	*RARβ*	*CRBP1*	*TIG1*	*RARβ*	*CRBP1*	*TIG1*
8	Female	73	Negative	2	1	0	II	Intestinal	MET	MET	MET	R	R	R
4	Male	69	Positive	4	1	0	IV	Diffuse	MET	MET	MET	R	R	R
20	Male	77	Negative	2	0	0	IB	Intestinal	MET	MET	—[c]	R	R	—[d]
10	Male	81	Positive	2	2	0	IIIA	Diffuse	MET	MET	—	—	R	—
6	Female	61	Negative	3	2	0	IIIB	Intestinal	MET	MET	—	—	—	—
31	Female	88	Positive	4	3	0	IV	Diffuse	MET	MET	—	R	R	—
3	Female	62	Positive	3	3	0	IV	Diffuse	MET	—	MET	R	—	R
15	Male	76	Positive	1	0	0	IA	Intestinal	MET	—	—	R	—	—
41	Male	61	Positive	2	1	0	II	Diffuse	MET	—	—	—	—	R
2	Male	69	Positive	2	1	0	II	Intestinal	MET	—	—	—	—	—
25	Male	65	Negative	3	1	0	IIIA	Diffuse	MET	—	—	R	—	—
16	Male	69	Positive	3	1	0	IIIA	Intestinal	MET	—	—	R	—	—
36	Male	70	Positive	4	0	0	IV	Intestinal	MET	—	—	—	—	—
9	Female	74	Negative	4	2	0	IV	Diffuse	MET	—	—	R	—	—
5	Male	34	Negative	4	2	0	IV	Intestinal	MET	—	—	R	—	—
37	Female	74	Positive	2	0	0	IB	Intestinal	—	MET	—	—	R	—
34	Female	54	Positive	3	0	0	II	Diffuse	—	MET	—	—	R	—
38	Male	76	Positive	2	1	0	II	Diffuse	—	MET	—	—	R	—
33	Male	63	Positive	2	1	0	II	Intestinal	—	MET	—	—	—	—
13	Female	77	Positive	3	0	0	II	Intestinal	—	MET	—	—	R	—
30	Male	74	Positive	3	1	0	IIIA	Diffuse	—	MET	—	—	—	—
17	Female	53	Positive	2	2	0	IIIA	Diffuse	—	MET	—	—	R	R
32	Female	40	Positive	3	3	0	IV	Diffuse	—	MET	—	—	—	—
11	Male	66	Negative	3	1	0	IIIA	Intestinal	—	—	MET	—	—	R
1	Male	71	Positive	2	0	0	IB	Intestinal	—	—	—	—	—	—
24	Male	75	Negative	2	0	0	IB	Intestinal	—	—	—	—	—	R
40	Male	72	Positive	2	0	0	IB	Intestinal	—	—	—	—	—	—
42	Male	73	Positive	2	0	0	IB	Diffuse	—	—	—	R	R	—
23	Female	69	Positive	2	1	0	II	Intestinal	—	—	—	R	R	—
29	Male	79	Positive	2	1	0	II	Diffuse	—	—	—	—	—	—
28	Male	75	Positive	3	2	0	IIIB	Diffuse	—	—	—	—	—	—
12	Male	85	Positive	3	1	0	IIIA	Diffuse	—	—	—	—	—	—
21	Male	78	Negative	3	1	0	IIIA	Diffuse	—	—	—	—	—	R
26	Male	87	Positive	3	2	0	IIIB	Diffuse	—	—	—	—	—	—
35	Male	78	Positive	3	1	0	IIIA	Diffuse	—	—	—	R	R	—
18	Female	57	Positive	3	1	0	IIIA	Intestinal	—	—	—	R	R	—
22	Male	54	Negative	3	2	0	IIIB	Intestinal	—	—	—	R	R	—
27	Female	67	Positive	3	3	0	IV	Diffuse	—	—	—	—	—	—
14	Male	72	Positive	3	3	0	IV	Diffuse	—	—	—	R	R	—
39	Male	71	Positive	3	3	0	IV	Diffuse	—	—	—	—	—	—
7	Male	72	Positive	3	3	0	IV	Intestinal	—	—	—	—	—	—
19	Male	58	Positive	3	3	0	IV	Intestinal	—	—	—	R	—	—

RARβ: retinoid acid receptor *β*; *CRBP1*: cellular retinol-binding protein 1; *TIG1*: tazarotine-induced gene 1; *H. pylori*; *Helicobacter pylori*; T: tumor; N: lymph node; M: metastasis; MET: methylated; R: reduced expression.

[a] Stage was classified according to the criteria of the International Union Against Cancer TNM classification system, 6th edition, 2002.

[b] Histology was classified according to the criteria of Lauren.

[c] Unmethylated.

[d] Not reduced.

acid signaling. DNA hypermethylation of *CRBP1* was found in 33% of GC samples and was associated with low levels of mRNA expression. DNA hypermethylation of *TIG1* was found in 10% of GC samples and also was associated with low levels of mRNA expression. In addition, Aza-dC treatment induced demethylation of both the *CRBP1* gene and the *TIG1* gene and restored expression of both mRNAs in MKN-28 cells, suggesting that hypermethylation of both *CRBP1* and *TIG1* plays an important role in transcriptional silenc-

TABLE 3
Correlation of DNA Methylation Status of Retinoic Acid Signaling-Associated Genes in Gastric Carcinoma

	Methylation status				
	RARβ		*CRBP1*		
Methylation status	Methylated	Unmethylated	Methylated	Unmethylated	*P* value[a]
CRBP1					
Methylated	6	8			0.5159
Unmethylated	9	19			
TIG1					
Methylated	3	1			0.1220
Unmethylated	12	26			
TIG1					
Methylated			2	2	0.5902
Unmethylated			12	26	

RARβ: retinoid acid receptor β *CRBP1*: cellular retinol-binding protein 1; *TIG1*: tazarotine-induced gene 1.
[a] Statistical significance determined using the Fisher exact test.

TABLE 4
Association between DNA Methylation of Retinoic Acid Signaling-Associated Genes and Clinicopathologic Parameters

	No. of patients (%)								
	RARβ			*CRBP1*			*TIG1*		
Characteristic	M	U	*P* value[a]	M	U	*P* value	M	U	*P* value
Age									
> 65 yrs	10 (33)	20	NS	9 (30)	21	NS	3 (10)	27	NS
≤ 65 yrs	5 (42)	7		5 (42)	7		1 (8)	11	
Gender									
Male	10 (34)	19	NS	6 (21)	23	0.0148	2 (7)	27	NS
Female	5 (38)	8		8 (62)	5		2 (15)	11	
H. pylori infection									
Positive	9 (28)	23	NS	11 (34)	21	NS	2 (6)	30	NS
Negative	6 (60)	4		3 (30)	7		2 (20)	8	
T classification[b]									
T1/T2	6 (38)	10	NS	7 (44)	9	NS	1 (6)	15	NS
T3/T4	9 (35)	17		7 (27)	19		3 (12)	23	
N status[b]									
N0	3 (30)	7	NS	4 (40)	6	NS	0 (0)	10	NS
N1/N2/N3	12 (38)	20		10 (31)	22		4 (13)	28	
Stage[b]									
Stage I/II	5 (31)	11	NS	7 (44)	9	NS	1 (6)	15	NS
Stage III/IV	10 (38)	16		7 (27)	19		3 (12)	23	
Histologic type[c]									
Intestinal	8 (40)	12	NS	6 (30)	14	NS	2 (10)	18	NS
Diffuse	7 (32)	15		8 (36)	14		2 (9)	20	

