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Original Paper

Histone H3 acetylation is associated with reduced p21$^{WAF1/CIP1}$ expression by gastric carcinoma

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Abstract

Histone acetylation appears to play an important role in transcriptional regulation. Inactivation of chromatin by histone deacetylation is involved in the transcriptional repression of several tumour suppressor genes, including *p21$^{WAF1/CIP1}$*. However, the *in vivo* status of histone acetylation in human cancers, including gastric carcinoma, is not well understood. This study shows that histone H3 in the *p21$^{WAF1/CIP1}$* promoter region is hypoacetylated and that this hypoacetylation is associated with reduced *p21$^{WAF1/CIP1}$* expression in gastric carcinoma specimens. Chromatin immunoprecipitation assays revealed that histone H3 was hypoacetylated in the *p21$^{WAF1/CIP1}$* promoter and coding regions in 10 (34.5%) and 10 (34.5%) of 29 gastric carcinoma specimens, respectively. Hypoacetylation of histone H4 in the *p21$^{WAF1/CIP1}$* promoter and coding regions was observed in 6 (20.7%) and 16 (55.2%) of 29 gastric carcinoma specimens, respectively. *p21$^{WAF1/CIP1}$* mRNA levels were associated with histone H3 acetylation status in the *p21$^{WAF1/CIP1}$* promoter region ($p = 0.047$) but not *p53* mutation status ($p = 0.460$). In gastric carcinoma cell lines, expression of p21$^{WAF1/CIP1}$ protein was induced by trichostatin A, a histone deacetylase inhibitor. This induction was associated with hyperacetylation of histone H3 in the *p21$^{WAF1/CIP1}$* promoter region. Hyperacetylation of histone H4 in the *p21$^{WAF1/CIP1}$* promoter region did not appear to be associated with increased expression. Induction of p21$^{WAF1/CIP1}$ protein expression was associated with hyperacetylation of histones H3 and H4 in the *p21$^{WAF1/CIP1}$* coding region. Expression of a dominant-negative mutant of p53 reduced expression of p21$^{WAF1/CIP1}$ protein. Histone H4 acetylation in both the promoter and coding regions of the *p21$^{WAF1/CIP1}$* gene in cells expressing dominant-negative p53 was less than half of that in cells expressing wild-type p53, whereas histone H3 acetylation in both the promoter and coding regions was slightly reduced (by approximately 20%) in cells expressing the dominant-negative p53. These findings provide evidence that alteration of histone acetylation occurs in human cancer tissue specimens such as those from gastric carcinoma.
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Introduction

A variety of genetic and epigenetic alterations are associated with gastric carcinoma (GC) [1,2]. We have reported reduced expression of p21$^{WAF1/CIP1}$ in 34% of GC tissues [3]. *p21$^{WAF1/CIP1}$* was identified through its activation by p53 [4], association with cyclin/cyclin-dependent kinase complexes [5], and increased expression during senescence [6]. Although p21$^{WAF1/CIP1}$ is activated in a p53-dependent manner in response to DNA damage to ensure cell-cycle arrest and DNA repair, various agents that promote differentiation can increase p21$^{WAF1/CIP1}$ expression in a p53-independent manner. We have also reported that p21$^{WAF1/CIP1}$ expression is induced by 9-*cis*-retinoic acid [7] and inhibition of telomerase [8], but we found no correlation between expression of p21$^{WAF1/CIP1}$ and abnormal accumulation of p53 in GC tissues [3].

Changes in DNA methylation patterns, such as hypermethylation of CpG islands, are observed frequently in human cancers [9]. Hypermethylation of CpG islands in promoters is associated with the silencing of some tumour suppressor genes [10]. Methylation and inactivation of various genes have been reported in GC [11,12]. Although hypermethylation of the *p21$^{WAF1/CIP1}$* promoter occurs in acute lymphoblastic leukaemia [13], the *p21$^{WAF1/CIP1}$* promoter is not hypermethylated in GC [14].

Several lines of evidence suggest that histone acetylation plays an important role in transcriptional

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regulation [15]. There appears to be a positive correlation between the level of histone acetylation at specific loci and transcriptional activity, and the recruitment of histone acetyltransferases and hyperacetylation of histones in promoter regions often correlate with transcriptional activation [16,17]. Histone hyperacetylation is thought to relax the chromatin structure and allow transcription factors to access promoter sequences [18,19]. Some genes, including *p21*$^{WAF1/CIP1}$ [20] and *hTERT* [21], are thought to be regulated by histone acetylation. We have reported that trichostatin A (TSA), a histone deacetylase (HDAC) inhibitor, induces p21$^{WAF1/CIP1}$ expression in GC cell lines [22].

Taken together, the currently available data suggest that reduced expression of p21$^{WAF1/CIP1}$ in GC tissues may be due to aberrant histone acetylation and not p53. Little is known, however, about the *in vivo* histone acetylation status in human cancers, including GC. To date, there are no reports of changes in promoter acetylation in human cancer specimens. Thus, we investigated the histone acetylation status of the *p21*$^{WAF1/CIP1}$ promoter region by means of chromatin immunoprecipitation (ChIP) assays with antibodies against the acetylated forms of histones H3 and H4. Because a recent study in yeast suggested that hypoacetylation of histones in coding regions is important for transcriptional inhibition [23], we investigated the histone acetylation status in the coding region of *p21*$^{WAF1/CIP1}$. We show for the first time that histone acetylation is altered in GC tissue specimens and that this can reduce *p21*$^{WAF1/CIP1}$ expression in a p53-independent manner.

