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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

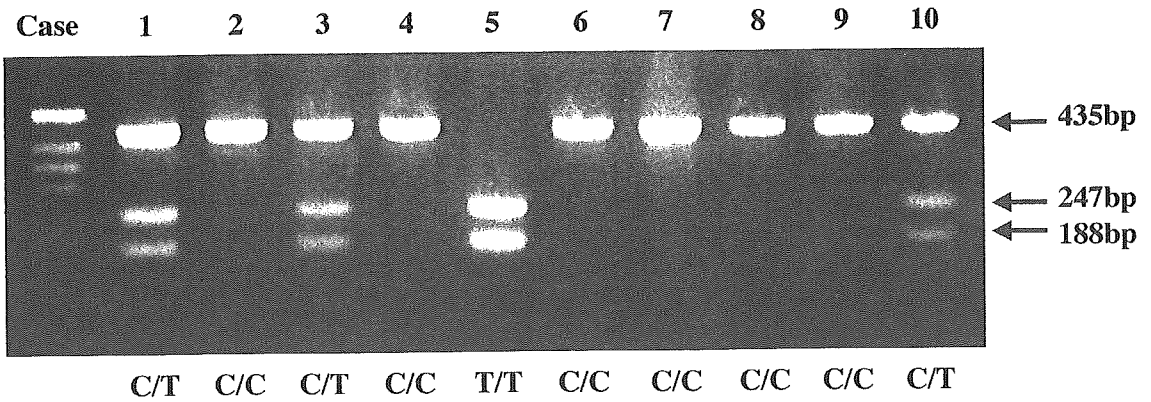

Fig. 1 PCR-RFLP analysis of the −1562 C/T polymorphism in ten patients with gastric cancer. The ethidium bromide-stained 2.5% NuSieve 3:1 agarose gel used for genotyping is shown. The target region (435 bp) of the *MMP-9* gene promoter was PCR amplified and digested with *SphI*, which cleaved the T allele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

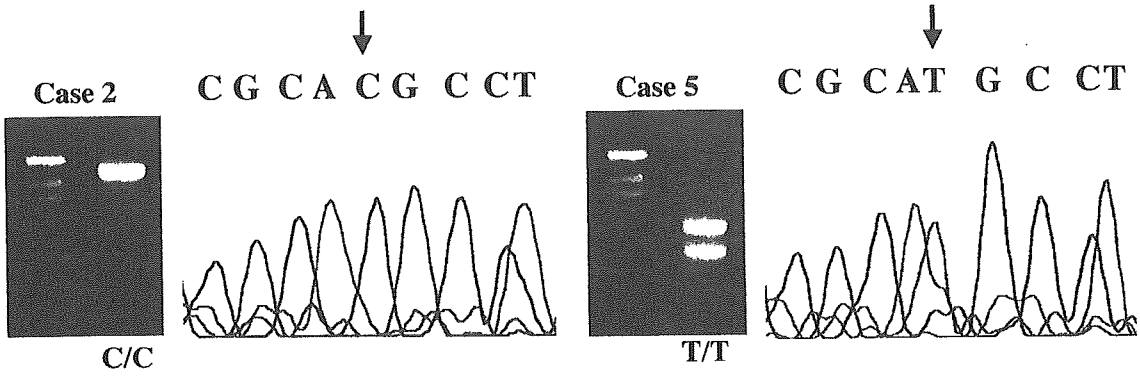

Table 1 *MMP-9* Genotype distributions in study subjects (*OR* odds ratio, *CI* confidence interval)

	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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Gene Expression Profile of Gastric Carcinoma: Identification of Genes and Tags Potentially Involved in Invasion, Metastasis, and Carcinogenesis by Serial Analysis of Gene Expression