RARβ: retinoid acid receptor β; *CRBP1*: cellular retinol-binding protein 1; *TIG1*; tazarotine-induced gene 1; M: methylated; U: unmethylated; NS: not significant; *H. pylori*: *Helicobacter* pylori.
[a] Statistical significance was determined using the Fisher exact test.
[b] Stage was classified according to the criteria of the International Union Against Cancer TNM classification of malignant tumors, 6th edition, 2002.[27]
[c] Histology was classified according to the criteria of Lauren.

ing in GC. We also confirmed that DNA methylation of *RARβ* is associated with low levels of mRNA expression in this study. It is important to note that there were several tumor samples with DNA hypermethylation that did not show low gene expression. This may be related to the extreme sensitivity of the MSP technique, which theoretically can detect as few as 0.1% of methylated cells.[28] In contrast, several samples showed low gene expression in the absence of DNA methylation. Alternative inactivating pathways, such

TABLE 5
Frequency of DNA Methylation of Retinoic Acid Signaling-Associated Genes in Gastric Carcinoma and Corresponding Nonneoplastic Mucosa

Measure	No. of patients (%)		*P* value[a]
	Methylated	Unmethylated	
RARβ methylation status			
Gastric carcinoma	15 (36)	27	0.1922
Corresponding nonneoplastic mucosa	6 (20)	24	
CRBP1 methylation status			
Gastric carcinoma	14 (33)	28	0.0002
Corresponding nonneoplastic mucosa	0 (0)	30	
TIG1 methylation status			
Gastric carcinoma	4 (10)	38	0.3932
Corresponding nonneoplastic mucosa	1 (3)	29	

RARβ: retinoid acid receptor β; *CRBP1*: cellular retinol-binding protein 1; *TIG1*: tazarotine-induced gene 1.
[a] Statistical significance was determine using the Fisher exact test.

TABLE 6
Association between DNA Methylation of Retinoic Acid Signaling-Associated Genes and mRNA Expression

Sample	No. of patients	Expression level[a]	*P* value[b]
RARβ			
Gastric carcinoma	42	6.22 ± 1.11[c]	
Methylated	15	2.40 ± 0.58	0.0087
Unmethylated	27	8.35 ± 1.56	
Corresponding nonneoplastic mucosa	42	10.24 ± 2.14	
CRBP1			
Gastric carcinoma	42	4.22 ± 1.83[c]	
Methylated	14	1.40 ± 0.28	0.0109
Unmethylated	28	5.63 ± 2.72	
Corresponding nonneoplastic mucosa	42	6.07 ± 0.91	
TIG1			
Gastric carcinoma	42	17.68 ± 5.59[d]	
Methylated	4	0.41 ± 0.05	0.0003
Unmethylated	38	19.50 ± 6.11	
Corresponding nonneoplastic mucosa	42	15.86 ± 5.18	

RARβ: retinoid acid receptor β; *CRBP1*: cellular retinol-binding protein 1; *TIG1*: tazarotine-induced gene 1.
[a] Mean ± standard error.
[b] Mann-Whitney *U* test.
[c] The units were arbitrary, and the *RARβ* and *CRBP1* mRNA expression levels were calculated by standardization with 1 μg total RNA of the MKN-1 cells, taken as 1.0.
[d] The units were arbitrary, and the *TIG1* mRNA expression levels were calculated by standardization with 1 μg total RNA of the MKN-7 cells, taken as 1.0.

as hemizygous deletion and alteration of transcription factors, may account for the low gene expression in these samples. The *RARβ* gene is located on chromosome 3p, and both the *CRBP1* gene and the *TIG1* gene are located on chromosome 3q. Loss of heterozygosity of chromosome 3p has been reported in 33% of GCs.[33]

DNA methylation of *RARβ* and *TIG1* was detected in corresponding nonneoplastic mucosa. Although the frequency of methylation of all 3 genes in GC was greater than in nonneoplastic tissue, statistical analysis revealed that only *CRBP1* was methylated significantly more frequently in tumor tissue compared with corresponding nonneoplastic mucosa. This suggests that DNA methylation of the *CRBP1* gene may contribute to gastric carcinogenesis. Conversely, there was no statistical difference between the *RARβ* and *TIG1* methylation status of tumor tissue and nonneoplastic tissue. Because the mRNA expression levels of these two genes in tumor tissues were correlated with DNA methylation, it is possible that these two genes may demonstrate monoallelic methylation in nonneoplastic tissue and biallelic methylation in tumors. Epi-

TABLE 7
Association between DNA Methylation of Retinoic Acid Signaling-Associated Genes and mRNA Expression

	No. cases	*RAR-beta* expression levels[a]	*P* value[b]
Gastric cancer	42	6.22 ± 1.11[c]	
Methylated	15	2.40 ± 0.58	0.0087
Unmethylated	27	8.35 ± 1.56	
Corresponding non-neoplastic mucosa	42	10.24 ± 2.14	
		CRBP1 expression levels	
Gastric cancer	42	4.22 ± 1.83[c]	
Methylated	14	1.40 ± 0.28	0.0109
Unmethylated	28	5.63 ± 2.72	
Corresponding non-neoplastic mucosa	42	6.07 ± 0.91	
		TIG1 expression levels	
Gastric cancer	42	17.68 ± 5.53[d]	
Methylated	4	0.41 ± 0.05	0.0003
Unmethylated	38	19.50 ± 6.11	
Corresponding non-neoplastic mucosa	42	15.86 ± 5.18	

[a] Mean ± SE
[b] Mann-Whitney *U*-test.
[c] The units are arbitrary, and we calculated the *RAR-beta* and *CRBP1* mRNA expression level by standardization with 1 μg total RNA of the MKN-1 cells, taken as 1.0.
[d] The units are arbitrary, and we calculated the *TIG1* mRNA expression level by standardization with 1 μg total RNA of the MKN-7 cells, taken as 1.0.

genetic changes, including DNA methylation, occur in premalignant and histologically normal gastric epithelium.[34] Recent evidence suggests that methylation of certain genes is associated with aging.[34] We confirmed that normal gastric mucosa from young, healthy individuals did not show hypermethylation of the three genes. Thus, aging may be involved in the methylation status of nonneoplastic mucosa. Age-related methylation of the *RARβ* and *TIG1* genes also may explain the possible link between aging and increased risk for GC. Because *CRBP1* methylation was not detected in corresponding nonneoplastic mucosa, *CRBP1* methylation may serve as a good biomarker for GC. It is known that *CRBP1* regulates intracellular retinoic acid transport, metabolism, and transcriptional activity. Thus, *CRBP1* affects the transcription of retinoic acid-responsive genes that regulate cell proliferation. It is possible that, even if *RARβ* is expressed, the loss of *CRBP1* may compromise retinoic acid metabolism by diminishing retinoic acid transport, resulting in blockage of the transcription of retinoic acid-responsive genes that regulate cell proliferation. In the current study, there was no clear association between DNA methylation of *RARβ* and *CRBP1*, indicating that inactivation of the two genes may not create a synergistic effect but may be a random event in gastric carcinogenesis. *TIG1* hypermethylation was detected in 10% of GC samples. In other malignancies, *TIG1* gene hypermethylation has been reported in 33–90% of samples.[22,23,35] Although *TIG1* has been identified as a potential tumor suppressor gene for prostate carcinoma,[24] the significance of *TIG1* inactivation in GC may be weak compared with that in other malignancies. In the current study, there was a significant difference between the methylation of *TIG1* in cell lines (6 of 8 cell lines; 75%) and in GC tissue samples (4 of 42 samples; 10%). The reason for this discrepancy is unclear. Because DNA methylation of *TIG1* reportedly has been correlated with tumor invasiveness in prostate carcinoma,[22] DNA methylation of *TIG1* may occur frequently in GC cell lines that were established from metastatic foci.[36]

DNA methylation occurs early in the multistep process of gastric carcinogenesis[9,37]; thus, it is believed that DNA methylation contributes to carcinogenesis but not to tumor progression. In the current study, although DNA methylation of the *CRBP1* gene was observed more frequently in female patients than in male patients, the other clinical characteristics, such as tumor stage, were not correlated with DNA methylation. In addition, the frequencies of methylation of three genes in GC were greater than those in corresponding nonneoplastic mucosa, indicating that DNA methylation of these genes may participate in gastric carcinogenesis but not in GC progression. In addition, although concordant hypermethylation of *RARβ* and *CRBP1*[19] and of *RARβ* and *TIG1*[22] has been reported, we found no statistically significant concordant hypermethylation among these

genes, and greater than 50% of the GC samples we studied showed DNA methylation of at least 1 of the 3 genes. These results suggest that alterations of retinoic acid signaling are involved widely in GC.