Materials and methods

Tissue samples

Twenty-nine GC tissue specimens from 29 patients were studied. The tissue specimens were obtained from Hiroshima University Hospital and affiliated hospitals. Tumours and corresponding non-neoplastic mucosae were removed surgically, frozen immediately in liquid nitrogen, and stored at −80 °C until use. All GCs were located in the middle third of the stomach. Tissues were embedded in OCT compound (Sakura Finetechnical Co, Ltd, Tokyo, Japan) and frozen sections were prepared. After we had confirmed microscopically that the tumour specimens consisted mainly (> 50%, on a nuclear basis) of carcinoma tissue and that non-neoplastic mucosa did not show any tumour cell invasion or significant inflammatory changes, samples from embedded tissues were used for ChIP assay, RNA extraction, and genomic DNA extraction. Histological classification of GC was performed according to the Lauren classification system [24]. Tumour staging was carried out according to the TNM stage grouping [25]. Because written informed consent was not obtained, all samples were cleared of any identifying information, for strict privacy protection, before histone acetylation status was analysed. This procedure is in accordance with the Ethical Guidelines for Human Genome/Gene Research enacted by the Japanese Government and was approved by the Ethics Review Committee of the Hiroshima University School of Medicine.

Cell culture and drug treatment

Eight cell lines derived from human GCs were used. The TMK-1 cell line was established in our laboratory from a poorly differentiated adenocarcinoma [26]. Five GC cell lines of the MKN series (MKN-1, adenosquamous cell carcinoma; MKN-7, MKN-28, and MKN-74, well-differentiated adenocarcinomas; and MKN-45, poorly differentiated adenocarcinoma) were kindly provided by Dr T Suzuki. The KATO-III and HSC-39 cell lines, which were established from signet ring cell carcinomas, were kindly provided by Dr M Sekiguchi and by Dr K Yanagihara [27], respectively. All cell lines were maintained in RPMI 1640 (Nissui Pharmaceutical Co, Ltd, Tokyo, Japan) containing 10% fetal bovine serum (FBS; BioWhittaker, Walkersville, ME, USA) in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. To analyse transcriptional activation of the *p21*$^{WAF1/CIP1}$ gene, MKN-28, MKN-74, and KATO-III cells were incubated for 5 days with 1 μM 5-aza-2′-deoxycytidine (Aza-dC; Sigma Chemical Co, St Louis, MO, USA) or for 24 h with 300 nM TSA (Wako, Tokyo, Japan).

Stable transfection

pCMV-p53mt135 expression vector (CLONTECH, Palo Alto, CA, USA) was transfected into MKN-74 cells with FuGENE6 (Roche Diagnostics, Mannheim, Germany). pCMV-p53mt135 expresses a dominant-negative mutant of p53. The *p53* and *p53mt135* genes differ by a G-to-A transition at nucleotide 1017. Stable transfectants were selected with 2 weeks of culture with 80 μg/ml G418 (Invitrogen Corp, Carlsbad, CA, USA). Clone number 5 expressed p21$^{WAF1/CIP1}$ protein at a level lower than that of the mock transfectant (see the Results section) and was used for further analyses of the dominant-negative mutant.

ChIP assay

The ChIP assay was performed as described previously [28]. Polymerase chain reaction (PCR) analysis of immunoprecipitated DNA was performed using primers specific for the 5′ upstream region of the *ACTB* gene. PCR product (15 μl) was loaded onto 8% non-denaturing polyacrylamide gels, separated by electrophoresis, stained with ethidium bromide, and visualized under UV light to confirm that there was no genomic DNA contamination of the no-antibody control. Quantitative PCR analysis of immunoprecipitated DNAs was performed by real-time PCR. The position

Table 1. Primer sequences for RT-PCR and ChIP

Primer sequence	Annealing temperature (°C)	Product size (bp)
Quantitative RT-PCR (*p21$^{WAF1/CIP1}$*)		
F: 5′-TGGAGACTCTCAGGGTCGAAA-3′	55	87
R: 5′-GGCGTTTGGAGTGGTAGAAATC-3′		
Quantitative RT-PCR (*ACTB*)		
F: 5′-TCACCGAGCGCGGCT-3′	55	60
R: 5′-TAATGTCACGCACGATTTCCC-3′		
ChIP-PCR (*p21$^{WAF1/CIP1}$* promoter region)		
F: 5′-GGGGCTTTTCTGGAAATTGC-3′	55	116
R: 5′-CTGGCAGGCAAGGATTTACC-3′		
ChIP-PCR (*p21$^{WAF1/CIP1}$* coding region)		
F: 5′-CGCTAATGGCGGGCTG-3′	55	60
R: 5′-CGGTGACAAAGTCGAAGTTCC-3′		
ChIP-PCR (*ACTB* 5′ upstream region)		
F: 5′-CCCACCCGGTCTTGTGTG-3′	55	72
R: 5′-GGGAAGACCCTGTCCTTGTCA-3′		

and sequences of primers, and annealing temperatures, are shown in Table 1 and Figure 1A. PCR was performed 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 done with the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). The relative histone acetylation level was determined from the threshold cycles for the promoter or coding region of the *p21$^{WAF1/CIP1}$* gene and the 5′ region of the *ACTB* gene. Reference samples (genomic DNA from MKN-1 cells) were included on each assay plate to verify plate-to-plate consistency. Plates were normalized to each other with these reference samples. The PCR amplification was performed in 96-well optical trays with caps according to the manufacturer's instructions. Quantitative PCRs were performed in triplicate for each sample primer set, and the mean of the three experiments was calculated as the relative quantification value. At the end of 40 PCR cycles, reaction products were separated electrophoretically on 8% non-denaturing polyacrylamide gels for visual confirmation of PCR products.