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ABSTRACT

Gastric carcinoma (GC) is one of the most common malignancies worldwide. To better understand the genetic basis of this disease, we performed serial analysis of gene expression (SAGE) on four primary GC samples and one associated lymph node metastasis. We obtained a total of 137,706 expressed tags (Gene Expression Omnibus accession number GSE 545, SAGE Hiroshima gastric cancer tissue), including 38,903 that were unique. Comparing tags from our GC libraries containing different stages and different histologies, we found several genes and tags that are potentially involved in invasion, metastasis, and carcinogenesis. Among these, we selected 27 genes and measured mRNA expression levels in an additional 46 GC samples by quantitative reverse transcription-PCR. Frequently overexpressed genes (tumor/normal ratio > 2) were *COL1A1* (percentage of cases with overexpression, 78.3%), *CDH17* (73.9%), *APOC1* (67.4%), *COL1A2* (58.7%), *YF13H12* (52.2%), *CEACAM6* (50.0%), *APOE* (50.0%), *REGIV* (47.8%), *S100A11* (41.3%), and *FUS* (41.3%). Among these genes, mRNA expression levels of *CDH17* and *APOE* were associated with depth of tumor invasion ($P = 0.0060$ and $P = 0.0139$, respectively), and those of *FUS* and *APOE* were associated with degree of lymph node metastasis ($P = 0.0416$ and $P = 0.0006$, respectively). In addition, mRNA expression levels of *FUS*, *COL1A1*, *COL1A2*, and *APOE* were associated with stage ($P = 0.0414$, $P = 0.0156$, $P = 0.0395$, and $P = 0.0125$, respectively). Quantitative reverse transcription-PCR analysis also showed a high level of *REGIV* expression (>100 arbitrary units) in 14 of 46 GC samples (30.4%) but not in noncancerous tissues. We detected V5-tagged RegIV protein in the culture media of cells transfected with pcDNA-RegIV-V5 by Western blot. Our results provide a list of candidate genes that are potentially involved in invasion, metastasis, and carcinogenesis of GC. *REGIV* may serve as a specific biomarker for GC.

INTRODUCTION

Gastric carcinoma (GC) is one of the most common human cancers. Despite improvements in cancer therapy, ~650,000 patients with GC die/year (1). A variety of genetic and epigenetic alterations are associated with GC (2–4). However, the underlying mechanism of gastric carcinogenesis is still poorly understood. To identify potential molecular markers for GC and to better understand the development of GC at the molecular level, comprehensive gene expression analysis may be useful. Although several large-scale gene expression studies with cDNA or oligonucleotide arrays have been performed in GC (5–8), they have used different platforms that varied in the number and identity of genes printed on them. On the other hand, serial analysis of gene expression (SAGE) analyzes 14-bp tags derived from defined positions of cDNAs without *a priori* knowledge of the sequence of the genes expressed (9). Thus, SAGE offers an unbiased, comprehensive gene expression profiling approach. Recently, three SAGE studies of GC were reported, and several up-regulated and down-regulated genes were identified (10–12). However, only one (10) or two samples (11, 12) were examined, and the relation to invasion and metastasis was not analyzed. In the present study, we performed SAGE analysis on four samples of GC of different stages and different histologies. In addition, we performed SAGE analysis on one lymph node metastasis of GC. We report here the identification of several genes and tags potentially involved in invasion, metastasis, and carcinogenesis of GC. Among these, we focused on the *REGIV* gene because this gene is frequently overexpressed in GC, and *REGIV* expression is narrowly restricted in noncancerous tissues. In addition, the amino acid sequence of the RegIV protein suggests that it may be secreted.