In conclusion, the results of the current study demonstrate that inactivation of retinoic acid signaling-associated genes, *RARβ*, *CRBP1*, and *TIG1*, due to DNA methylation occurs frequently in GC. Because DNA methylation can be reversed by demethylating agents, such as Aza-dC, it may be better to use a combination of retinoids and demethylating agents for therapeutic strategies in patients with GC.

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ORIGINAL PAPER

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A single nucleotide polymorphism in the *MMP-9* promoter affects tumor progression and invasive phenotype of gastric cancer

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Abstract *Purpose*: Matrix metalloproteinase-9 (MMP-9, gelatinase B) plays a key role in cancer invasion and metastasis by degradating the extracellular matrix (ECM) and basement membrane barriers. A cytosine (C)-thymidine (T) single nucleotide polymorphism (SNP) at position −1562 in the *MMP-9* promoter is reported to affect expression of this gene. The purpose of this study was to investigate the relation between the −1562 C/T polymorphism and the development and progression of gastric cancer. *Methods*: The study population included 177 gastric cancer patients and 224 healthy control subjects. The SNP in the *MMP-9* promoter was analyzed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and sequencing. Genotype frequencies were compared between patients and controls, and the association of genotypes with clinicopathological features was studied. *Results*: Genotype frequencies in gastric cancer patients were similar to those in control subjects ($P = 0.223$). However, significant association was found between degree of tumor invasion, clinical stage, and lymphatic invasion and the *MMP-9* polymorphism in gastric cancer patients ($P < 0.05$, for each). *Conclusions*: Our results indicate that the T allele in the *MMP-9* promoter is associated with the invasive phenotype of gastric cancer.

Keywords MMP-9 · SNP · Gastric cancer · Depth of tumor invasion · Lymphatic invasion

Abbreviations MMP-9: Matrix metalloproteinase-9 · SNP: Single nucleotide polymorphism · C: Cytosine · T: Thymidine · RFLP: Restriction fragment length polymorphism · ECM: Extracellular matrix · PCR: Polymerase chain reaction · OR: Odds ratio · CI: Confidence interval

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Introduction

Gastric cancer is one of the most common malignancies in the world and is the leading cause of death in Japan. Poor prognosis reflects the invasive and metastatic capabilities of cancer cells. Degradation of the extracellular matrix (ECM) and basement membrane barriers by matrix metalloproteinases (MMPs) plays an important role in tumor invasion and metastasis (Forget et al. 1999; Kohn and Liotta 1995; Liotta et al. 1991). MMP-9 (92-kDa gelatinase, type IV collagenase) is a member of the family of MMP genes, which encode zinc-dependent enzymes that break down ECM through the degradation of type IV collagen (Nagase and Woessner 1999), and promote tumor cell invasion. The prognostic value of MMP-9 expression by tumor tissues has been reported in relation to a variety of cancers (McDonnell and Matri-

sian 1990; Kallakury et al. 2001; Baker and Leaper 2003; Tanioka et al. 2003; Ozalp et al. 2003; Sakata et al. 2004).

Zhang et al. (Zhang et al. 1999) reported that a cytosine (C)-to-thymidine (T) transition at nucleotide −1562 in the *MMP-9* gene promoter generates low-activity (C/C) and high-activity (C/T, T/T) promoter genotypes, which influence gene transcription. This polymorphism is associated with the severity of coronary atherosclerosis in patients with coronary artery disease. However, there have been no studies of the relation between this polymorphism and malignancies. Various genetic and epigenetic alterations are associated with gastric carcinoma (Yasui et al. 2000; Oue et al. 2002; Oue et al. 2003). We previously reported that several polymorphisms are significantly associated with gastric cancer (Kuraoka et al. 2003; Matsumura et al. 2004). With respect to the role of the C/T polymorphism in transcriptional activity and degradation of ECM, we hypothesized that this polymorphism might also act as a genetic modifier in the development and progression of gastric cancer. Therefore, we conducted a case-control study to investigate the association between the different *MMP-9* promoter alleles and gastric cancer. Moreover, we examined the relation between the C/T polymorphism and the clinicopathological features of gastric cancer patients.

Materials and methods

Samples

A total of 401 peripheral blood samples from 224 healthy control subjects and 177 gastric cancer patients were used in the present study. Control subjects were randomly selected from among individuals visiting hospitals for regular health checks or because of symptoms such as appetite loss or epigastralgia. Control subjects were confirmed to be free of malignancy by gastrointestinal endoscopy and biopsy. Gastric cancer patients underwent surgical or endoscopic mucosal resection (EMR) at Hiroshima University Hospital or Hofu Institute of Gastroenterology. Gastric cancer patients were 129 men and 48 women; the median age was 65.1 ± 11.7 years. Gastric cancer was classified histologically according to the criteria of Lauren (Lauren 1965); 103 patients had intestinal type gastric cancer, and 74 patients had diffuse type. Intestinal type and diffuse type correspond to well-differentiated type and poorly differentiated type, respectively, of the histological classification system of the Japanese Gastric Cancer Association (Japanese Gastric Cancer Association 1998). Gastric cancer patients were grouped according to TNM classification (Sobin and Wittekind 2002) on the basis of the post-operative histopathological evaluation. Moreover, patients were assigned to two subgroups on the basis of whether they were positive or negative for lymphatic invasion or venous invasion at the time of diagnosis (Japanese Gastric Cancer Association 1998). All patients and control subjects gave written informed consent prior to enrollment in the study. The Human Genome Research Ethics Screening Committee of Hiroshima University School of Medicine approved the study.

DNA extraction and polymerase chain reaction-restriction fragment length polymorphism (PCR–RFLP) analysis

A genomic DNA purification kit (Promega, Madison, Wisc., USA) and a QIAamp 96 DNA Blood kit (QIAGEN, Valencia, Calif., USA) were used for DNA extraction. PCR- RFLP assay was used to determine MMP-9 genotypes as previously described (Zhang et al. 1999). To analyze the −1562 C/T polymorphism, we amplified a region of the *MMP-9* promoter with forward primer 5'-GCC TGG CAC ATA GTA GGC CC-3' and reverse primer 5'-CTT CCT AGC CAG CCG GCA TC −3' (Zhang et al. 1999). 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 $MgCl_2$, 0.3 μM of each primer, and 0.75 units of AmpliTaq Gold (Perkin-Elmer, Norwalk, Conn., USA). Amplification conditions were an initial activation step of 94 °C for 10 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 59 °C for 30 s, and extension at 72 °C for 30 s. The 435 bp fragment was then digested with *SphI* (New England BioLabs, Beverly, Mass., USA) overnight at 37 °C. *SphI* does not digest the C allele (435 bp) but generates 188 bp and 247 bp fragments for the T allele. Digests were separated by electrophoresis on 2.5% NuSieve 3:1 agarose (FMC BioProducts, Rockland, Me., USA) gels for 60 min at 100 V. Heterozygotes had a combination of both alleles (435 bp, 247 bp, and 188 bp bands).