Quantitative reverse transcription (RT)-PCR analysis of GC tissues

Total RNA was extracted with an RNeasy Mini Kit (QIAGEN, Hilden, Germany), and 1 µg of total RNA was converted to cDNA with a First Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, Uppsala, Sweden). To analyse expression of the *p21$^{WAF1/CIP1}$* gene in GC tissue specimens, real-time RT-PCR was performed as described previously [29]. Primer sequences and annealing temperatures are shown in Table 1. PCR was performed with the SYBR Green PCR Core Reagents Kit (Applied Biosystems). Reference samples (MKN-1) were included on each assay plate to verify plate-to-plate consistency.

Western blot analysis of GC cell lines

Preparation of whole cell lysates from GC cell lines and western blotting were performed as described previously [30]. Protein concentrations were determined by Bradford protein assay (Bio-Rad, Hercules, CA, USA) with bovine serum albumin (BSA) used as the standard. Lysates (20 µg) were solubilized in Laemmli's sample buffer by boiling and then subjected to 10% SDS-PAGE followed by electrotransfer onto a nitrocellulose filter. Anti-p21$^{WAF1/CIP1}$ monoclonal antibody was purchased from PharMingen (San Diego, CA, USA). Peroxidase-conjugated anti-mouse IgG was used in the secondary reaction. The immunocomplex was visualized with an ECL Western Blot Detection System (Amersham Pharmacia Biotech). The quality and amount of each protein sample on the gel were confirmed by detection with anti-beta-actin antibody (Sigma). Autoradiographic signal intensities of the p21$^{WAF1/CIP1}$ bands on western blots were determined by densitometric scanning and normalization of these signals to those of the internal control (beta-actin).

DNA extraction and p53 mutation analysis

To examine mutations in the *p53* gene, genomic DNAs were extracted from GC specimens with a genomic DNA purification kit (Promega, Madison, WI, USA). Exons 5–8 of the *p53* gene were amplified by PCR with ten sets of primers as described previously [31]. The PCR products were purified and sequenced directly with the ABI Prism Dye Terminator Cycle Sequencing Kit (Applied Biosystems) and an ABI Prism 310 DNA Sequencer (Applied Biosystems).

Statistical methods

Differences were analysed statistically by Fisher's exact and Mann–Whitney *U*-tests. *p* values less than 0.05 were considered statistically significant.

Results

Histone acetylation status in GC tissues

To examine the *in vivo* status of histone acetylation and expression of $p21^{WAF1/CIP1}$, $p21^{WAF1/CIP1}$ mRNA levels were measured by quantitative RT-PCR (Figure 1B) and acetylation of histones H3 and H4 by ChIP in 29 GC specimens (Figure 1C). The ratio of histone acetylation levels in GC specimens relative to those in non-neoplastic mucosae (T/N) was calculated. A T/N of less than 0.5 was considered to represent hypoacetylation, and a T/N of greater than 2.0 was considered to represent hyperacetylation. Hypoacetylation of histone H3 in the promoter