MATERIALS AND METHODS

Tissue Samples. For SAGE analysis, four primary GC samples and 1 associated lymph node metastasis were used (Table 1). We confirmed microscopically that the tumor specimens consisted mainly (>80%) of carcinoma tissue with the exception of S219T. For quantitative reverse transcription-PCR (RT-PCR), 46 GC samples and corresponding nonneoplastic mucosa samples were used. Of the 46 GC samples, lymph node metastasis samples were available for 9. The samples were obtained at surgery at Hiroshima University Hospital and affiliated hospitals. Noncancerous samples of the heart, aorta, lung, tongue, esophagus, stomach, duodenum, ileum, colon, liver, gallbladder, pancreas, kidney, urinary bladder, thyroid gland, adrenal gland, spleen, skin, endometrium, and lymph node were obtained at autopsy from a 28-year-old woman diagnosed with multiple sclerosis. Samples were frozen immediately in liquid nitrogen and stored at −80°C until use. Histological classification of GC was performed according to the Lauren classification system (13). In addition, diffuse-type GC samples were additionally classified into diffuse-adherent and diffuse-scattered subtypes (14). Tumor staging was carried out according to the Tumor-Node-Metastasis Stage Grouping (15).

SAGE. SAGE was performed according to SAGE protocol version 1.0e, June 23, 2000. Tags were extracted from the raw sequence data with SAGE2000 analysis software version 4.12 kindly provided by Dr. Kenneth W. Kinzler (The Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine). Clinicopathological details of the 5 samples are shown in Table 1. To identify genes involved in tumor progression, we analyzed 2 GC samples (W226T and W246T). Both samples were classified as intestinal type GC. However, W226T was early, and W246T was advanced. Early GC is limited to the mucosa or the mucosa and submucosa, regardless of nodal status (16). We confirmed microscopically that these 2 samples showed similar histological features (Fig. 1). To identify genes involved in tumor metastasis, we analyzed 1 GC sample (P208T) and its lymph node metastasis (P208L). Histologically, these samples were classified as diffuse-adherent type, and we confirmed microscopically that both the primary tumor (P208T) and the metastatic tumor (P208L) contained few stromal cells and lymphocytes (Fig. 1). Scirrhous-type GC belongs to the diffuse-scattered type, often occurs in young women, and is characterized by extensive fibrous stroma, infiltrative and rapid growth, and poor prognosis (17). Sample S219T was a scirrhous-type GC showing scattering growth in an abundant fibrous stroma (Fig. 1). To

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Table 1 *Clinicopathological details of the 5 samples analyzed by serial analysis of gene expression*

Sample name	Sample type	Sex	Age (yrs)	Tumor[a]	Node[a]	Metastasis[a]	Stage[a]	Histological classification[b]	Total number of tags	Number of unique tags in each sample
W226T	Primary	Male	59	1	0	0	IA	Intestinal	43,908	16,082
W246T	Primary	Male	44	2	2	0	IIIA	Intestinal	32,174	12,792
S219T	Primary	Female	29	3	3	0	IV	Diffuse-scattered	34,660	14,576
P208T	Primary	Male	60	4	3	0	IV	Diffuse-adherent	11,582	6,135
P208L	Lymph node metastasis of P208T								15,382	7,425
									137,706[c]	38,903[d]

[a] Tumor staging of gastric carcinoma (GC) were done according to the Tumor-Node-Metastasis Stage Grouping (15).
[b] Histological classification of GC was done according to the Lauren classification system (13). In addition, diffuse-type GC were additionally classified into diffuse-adherent and diffuse-scattered subtypes (14).
[c] Total number of tags of 5 GC samples.
[d] Total number of unique tags among 5 GC samples.

permit direct comparison, each library was normalized to a total of 1,000,000 tags.

Cluster Analysis. The Cluster and TreeView computer programs were obtained from online resources.[1] We compared SAGE tags from 4 primary GC samples with those from samples of normal gastric epithelia [GSM784, SAGE normal gastric body epithelial (10)], available from SAGEmap (18).[2] We also compared SAGE tags from 2 primary GC samples, also available from SAGEmap [GSM757, SAGE gastric cancer-G234 (10) and GSM2385, SAGE gastric cancer-G189] with those from normal gastric epithelia (GSM784) and obtained the 20 most up-regulated and 20 most down-regulated tags. This produced a dataset of 128 tags. These data were imported into the Cluster program and were log-transformed, and complete linkage clustering was performed.