Sequencing analysis of PCR products

The PCR products were purified and cloned into pCR2.1 (Invitrogen, Carlsbad, Calif., USA). Plasmid DNA was extracted from individual clones by alkaline lysis plasmid minipreparation. The cloned PCR fragments obtained from each sample were sequenced with both M13 reverse and M13 forward primers and the PRISM AmpliTaq DNA Polymerase FS Ready Reaction Dye Terminator Sequencing Kit (PerkinElmer ABI, Foster City, Calif., USA). Reamplified DNA fragments were purified with Centri-Sep Columns (Princeton Separations, Adelphia, N.J., USA) and sequenced with an ABI PRISM 310 Genetic Analyzer (PerkinElmer ABI).

Helicobacter pylori status

Confirmation of *H. pylori* status was based on combinations of histology, ^{13}C-urea breath test, and serum anti-*H. pylori* IgG antibody. One hundred and seventeen

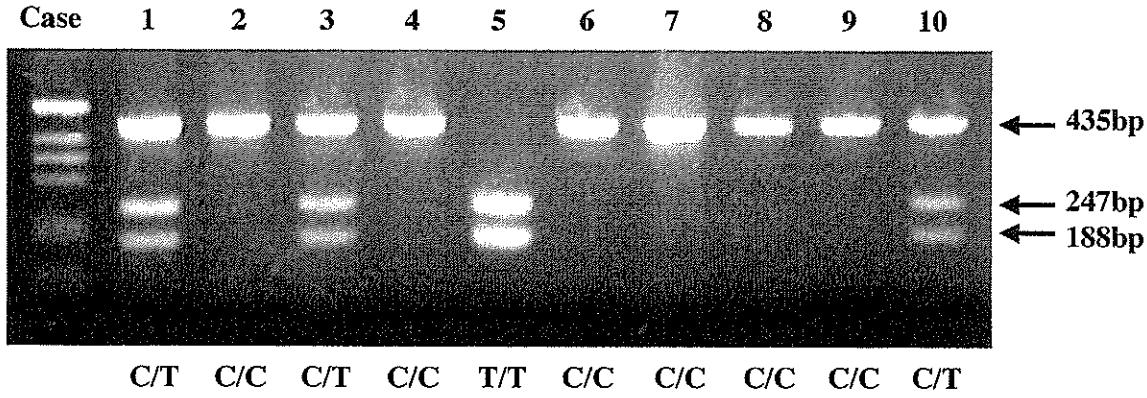

Fig. 1 PCR-RFLP analysis of the −1562 C/T polymorphism in ten patients with gastric cancer. The ethidium bromide-stained 2.5% NuSieve 3:1 agarose gel used for genotyping is shown. The target region (435 bp) of the *MMP-9* gene promoter was PCR amplified and digested with *SphI*, which cleaved the T allele to generate two fragments (247 bp and 188 bp) are indicated, but did not cut the C allele (435 bp). Numbers above the panel are case numbers. Genotypes are indicated below each case

of 224 healthy control subjects and 73 of 177 gastric cancer patients underwent these examinations. Four biopsy specimens were taken, two from the greater curvature of the antrum and two from the upper body of the stomach. When lesions suspected to be cancerous were noted, additional biopsies were performed. Of these four specimens were fixed in formalin and assessed for *H. pylori* by Giemsa staining. The cutoff value of ^{13}C-urea breath test was 3.5‰. If one or more test were positive, patient was judged to be infected with *H. pylori*. If all *H. pylori* tests were negative, the patient was considered uninfected.

Statistical analysis

Statistical analysis was performed with the use of Fisher's exact test. $P < 0.05$ was considered statistically significant. Odds ratios (ORs) and 95% confidence intervals (95% CIs) were used to estimate the risk of association with genotypes. ORs for the genotypes were calculated by the logistic regression model, with adjustments for age and sex; logistic regression analysis was performed for the association between genotypes and clinicopathological factors (SPSS 11.0, SPSS, Chicago, Ill., USA).

Results

Genotyping and genotype distributions in gastric cancer patients and control subjects

We examined the −1562 C/T polymorphism in the *MMP-9* gene promoter by PCR-RFLP in gastric cancer patients and control subjects. Three PCR-RFLP patterns were detected (Fig. 1). Patients 2, 4, 6, 7, 8, and 9 had a single 435 bp band (C/C genotype), and patient 5 had 247 bp and 188 bp bands (T/T genotype). Patients 1, 3, and 10 had all three bands, indicating the heterozygous genotype (C/T). Subsequent sequencing of representative cases confirmed the genotypes of patients 2 and 5 (Fig. 2). Distribution of the −1562 C/T polymorphism genotypes in gastric cancer patients and control subjects is shown in Table 1. Distribution of genotypes in controls was in good agreement with Hardy-Weinberg equilibrium; distribution in cases also agreed with Hardy-Weinberg equilibrium. Frequency of the T allele (C/T and T/T) was similar between gastric cancer patients (24.9%) and control subjects (30.3%; $P = 0.22$, OR = 0.75, 95% CI = 0.48–1.18). In addition, genotype frequencies (C/C vs C/T + T/T) did not differ by sex, age, or *H. pylori* status.

Fig. 2 Representative electropherogram for each genotype for the −1562 C/T polymorphism of *MMP-9*. Genotype of the SNP was C/C in case 2 and T/T in case 5

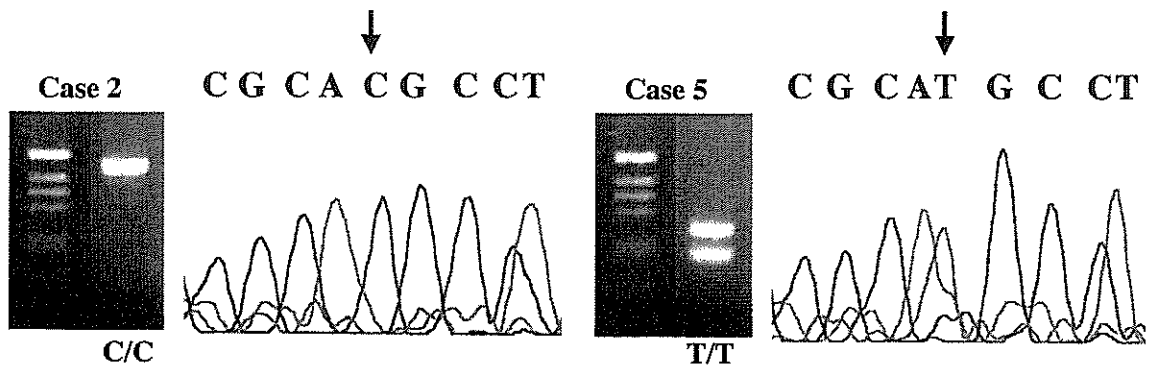

Table 1 *MMP-9* Genotype distributions in study subjects (*OR* odds ratio, *CI* confidence interval)