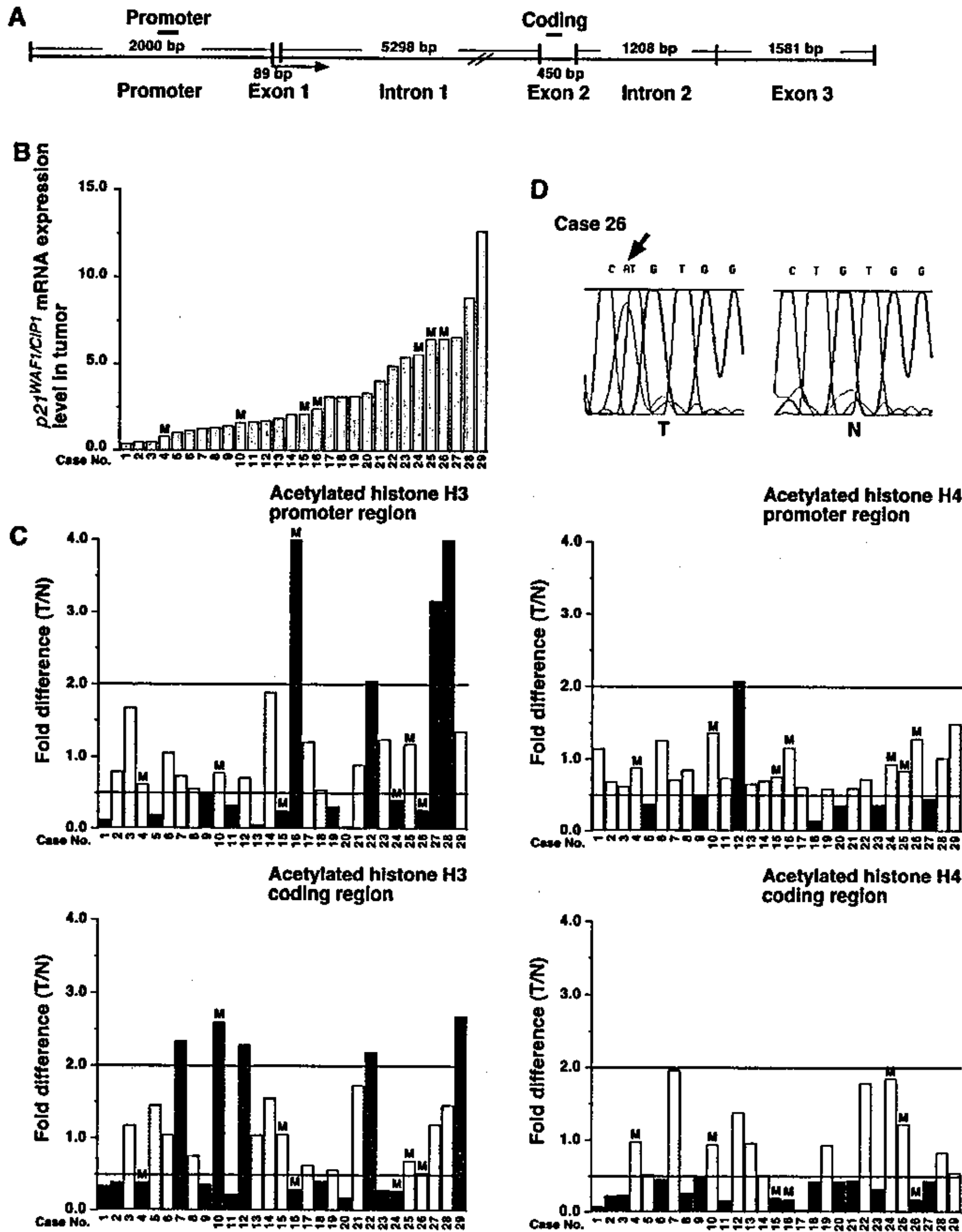

Figure 1. Expression and acetylation of the $p21^{WAF1/CIP1}$ gene and *p53* mutation status in GC tissues. (A) Schematic representation of the human $p21^{WAF1/CIP1}$ gene. Positions of the primer pairs used in the present study are indicated as black bars (Promoter and Coding). (B) Quantitative RT-PCR analysis of $p21^{WAF1/CIP1}$. Units are arbitrary and we calculated $p21^{WAF1/CIP1}$ mRNA levels by standardization against 1 µg of total RNA from MKN-1 cells, which was taken as 1.0. The 29 GC tissues are sorted by increasing $p21^{WAF1/CIP1}$ expression. M indicates specimens carrying *p53* mutations. (C) ChIP analysis of histones H3 and H4 in the $p21^{WAF1/CIP1}$ promoter and coding regions in 29 GC tissues. Fold change indicates the ratio of $p21^{WAF1/CIP1}$ acetylation level in GC to that in corresponding non-neoplastic mucosa (T/N). We considered a T/N < 0.5 (red bars) to indicate hypoacetylation and a T/N > 2.0 (green bars) to indicate hyperacetylation. (D) Sequencing analysis of the *p53* gene (case 26, exon 5). There is a mutation in codon 145 (CTG to CAG, arrow)

and coding regions of $p21^{WAF1/CIP1}$ was observed in 10 (34.5%) and 10 (34.5%) of 29 specimens, respectively. Hypoacetylation of histone H4 in the promoter and coding regions was found in 6 (20.7%) and 16 (55.2%) of 29 specimens, respectively. $p21^{WAF1/CIP1}$ mRNA levels in tumour tissues with histone H3 hypoacetylation in the promoter region were significantly lower than those in specimens with histone H3 hyperacetylation ($p = 0.047$; Mann–Whitney U-test), whereas $p21^{WAF1/CIP1}$ levels in tumour tissues were not associated with histone H3 acetylation status in the coding region ($p = 0.540$; Mann–Whitney U-test, Table 2). No association was found between $p21^{WAF1/CIP1}$ levels and histone H4 acetylation status in either the promoter or the coding regions of $p21^{WAF1/CIP1}$.

The correlation was then examined between $p53$ mutation status and $p21^{WAF1/CIP1}$ mRNA expression, and histone acetylation. Representative results of $p53$ sequencing analysis are shown in Figure 1D, and the results of $p53$ mutation analyses are summarized in Table 3. $p53$ gene mutation was detected in 10 (34.5%) of 29 specimens. Of the ten mutations, seven were missense mutations and three were silent mutations. The seven missense mutations were analysed further. The level of $p21^{WAF1/CIP1}$ expression was not associated with $p53$ mutation status ($p = 0.460$; Mann–Whitney U-test), and $p53$ mutation status did not correlate with histone acetylation status (data not shown). Histone acetylation status was not associated with T grade (depth of tumour invasion), N grade (degree of lymph node metastasis), tumour stage, or histological type (data not shown).

TSA induced $p21^{WAF1/CIP1}$ expression and histone H3 hyperacetylation

To confirm the correlation between reduced $p21^{WAF1/CIP1}$ expression and hypoacetylation of histones, $p21^{WAF1/CIP1}$ expression and histone acetylation status were examined in eight GC cell lines. Levels of p21$^{WAF1/CIP1}$ protein were measured by western blot analysis (Figure 2A). Levels of p21$^{WAF1/CIP1}$ in GC cell lines were classified into three groups. MKN-45 and MKN-74 showed high levels of expression; MKN-28, TMK-1, and HSC-39 showed intermediate expression; and MKN-1, MKN-7, and KATO-III showed low expression. One cell line was selected from each group (MKN-74, MKN-28, and KATO-III) for further analysis. The effects of TSA and Aza-dC on p21$^{WAF1/CIP1}$ protein expression were examined by western blot analysis (Figure 2B). MKN-28 (mutant-type p53), MKN-74 (wild-type p53), and KATO-III (p53 null) cells were cultured with or without TSA for 24 h or Aza-dC for 5 days. Treatment with TSA increased the p21$^{WAF1/CIP1}$ protein levels in all three cell lines, whereas the p53 protein levels did not change. TSA induced 5.2-fold and 6.1-fold increases in p21$^{WAF1/CIP1}$ protein levels in MKN-28 and KATO-III cells, respectively, whereas TSA yielded only a 1.4-fold increase in p21$^{WAF1/CIP1}$ protein levels in MKN-74 cells. Treatment with Aza-dC had no effect on p21$^{WAF1/CIP1}$ protein expression in any of the cell lines (Figure 2B). ChIP assay was carried out to investigate acetylation of histones H3 and H4 associated with the $p21^{WAF1/CIP1}$ gene (Figure 2C). TSA increased acetylation of histone H3 in both the promoter and coding regions of $p21^{WAF1/CIP1}$ in both MKN-28 and KATO-III cells. Histone H4 acetylation in the $p21^{WAF1/CIP1}$ promoter region in MKN-28 cells was increased slightly in response to TSA, whereas that in KATO-III cells was increased significantly. Histone H4 acetylation in the coding region was increased markedly by TSA in both MKN-28 and KATO-III

Table 3. Summary of $p53$ mutations

Case No	Location	Codon	Sequence change	Amino acid
4	Exon 7	245	GGC to GTC	Gly to Val
5	Exon 5a	129	GCC to GCT	Ala to Ala
10	Exon 5b	160	ATG to ATA	Met to Ile
15	Exon 7	251	ATC to AAC	Ile to Asn
16	Exon 5b	160	ATG to ACG	Met to Thr
18	Exon 5a	129	GCC to GCT	Ala to Ala
21	Exon 7	240	AGT to AGC	Ser to Ser
24	Exon 5b	173	GTG to GCG	Val to Ala
25	Exon 5a	128	CCT to ACT	Pro to Thr
26	Exon 5a	145	CTG to CAG	Leu to Gln

Table 2. Association between $p21^{WAF1/CIP1}$ mRNA levels and histone acetylation status

		No of cases	$p21^{WAF1/CIP1}$ mRNA level (mean ± SE)*	p value†
Histone H3 acetylation status in	Hypo	10	2.71 ± 0.62	0.047
promoter region	Hyper	4	5.71 ± 1.36	
Histone H3 acetylation status in	Hypo	10	2.47 ± 0.60	0.540
coding region	Hyper	5	4.43 ± 2.16	
Histone H4 acetylation status in	Hypo	6	3.50 ± 0.89	0.617
promoter region	Hyper	1	1.71	
Histone H4 acetylation status in	Hypo	16	2.73 ± 0.511	—
coding region	Hyper	0	—	

* The units are arbitrary and we calculated the $p21^{WAF1/CIP}$ mRNA expression level by standardization against 1 μg of total RNA from MKN-1 GC cells taken as 1.0. SE = standard error.
† Mann–Whitney U-test. Hypo = hypoacetylation; Hyper = hyperacetylation.