	Controls[a] (%) (n = 224)	Patients (%) (n = 177)	P value[b]	Crude OR[c] (95% CI)
Overall				
C/C	156 (69.7)	133 (75.1)		
C/T	63 (28.1)	38 (21.5)		
T/T	5 (2.2)	6 (3.4)	0.223	0.758 (0.486–1.182)
Sex				
Male				
C/C	73 (68.2)	97 (75.2)		
C/T	32 (29.9)	28 (21.7)		
T/T	2 (1.9)	4 (3.1)	0.235	0.708 (0.400–1.252)
Female				
C/C	83 (70.9)	36 (75.2)		
C/T	31 (26.5)	10 (20.8)		
T/T	3 (2.6)	2 (4.2)	0.597	0.813 (0.378–1.748)
Age (years)				
<65				
C/C	78 (67.8)	54 (71.1)		
C/T	35 (30.4)	20 (26.3)		
T/T	2 (1.8)	2 (2.6)	0.636	0.858 (0.456–1.613)
≥65				
C/C	78 (71.6)	79 (78.2)		
C/T	28 (35.7)	18 (17.8)		
T/T	3 (2.7)	4 (4.0)	0.267	0.701 (0.373–1.316)
H. pylori infection				
Negative	n = 42	n = 29		
C/C	32 (76.2)	25 (86.2)		
C/T	9 (21.4)	3 (21.7)		
T/T	1 (2.4)	1 (3.5)	0.297	0.512 (0.143–1.827)
Positive	n = 75	n = 44		
C/C	55 (73.3)	32 (72.7)		
C/T	19 (25.3)	12 (27.3)		
T/T	1 (1.4)	0 (0.0)	0.942	1.031 (0.446–2.383)

[a]Observed genotype distribution of controls was in agreement with Hardy-Weinberg equilibrium
[b]Association was analyzed by Fisher's exact test. P are values for C/T + T/T genotype relative to C/C genotype
[c]Odds ratios are for C/T + T/T genotypes relative to C/C genotype

Association between genotypes and clinicopathological features

We next analyzed the association between the –1562 C/T genotypes and clinicopathological features among gastric cancer patients (Table 2). Gastric cancer patients with the T allele (C/T and T/T) showed deeper invasion over the submucosal (sm) layer than did patients with the C/C genotype (crude OR = 2.61, 95% CI = 1.07–6.33). Advanced-stage gastric cancers were significantly more frequent in patients with the T allele (C/T and T/T) than in patients with the C/C genotype (stage I vs stages II, III, and IV, crude OR = 2.25, 95% CI = 1.12–4.50). Furthermore, there was a significant difference in genotype distribution (C/T + T/T vs C/C) with respect to lymphatic invasion (lymphatic invasion positive vs negative, crude OR = 2.27, 95% CI = 1.09–4.73), and risk of venous invasion of gastric cancer was increased (enhanced risk = 1.98) in patients with the T allele (venous invasion positive vs negative, crude OR = 1.98, 95% CI = 0.99–3.96). Histological classification (diffuse type vs intestinal type) and lymph node metastasis was not significantly associated with genotype. Logistic regression analysis revealed that genotype associations with depth of tumor invasion (adjusted OR = 2.61, 95% CI = 1.07–6.34), TNM classification (adjusted OR = 2.26, 95% CI = 1.12–4.54), and lymphatic invasion (adjusted OR = 2.27, 95% CI = 1.09–4.73) remained significant even after adjustments for age and sex.

Discussion

In the present study, we examined whether risk of gastric cancer is associated with the –1562 C/T polymorphism in the promoter of *MMP-9* in a Japanese population. It has been suggested that carcinogenesis is a multicellular and multistage process in which breakdown of the microenvironment is required for conversion of normal tissue to tumor (Park et al. 2000). Although we hypothesized that the *MMP-9* polymorphism alters the microenvironment and may be involved in the process of carcinogenesis, the allele frequencies in the gastric cancer patients were similar to those in controls. Our findings suggest that the T allele does not enhance susceptibility to the development of gastric cancer. In contrast, we found a significant association between this polymorphism and clinicopathological features, specifically depth of tumor invasion, TNM classification, and lymphatic invasion. The T allele was detected more frequently in patients with advanced-stage gastric cancers than in those with the C/C genotype (OR = 2.26). Moreover, lymphatic invasion was significantly enhanced in gastric cancer patients with the T allele. However, lymph node metastasis showed no signifi-

Table 2 Association between −1562 C/T polymorphism in *MMP-9* promoter and clinicopathological features of gastric cancer

	Genotype (%)			OR[a] (95%CI)	
	C/C	C/T	T/T	Crude	Adjusted[b]
Age (year)					
<65 (n = 76)	54 (71.1)	20 (26.3)	2 (2.6)		
≥65 (n = 101)	79 (78.2)	18 (17.8)	4 (4.0)	0.683 (0.345–1.356)	
Sex					
Male (n = 129)	97 (75.2)	28 (21.7)	4 (3.1)		
Female (n = 48)	36 (75.0)	10 (20.8)	2 (4.2)	0.990 (0.460–2.129)	
Histological classification[c]					
Intestinal (n = 103)	81 (78.6)	18 (17.5)	4 (3.9)		
Diffuse (n = 74)	52 (70.3)	20 (27.0)	2 (2.7)	1.558 (0.784–3.093)	1.601 (0.778–3.297) P = 0.201
Depth[d]					
m (n = 51)	44 (86.3)	6 (11.8)	1 (1.9)		
sm ~ (n = 126)	89 (70.6)	32 (25.4)	5 (4.0)	2.613 (1.079–6.331)	2.610 (1.074–6.340) P = 0.034
Lymphatic invasion[e]					
Negative (n = 78)	65 (83.3)	12 (15.4)	1 (1.3)		
Positive (n = 99)	68 (68.7)	26 (26.3)	5 (5.0)	2.279 (1.097–4.736)	2.274 (1.092–4.736) P = 0.028
Venous invasion[e]					
Negative (n = 95)	77 (81.1)	16 (16.8)	2 (2.1)		
Positive (n = 82)	56 (68.3)	22 (26.8)	4 (4.9)	1.986 (0.994–3.969)	1.984 (0.993–3.967) P = 0.053
Lymph node metastasis					
N (-) (n = 114)	89 (78.1)	22 (19.3)	3 (2.6)		
N (+) (n = 63)	44 (69.8)	16 (25.4)	3 (4.8)	1.537 (0.765–3.088)	1.537 (0.761–3.102) P = 0.230
TNM classification[f]					
Stage I (n = 99)	81 (81.8)	16 (16.2)	2 (2.0)		
Stage II, III, IV (n = 78)	52 (66.7)	22 (28.2)	4 (5.1)	2.250 (1.123–4.507)	2.260 (1.124–4.547) P = 0.022
H. pylori infection[g]					
Negative (n = 29)	25 (86.2)	3 (10.3)	1 (3.5)		
Positive (n=44)	32 (72.7)	12 (27.3)	0 (0.0)	0.427 (0.123–1.484)	0.416 (0.117–1.473) P=0.174

[a]Odds ratios (ORs) and 95% confidence intervals (CIs) for clinicopathological features with reference to the *MMP-9*promoter polymorphism (C/T + T/T to C/C genotypes)
[b]Adjusted for age and sex, with a logistic regression model
[c]Gastric cancer classified histologically according to the criteria of Lauren
[d]Depth of tumor invasion. (*m* tumour without invasion of the submucosa. *sm*~ tumour invades over the submucosal layer)
[e]Lymphatic invasion and venous invasion classified according to the criteria of Japanese Classification of Gastric Carcinoma, 2nd English edn
[f]TNM grades were according to the criteria of the TNM Classification of Malignant Tumors, 6th edn
[g]*H. pylori*status was based on histology, ^{13}C-urea breath test, and serum anti-*H. pylori* IgG antibody

cantly associated with genotype. Establishment of metastasis requires the serial processes such as invasion, migration, implantation, and regrowth of cancer cells at the metastatic site. The *MMP-9* polymorphism may affect the initial invasion step of lymph node metastasis. The present findings support our hypothesis that the −1562 C/T polymorphism may have a profound impact on progression and invasion of gastric cancer. To our knowledge, this is the first study to investigate the association of the −1562 C/T polymorphism of *MMP-9* with development and malignant phenotypes of gastric cancer.