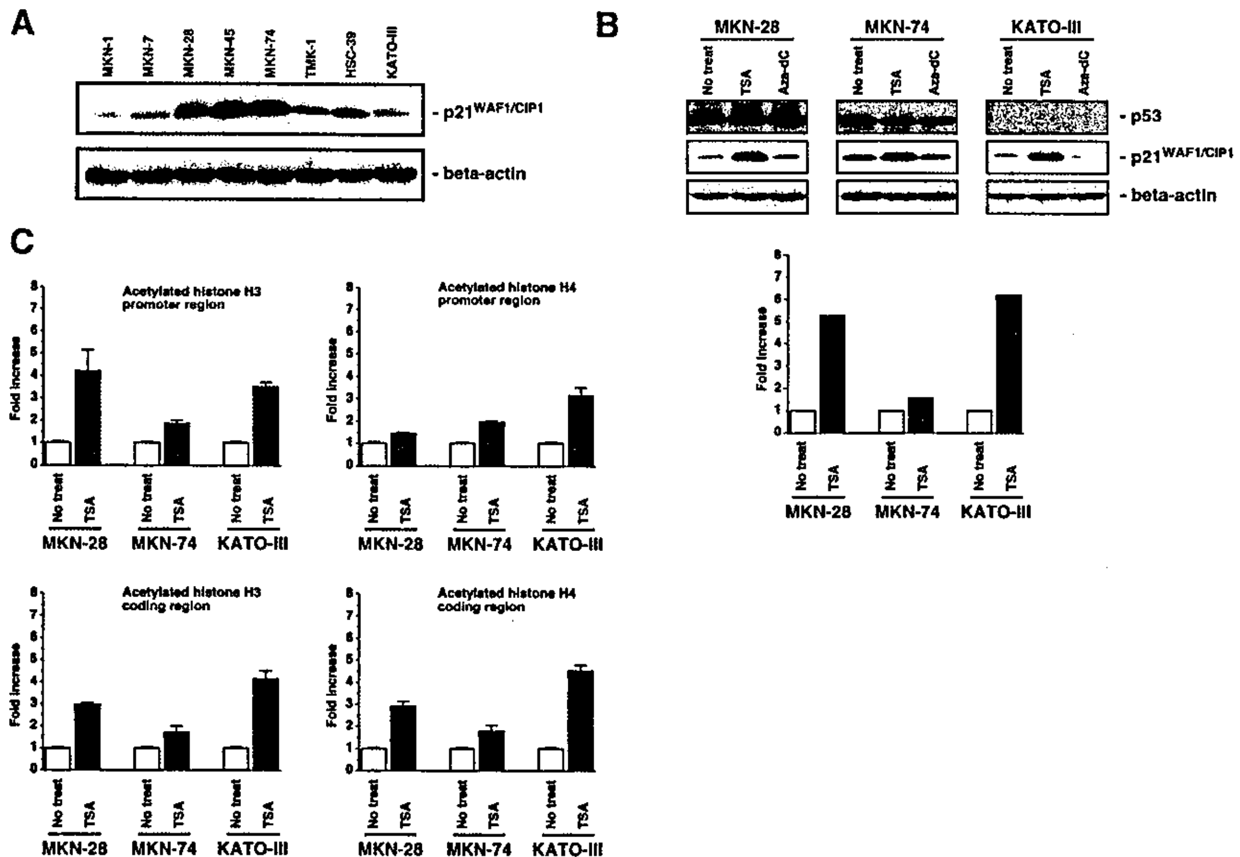

Figure 2. p21$^{WAF1/CIP1}$ protein expression and histone acetylation status in GC cell lines. (A) Western blot analysis of p21$^{WAF1/CIP1}$ in eight GC cell lines. MKN-45 and MKN-74 cells showed high expression. MKN-28, TMK-1, and HSC-39 cells showed intermediate expression. MKN-1, MKN-7, and KATO-III cells showed low expression. (B) Western blot analysis of p53 and p21$^{WAF1/CIP1}$ in three GC cell lines cultured with or without TSA for 24 h or Aza-dC for 5 days. In all three cell lines, TSA treatment induced p21$^{WAF1/CIP1}$ expression, whereas Aza-dC treatment did not. The relative p21$^{WAF1/CIP1}$ band intensity normalized to that of beta-actin is indicated in the lower panel. (C) ChIP analyses of the relative levels of histones H3 and H4 in the *p21*$^{WAF1/CIP1}$ promoter and coding regions in three GC cell lines. The value is the mean of three independent ChIP experiments. Error bars indicate the standard error (SE) of the mean. Note that acetylation of histone H4 in the promoter region does not increase significantly after TSA treatment in MKN-28 cells

cells. In MKN-74 cells, acetylation of histones H3 and H4 in both the promoter and coding regions was increased approximately 2.0-fold.

Inhibition of p53-induced hypoacetylation of histone H4

To investigate the effect of p53 on p21$^{WAF1/CIP1}$ protein levels, the MKN-74 cell line was stably transfected with a vector expressing a dominant-negative mutant of p53, and p21$^{WAF1/CIP1}$ protein levels were determined by western blot analysis (Figure 3A). p21$^{WAF1/CIP1}$ levels in p53 mutant cells were less than half those in wild-type cells. ChIP was also used to analyse histone acetylation levels (Figure 3B). Histone H4 acetylation levels in both the promoter and coding regions of cells expressing dominant-negative p53 were less than half those in cells expressing wild-type p53, whereas histone H3 acetylation levels in both the promoter and coding regions were reduced slightly (approximately 20%) in cells expressing dominant-negative p53.

Discussion

A variety of genetic and epigenetic alterations are associated with GC. Histone acetylation and DNA methylation appear to play important roles in transcriptional regulation; however, little is known about changes in histone acetylation in human cancers such as GC. In the present study, we investigated the histone acetylation status in regions of the *p21*$^{WAF1/CIP1}$ gene in GC tissues and GC cell lines.

We found that histones H3 and H4 in both the promoter and coding regions are hypoacetylated in GC tissues. *p21*$^{WAF1/CIP1}$ mRNA levels are associated with histone H3 acetylation status in the promoter region, suggesting that nucleosome conformation was altered due to histone H3 hypoacetylation and that access of transcriptional regulatory proteins to chromatin might be reduced in GC tissues. It is possible that histone hypoacetylation and reduced *p21*$^{WAF1/CIP1}$ expression were the result of the p53 mutation because previous studies have shown an interaction between p53 and chromatin-modifying enzymes.

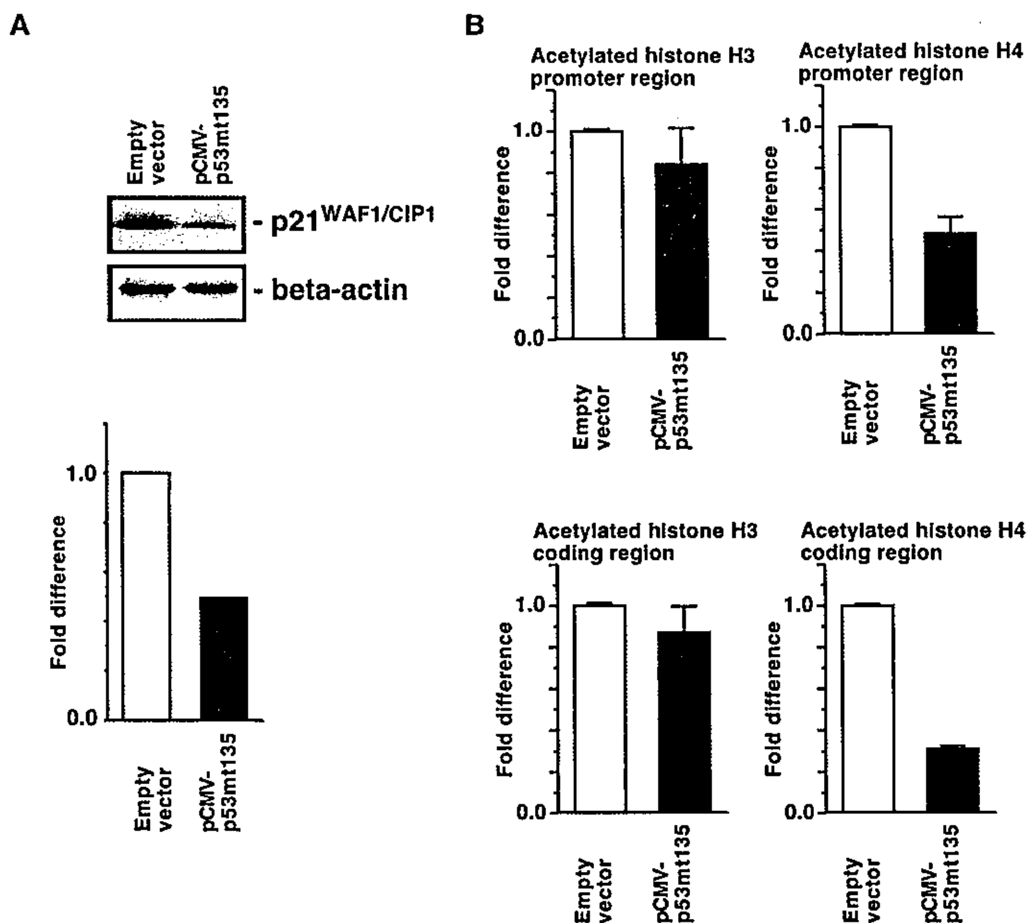

Figure 3. Effect of a dominant-negative (DN) mutant of p53 in MKN-74 cells. (A) Western blot analysis of p21$^{WAF1/CIP1}$ (upper panel). Expression of DN p53 reduced p21$^{WAF1/CIP1}$ expression. p21$^{WAF1/CIP1}$ band intensity normalized against beta-actin is indicated in the lower panel. (B) ChIP analyses of relative levels of histones H3 and H4 in the *p21*$^{WAF1/CIP1}$ promoter and coding regions. The value is the mean of three independent ChIP experiments. Error bars are the standard error (SE) of the mean. Note that expression of DN p53 reduced acetylation of histone H4 in both the promoter and coding regions of *p21*$^{WAF1/CIP1}$

Several acetyltransferases act as p53 co-activators and regulate the transcriptional activity of p53 [32,33]. In addition, we showed that a dominant-negative p53 mutant affects histone acetylation in MKN-74 cells. However, in our study, *p53* mutation status correlated with neither histone acetylation status nor *p21*$^{WAF1/CIP1}$ expression in GC tissues. Because several factors, such as transforming growth factor beta and nerve growth factor, have been reported to activate transcription of *p21*$^{WAF1/CIP1}$ [34], we cannot rule out the possibility that they cause hypoacetylation of histones in the *p21*$^{WAF1/CIP1}$ promoter. However, altered hypoacetylation of histone H3 in GC tissues does not appear to be due to a mutant form of p53 because p53 appears to affect acetylation of only histone H4 [35]. We also showed that expression of a dominant-negative p53 mutant suppresses p21$^{WAF1/CIP1}$ expression and that acetylation of histone H4 in the promoter is reduced significantly. Taken together, our data indicate that aberrant hypoacetylation of histones in the *p21*$^{WAF1/CIP1}$ promoter occurs in GC.

We found no association between *p21*$^{WAF1/CIP1}$ expression and histone H4 acetylation status in the *p21*$^{WAF1/CIP1}$ promoter in GC tissues and MKN-28 cells. In MDA-MB-435 cells, trapoxin (TPX), an HDAC inhibitor, significantly increases acetylation of histone H3 in the *p21*$^{WAF1/CIP1}$ promoter, whereas TPX does not significantly affect acetylation of histone H4 [36]. Similar results have been reported in HDAC1-null embryonic stem cells [37]. These results indicate that hyperacetylation of histone H4 in the *p21*$^{WAF1/CIP1}$ promoter region is not an absolute requirement for *p21*$^{WAF1/CIP1}$ expression.