The association between the *MMP-9* polymorphism and invasive phenotype of gastric cancer is consistent with the biological function of MMP-9. Overexpression of MMP-9 has been observed in a variety of cancers including gastric cancer, breast cancer, prostate cancer, brain cancer, melanoma, and lymphoma (Murray et al. 1998; Hujanen et al. 1994; Jones et al. 1999; Rao et al. 1993; Sehgal et al. 1996; Chicoine et al. 2002). In gastric cancer, expression of MMP-9 is associated with pathological features such as TNM stage, lymphatic invasion, and tumor depth (Zhang et al. 2003). Furthermore, expression of MMP-9 is associated with poor prognosis in renal cell carcinoma patients (Kallakury et al. 2001). In breast cancer patients, plasma MMP-9 activity is also associated with prognosis (Baker and Leaper 2003). These findings clearly indicate that MMP-9 plays an important role in tumor progression and invasion. Moreover, the C/T polymorphism in *MMP-9* has functional significance. The *MMP-9* promoter with the T allele shows significantly higher transcriptional activity than the C allele in cultured macrophages, and this polymorphism influences the atherosclerotic phenotype (Zhang et al. 1999). The *MMP-9* gene promoter contains binding sites for AP-1, NF-kB, Sp-1, and Ets

transcription factors (Gum et al. 1996). In particular, Ets-1 expression is upregulated together with MMP-9 (Behrens et al. 2001), and this upregulation correlates with tumor invasion in gastric cancer (Nakayama et al. 1996). This polymorphism is located within a transcription factor binding site, and that changing the allele may affect transcription factor binding and therefore expression of MMP-9 (Zhang et al. 1999). We also found the *MMP-9* polymorphism to be associated with depth of tumor invasion, lymphatic invasion, and TNM classification of gastric cancer. Taken together, these results suggest that the T allele may affect expression of MMP-9 in gastric cancer, increasing ECM degradation and leading to subsequent invasion of cancer cells.

Conclusion

In conclusion, our findings suggest that the T allele of the *MMP-9* promoter may affect expression of MMP-9 and is closely related to the invasive phenotype of gastric cancer. Recently, in vitro studies showed that MMP-9 production is increased more than two times by tumor necrosis factor-alpha (TNF-alpha) (Ferrand et al. 2002) and more than seven times by gastrin (Wroblewski et al. 2002). Moreover, Chicoine et al. (Chicoine et al. 2002) reported that MMP-9 expression is controlled by the methylation status of the *MMP-9* promoter. Further studies of the association between the levels of MMP-9 expression and the −1562 C/T polymorphism in gastric cancer patients are needed with increased numbers of cases to confirm our findings.

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ORIGINAL ARTICLE

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G1P3, an interferon inducible gene 6-16, is expressed in gastric cancers and inhibits mitochondrial-mediated apoptosis in gastric cancer cell line TMK-1 cell

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Abstract Expression of an interferon inducible gene 6-16, G1P3, increases not only in type I interferon-treated cells but also in human senescent fibroblasts. However, the function of 6-16 protein is unknown. Here we report that 6-16 is 34 kDa glycosylated protein and localized at mitochondria. Interestingly, 6-16 is expressed at high levels in gastric cancer cell lines and tissues. One of exceptional gastric cancer cell line, TMK-1, which do not express detectable 6-16, is sensitive to apoptosis induced by cycloheximide (CHX), 5-fluorouracil (5-FU) and serum-deprivation. Ectopic expression of 6-16 gene restored the induction of apoptosis and inhibited caspase-3 activity in TMK-1 cells. Thus 6-16 protein has anti-apoptotic function through inhibiting caspas-3. This anti-apoptotic function is expressed through inhibition of the depolarization of mitochondrial membrane potential and release of cytochrome c. By two-hybrid screening, we found that 6-16 protein interacts with calcium and integrin binding protein, CIB/KIP/Calmyrin (CIB), which interacts with presenilin 2, a protein involved in Alzheimer's disease. These protein interactions possibly play a pivotal role in the regulation of apoptosis, for which further detailed analyses are need. These results overall indicate that 6-16 protein may have function as a cell survival protein by inhibiting mitochondrial-mediated apoptosis.

Keywords Interferon inducible protein 6-16 · G1P3 · Mitochondria · Apoptosis · CIB · Survival gene · Bcl-2

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Introduction

Interferon inducible gene 6-16, G1P3, was first identified as one of the genes that are induced by interferon α and β [1]. It locates on chromosome 1p35 [2] and produces three types of mRNA by differential splicing between exon2 and exon3 [3]. The most abundant mRNA (B type) encodes a 14 kDa hydrophobic protein of 130 amino acids. Although extensive studies on the upstream regulatory region of 6-16 gene are well reported [4], little is known that the function or role of 6-16 protein in interferon treated cells. We isolated several cDNA clones that are expressed at higher level in senescent human fibroblasts than in young counterparts and found that one of these cDNA clones is 6-16 [5]. One of the characteristics of senescent human fibroblasts is that the cells do not proliferate but are viable for relatively long period of time, usually for more than a year in a

culture dish, without apparent apoptosis. SV40 large T antigen overcomes normal senescent pathway by inactivating some proteins, which are associated with cell proliferation, such as tumor suppressor gene p53 and pRB proteins. Interestingly, 6-16 is still expressed in SV40-transformed cells after extending their life-span [6]. 6-16 expression may be independent from p53 pathway and/or pRB pathway. Interestingly, we found that human senescent fibroblasts express 6-16 by producing interferon-β by autocrine mechanism. Interferon regulatory factor 1, IRF-1, is a major regulator of the interferon signaling pathway. Treatment of anti-IRF-1 antibody to human senescent cells or life-extended SV40-transformed fibroblasts resulted in down-regulation of 6-16 expression [6]. It is still unknown how senescent cells are protected from apoptosis, because senescent cells could be attached to the dish and are viable for more than 1 year with just a medium change; hence, it is believed that some anti-apoptotic gene may be protecting the senescent cells from apoptosis.

Attenuation of apoptosis appears recession for establishment and maintenance of transformed phenotype [7]. We found that 6-16 gene is expressed at a high level in immortalized cells and in gastrointestinal tumor cells. These data suggest that an increase in expression of 6-16 is associated with attenuation of apoptosis.

Mitochondria is an important organelle for the control of apoptosis, in addition to the role as the center of energy metabolism, and influence the commitment of cell death by regulating the mitochondrial permeability and membrane potential [8–11]. Bcl-2, an anti-apoptotic protein, is known to be located on mitochondria and expressed at high level in some tumor cells and tissues [11]. We report here that 6-16 is relatively expressed in cancer cells and tissues, and 6-16 is a novel anti-apoptotic protein located in the mitochondria and can be a new target for cancer chemotherapy and mitochondrial diseases.