HDAC inhibition appears to influence histone H3 hyperacetylation, whereas p53 appears to affect histone H4 hyperacetylation. Our present results also suggest that acetylated histones H3 and H4 have distinct roles. Distinct roles for acetylation of histones H3 and H4 have been reported in yeast [38]. Although a number of studies have shown induction of *p21*$^{WAF1/CIP1}$ by HDAC inhibitors, p53, and Sp1 [33,35,39], the significance of distinct roles for acetylation of histones H3 and H4 is not clear in human cancer cells. Further studies may reveal the functional significance of the acetylation of histones H3 and H4.

We also investigated the histone acetylation status in the coding region of $p21^{WAF1/CIP1}$. In GC cell lines, expression of $p21^{WAF1/CIP1}$ protein is associated with acetylation of histones H3 and H4 in the $p21^{WAF1/CIP1}$ coding region. This is consistent with the idea that transcript elongation and histone acetylation are needed to form and maintain, respectively, the relaxed structure of transcribing nucleosomes [40]. In contrast to our findings in cell lines, we found no association between $p21^{WAF1/CIP1}$ expression and histone acetylation status in the coding region in GC tissues. However, our data do show that histone H4 in the $p21^{WAF1/CIP1}$ coding region is frequently hypoacetylated in GC tissues. Although there have been many studies of promoter histone acetylation, the function of histone acetylation in coding regions is poorly understood. The significance of histone H4 hypoacetylation in the coding region of $p21^{WAF1/CIP1}$ remains unclear, but it is possible that it contributes to a change in nucleosome conformation. Further studies are needed to elucidate the function of histone acetylation in coding regions.

In conclusion, we have shown that histones H3 and H4 in both the promoter and coding regions of the $p21^{WAF1/CIP1}$ gene are frequently hypoacetylated in GC tissues. Hypoacetylation of histone H3 in the promoter region is associated with reduced expression of $p21^{WAF1/CIP1}$ in a p53-independent manner. Clinical trials of HDAC inhibitors as cancer therapeutics are underway [41,42]. Although we did not investigate the anti-tumour activity of $p21^{WAF1/CIP1}$ and HDAC inhibitors in GC, our data provide supporting evidence for the idea that inhibition of HDAC may be an effective therapy for 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 postoperative 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

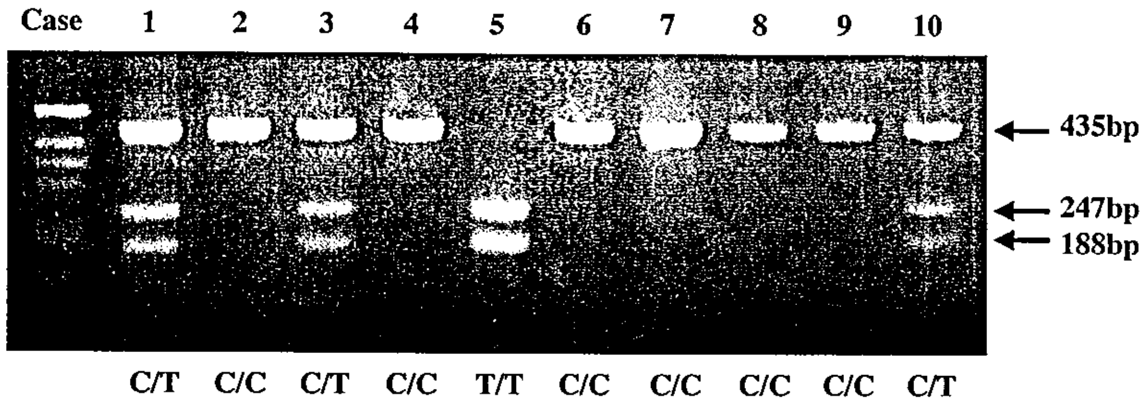

Fig. 1 PCR-RFLP analysis of the –1562 C/T polymorphism in ten patients with gastric cancer. The ethidium bromide-stained 2.5% NuSieve 3:1 agarose gel used for genotyping is shown. The target region (435 bp) of the *MMP-9* gene promoter was PCR amplified and digested with *SphI*, which cleaved the T allele 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

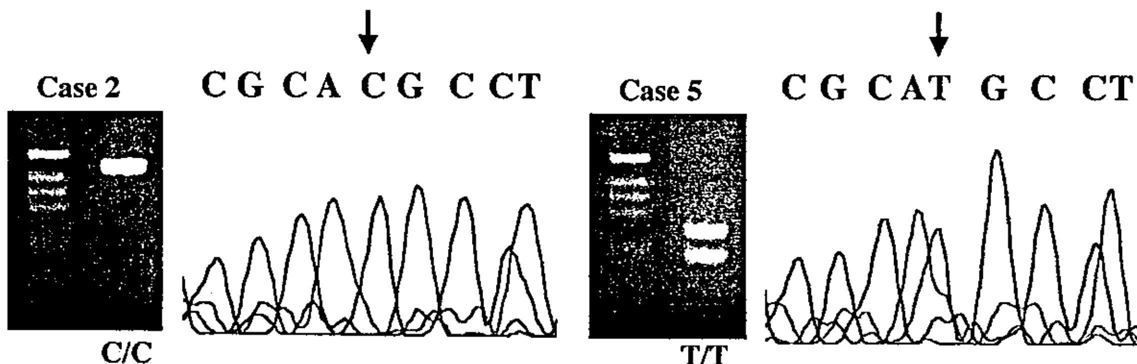

Table 1 *MMP-9* Genotype distributions in study subjects (*OR* odds ratio, *CI* confidence interval)

	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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