Materials and methods

Northern blotting and RT-PCR

RNA isolation and Northern blotting were performed as described previously [6]. All 6-16 splice valiants are recognized by the probe for Northern analysis. RT-PCR was performed according to the protocol with the Thermoscript one-step RT-PCR system (Invitrogen, USA). For the detection of B and C type of 6-16 spliced valiants, we used 5′-GGGTGGAGGCAGGTGAGAATGCGG-3′ as the forward primer and 5′-TGACCTTCATGGCCGTCGGAGGAG-3′ as the reverse primer. Samples were incubated at 50°C for 30 min and denatured at 94°C for 2 min, and then cycled 32 times with 30 s at 94°C and 30 s at 62°C followed by a final extension step of 5 min at 68°C. GAPDH was used for internal control for validating RNA amounts as described before [12].

In situ mRNA hybridization analysis

In situ mRNA hybridization (ISH) was performed as described previously [13] with minor modification. Briefly, an interferon inducible gene 6-16-specific oligonucleotide probe was designed complementary to the 5′-end of human 6-16 mRNA transcript (GenBank NM022873). The DNA oligonucleotide sequence 5′-CGCCGCCCCCATTCAGGA-3′ was of the antisense orientation and hence complementary to 6-16 mRNA. To verify the integrity and lack of degradation of mRNA in each sample, we used a d(T) oligonucleotide. All DNA probes were synthesized with six biotin molecules (hyperbiotinylated) at the 3′-end via direct coupling using standard phosphoramidite chemistry (Research Genetics, Huntsville). ISH was carried out using the Microprobe manual staining system (Fisher Scientific, Pittsburgh). A positive reaction in this assay stained red. Control for endogenous alkaline phosphatase included treatment of sample in the absence of the biotinylated probe and use of chromogen alone. To check the specificity of the hybridization signal, the following controls were used: RNAse pretreatment of tissue sections, substitution of the antisense probe with a biotin-labeled sense probe, and competition assay with unlabeled antisense probes. No or markedly decreased signals were obtained after either of these treatments.

Antibody

Rabbit polyclonal antibody against human 6-16 was raised against synthetic peptide (YATHKYLDSEEDEE) corresponding to amino acid residues 117–130 of human 6-16, and was purified by MAbTrap GII affinity chromatography kit (Amersham Bioscience, USA). This antibody was sufficient for immunoblotting but insufficient for immunoprecipitation and immunostaining of 6-16 protein. Another rabbit polyclonal anti-human 6-16 antibody (OT904-1A) was also prepared against synthesis peptides (VEAGKKKCSESSDSG) corresponding to amino acid residues 21–35 of human 6-16. This antibody was sufficient for immunostaining. Mouse monoclonal anti-human CIB antibody was kindly provided by Leslie V. Parise. Anti-cytochrome c antibody (clone 7H8.2C12 which recognizes the denatured form of human, mouse and rat cytochrome c; BD Pharmingen, USA), monoclonal anti-human Bcl-2 antibody (clone124, Upstate Biotechnology, USA) and polyclonal anti-Bax antibody (Upstate Biotechnology) were used for immunoblot and immunoprecipitation. Anti-cytochrome c antibody (clone 6H2.B4 which recognizes the native form of human, mouse and rat cytochrome c; BD Pharmingen) was used for immunostaining. Polyclonal anti-GST rabbit antibody (New England Biolabs, USA) and polyclonal anti-MBP antibody (kindly provided by Dr. M. Nakata, Sumitomo Electric Industries, Japan) were used for immunoblot.

Cell culture, PI staining, caspase-3 activity assay, and mitochondrial membrane potential assay

The 0.8 kb fragment encoding full length 6-16 was inserted in the pCXN vector, which was digested with Xho I and blunted with klenow fragment. To establish stable transfected TMK-1 cells expressing 6-16 (TMK-1-6-16), TMK-1 cells were transfected with pCXN/6-16 vector and were selected for neomycin resistance with 200 μg/ml Geneticin (Invitrogen, USA). Gastric cancer cell lines, TMK-1, TMK-1-6-16 and MKN-28 were cultured at 37°C and 5% CO_2 in RPMI 1640 (Invitrogen) supplemented with 10% FCS and Antibiotic-Antimycotic (Invitrogen). A normal human fetal fibroblast strain, TIG-3, was cultured in Dulbecco's modified Eagle medium (Invitrogen) supplemented with 10% FCS and Antibiotic-Antimycotic (Invitrogen).

Apoptosis was induced by actinomycin D (10, 20 and 40 μg/ml) for 24 h, cycloheximide (10, 30 and 100 μM) for 6 h, H_2O_2 (0.5, 5 and 50 μM) for 6 h, etoposide (200, 400 and 600 μM) for 24 h, bleomycin (100, 200 and 400 μg/ml) for 24 h, 5-FU (100, 200 and 400 μg/ml) for 24 h, aphidicolin (100, 200 and 400 μM) for 24 h or serum-deprivation for 60 h. Cells were collected and stained with PI-RNase solution (BD Biosciences, USA), and were analyzed for DNA content by Flow cytometry on a FACS Calibur. For DNA ladder analysis, apoptotic DNA was extracted by lysis buffer (50 mM Tris-HCl pH7.5/ 20 mM EDTA/1% NP40), and run on 2% agarose gel.

For Caspase-3 activity analysis, cells were grown in six-well plates overnight, and were treated with CHX (10 and 30 μM) for 6 h or with 5-FU (80 μg/ml) for 24 h. Cells were collected and incubated with PhiPhiLux substrate buffer (OncoImmunin Inc., Gaithersbury, MD, USA) for 60 min at 37°C in 5% CO_2 incubator, and were analyzed by Flow cytometry on a FACS Calibur.

For mitochondrial membrane potential analysis, cells were grown in six well plate overnight, and were treated with CHX (10 and 30 μM) or 5-FU (50 and 100 μg/ml) for an hour. Cells were collected and incubated with Mitosensor reagent buffer (Clontech) for 30 min at 37°C in CO_2 incubator, and were analysis by Flow cytometry on a FACS Calibur.

Immunofluorescence analysis

Cells grown on eight-well Lab-Tek chamber slides (Nalge Nunc International, Napaperville, IL) were fixed in 4% paraformaldehyde / PBS (pH 7.4) for 20 min and permeabilized in blocking buffer (0.2% Triton X-100/ 3% BSA/PBS) for 30 min. The cells were incubated for 1 h at room temperature with mouse anti cytochrome c monoclonal antibody (6H2.B4) diluted 1:100, or with rabbit anti-human 6-16 polyclonal antibody (OT904-1A) diluted 1:100 in blocking buffer. The cells were washed four times in PBS and incubated with Alexa FluorTM 488 goat anti-mouse IgG conjugate (Molecular Probes, USA) or with Alexa FluorTM 488 goat anti-rabbit IgG conjugate (Molecular Probes) diluted 1:200 in blocking buffer for an hour at room temperature. Immunofluorescent staining was analyzed using a confocal laser-scanning microscope (LSM510, Carl Zeiss, Germany).

Mitochondria were labeled in intact cells with Mito-Tracker CM-H_2XRos (Molecular Probes). Cells were incubated in the MitoTracker medium (final concentration 500 nM) for 45 min before finishing to treat with CHX or 5-FU. Then, the cells were fixed, permeabilized and double-labeled with anti-cytochrome c antibody (6H2.B4) as described above.

Cell fractionation, immunoprecipitation and immunoblot analysis

For isolation of cellular fraction, cells were suspended in sucrose-supplemented extraction buffer (SCEB, 300 mM sucrose, 10 mM HEPES pH 7.4, 50 mM KCl, 5 mM EGTA, 5 mM $MgCl_2$, 1 mM DTT, protease cocktail), left on ice for 30 min, and homogenized by 70 strokes in an ice-cold Dounce homogenizer. Unbroken cells and nuclei were pelleted by centrifugation for 10 min at 2,000×*g*, and the supernatant was further spun at 13,000×*g* for 20 min to separate mitochondria-rich fraction from cytosol fraction. The pellet was resuspended in 50 μl of SCEB (mitochondria fraction), and the supernatant was cytosolic fraction.

For co-immunoprecipitation experiments, cells were lysed in NP-40 lysis buffer (50 mM Tris-HCl, pH7.4, 120 mM NaCl, 1% NP-40) containing protease inhibitor cocktail (Roche, USA). Antigen-antibody reaction was performed by incubating 0.5 ml (500 μg protein) of the cell extract with rabbit anti-human 6-16 antibody (117–130), mouse anti-CIB antibody, rabbit anti-Bax antibody or mouse anti-Bcl-2 antibody overnight at 4°C. The immunocomplex were incubated with 50 μl protein-G-Sepharose for 3 h at 4°C, and the beads were washed three times with NP-40 lysis buffer and boiled in Laemmli buffer.

For immunoblot analysis, the proteins were separated on 13% or 15% SDS-PAGE, and transferred to Immobilon-P (Millipore) and immunoblotted with anti-6-16 antibody (117–130), anti-CIB antibody, anti-Bcl-2 antibody, anti-Bax antibody or anti-cytochrome c antibody (7H8.2C12) diluted 1:500. The signal was detected using ECL-Plus (Amersham Bioscience).

Yeast two-hybrid assay

Dr Y. Takai (Osaka University, Suita, Japan) kindly supplied yeast L-40 strain and pBTM116/HA for yeast two-hybrid screening. A strain of L40 carrying pBTM116/6-16 was transformed with pGADGH HeLa cDNA library (Clontech). Approximately 1×10^6 transformants were screened for the growth on SD medium

plated lacking Trp, Leu, and His as evidenced by transactivation of a LexA-HIS3 reporter gene and His prototrophy. His+ colonies were scored for β-galactosidase activity. Plasmids harboring cDNAs were recovered from positive colonies and introduced by electroporation into *E. coli* HB101 on the M9 plate lacking Leu. Then the plasmids were recovered from HB101 and transformed again into L40 containing pBTM116HA/6-16. The nucleotide sequences of plasmid DNAs were determined.

Interaction of proteins in vitro

6-16-pGEX-2T (Amersham Bioscience) or CIB-pMAL-C2 (New England Biolabs) expression vectors were constructed to produce GST-6-16 and MBP-CIB proteins. GST-6-16 and MBP-CIB proteins were purified from *E. coli* transformed with their expression vectors treated with 0.1 M IPTG by using amylose resin (New England Biolabs) or glutatione Sepharose 4B (Amersham Bioscience). GST-6-16 protein (20 pmol) was incubated with MBP-CIB protein (40 pmol) in 40 μl of reaction buffer (20 mM Tris-HCl pH7.5, 1 mM DTT and 0.05% CHAPS) for 1 h at 4°C. After glutathione Sepharose 4B (Amersham Bioscience) was added and further incubated for 1 h, the precipitates were washed three times and subjected to immunoblot analysis using anti-MBP and anti-GST rabbit polyclonal antibody.

Results

6-16 was expressed in gastric cancer cells and tissues

We examined expression levels of 6-16 mRNA in eight gastric cancer cell lines (MKN-1, MKN-7, MKN-28, MKN-45, MKN-74, TMK-1, HSC-39 and KATO III) by Northern blot analysis. Seven out of eight gastric

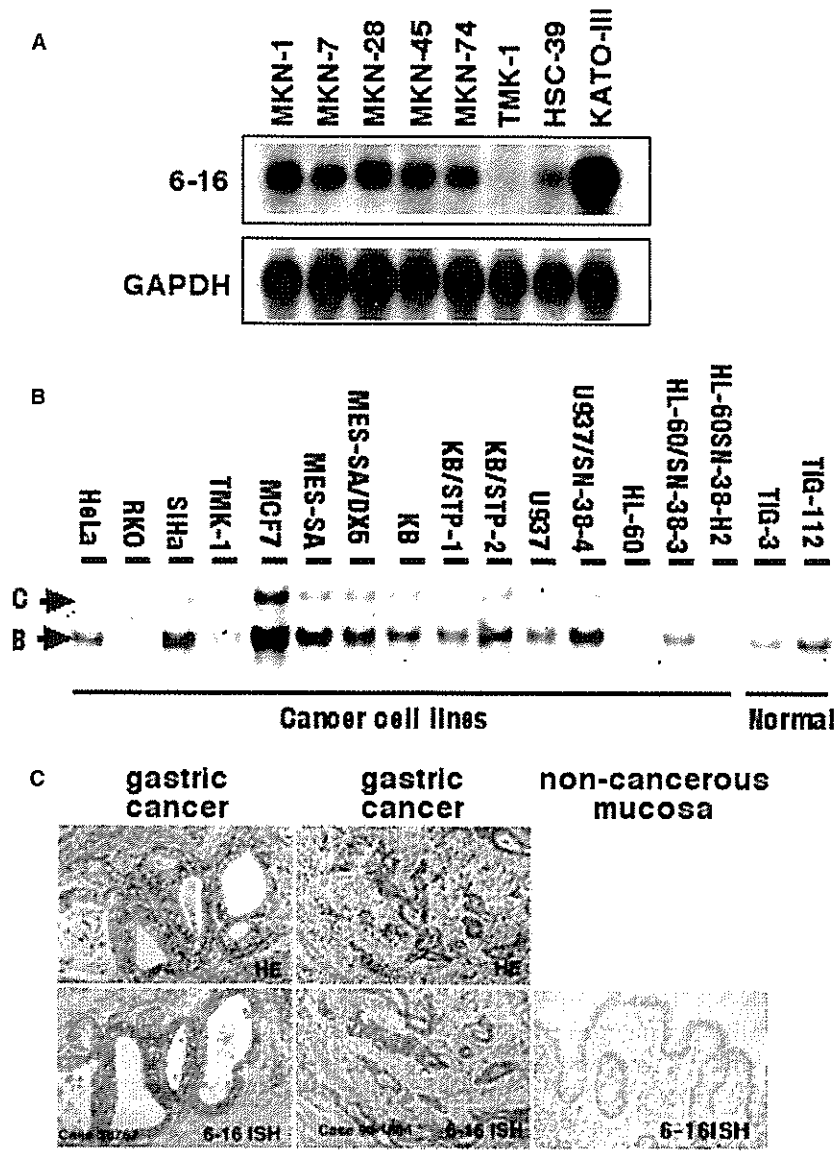

Fig. 1 Expression of 6-16 mRNA in gastric cancer cells and carcinoma tissues. **a** Expression of 6-16 mRNA was detected by Northern blot analysis with 10 μg total RNA in eight gastric cancer cell lines. Hybridization of a G3PDH (control) probe to the same filter membrane is depicted in the *lower panel*. **b** Expression of 6-16 mRNA was detected by RT-PCR analysis. 0.2 μg of RNA were used for RT-PCR reaction. PCR products were run on 6% acrylamide gel electrophoresis and staining with CYBR Green I nucleic acid staining. **c** Surgically resected adenocarcinomas of the stomach were examined on in situ hybridization using the microprobe manual staining system (Fisher Scientific). 6-16-specific anti-sense oligonucleotide DNA probe (5′-GCA CGC CGC CCC CAT TCA GGA TCG CAG-3′) was designed. *lower panel* 6-16 in situ hybridization. *upper panel* hematoxylin-eosin (HE) staining