Quantitative RT-PCR Analysis. 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). PCR was performed with a 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 (19). The sequences primer are listed in Supplementary Table 1. We calculated the ratio of target gene mRNA expression levels between GC tissue (T) and corresponding nonneoplastic mucosa (N). T/N ratios > 2-fold were considered to represent overexpression. Genes with T/N ratios > 2 in >40% of the samples examined were defined as frequently up-regulated genes.

Cell Lines, Expression Vector, and Western Blot. Two cell lines derived from human GC were used. MKN-28 was kindly provided by Dr. Toshimitsu Suzuki. HSC-39 was kindly provided by Dr. Kazuyoshi Yanagihara (20). All cell lines were maintained in RPMI 1640 (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 10% fetal bovine serum (BioWhittaker, Walkersville, MD) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. For constitutive expression of the *RegIV* gene, cDNA was PCR amplified and subcloned into pcDNA 3.1 (Invitrogen Corp., Carlsbad, CA) in-frame with a COOH-terminal V5 epitope tag. MKN-28 cells were transfected transiently with *REGIV* cDNA using FuGene6 Transfection Reagent (Roche Diagnostics Co., Indianapolis, IN) according to the manufacturer's instructions. For Western blot analysis, cells and culture media from MKN-28 cells transfected with pcDNA 3.1 or pcDNA-RegIV-V5 were lysed as described previously (21). The culture media was concentrated with a PROTEIN concentrate kit (Takara Bio, Inc., Shiga, Japan). The lysates (40 μg) were solubilized in Laemmli sample buffer by boiling and then were subjected to 15% SDS-PAGE followed by electrotransfer onto a nitrocellulose filter. Anti-V5 monoclonal antibody was purchased from Invitrogen Corp. Peroxidase-conjugated antimouse IgG was used as the secondary antibody. The immune complex was visualized with an ECL Western blot detection system (Amersham Pharmacia Biotech).

Statistical Methods. Statistical analyses were performed with the Mann-Whitney *U* test. *P* of <0.05 was regarded as statistically significant.

[1]Internet address: http://www.microarrays.org/software.html.
[2] Internet address: http://www.ncbi.nlm.nih.gov/SAGE/.

RESULTS

Generation of SAGE Data. A total of 137,706 tags was generated, including 38,903 that were unique. The numbers of tags and unique tags are shown in Table 1. Sequence data from our SAGE libraries are publicly available at SAGEmap (GEO accession number GSE 545, SAGE Hiroshima gastric cancer tissue).

Comparison of Expression Patterns in GCs and Normal Stomach. We compared SAGE tags from 4 primary GC samples with those from normal gastric epithelia (GSM784). The 20 most up-regulated tags and 20 most down-regulated tags in each GC are shown in Supplementary Table 2. Among the up-regulated tags, 12 were commonly up-regulated in both W226T (intestinal type) and W246T (intestinal type). These tags included *lysozyme* (*LYZ*), *trefoil factor 3* (*TFF3*), *aldolase A* (*ALDOA*), and *S100 calcium-binding protein*, which may participate in the genesis of intestinal type GC. P208T (diffuse-adherent type) and S219T (diffuse-scattered type) showed many different tags from those of W226T and W246T. The down-regulated tags were similar in all 4 GC samples and included *lipase* (*LIPF*), *pepsinogen* (*PGA5*), and *antrum mucosa protein* (*AMP18*), which are expressed physiologically in normal gastric glands.

The SAGE data were also analyzed by a clustering algorithm to delineate patterns in the expression of 128 tags among all four libraries (our four GC libraries, our one lymph node metastasis library, and three libraries available from SAGEmap[2]; Fig. 2). These tags were selected as described in "Materials and Methods." Clusters of coexpressed tags suggested that the two intestinal type GC libraries (W226T and W246T), despite being derived from 2 different patients at different stages, were the most similar to each other. The primary GC (P208T) and its lymph node metastasis (P208L) appeared not to be similar to each other. To identify ideal biomarkers for GC, we focused on a cluster of 14 tags, the expression of which was up-regulated in 6 GC samples (our four GC libraries plus two GC libraries available from SAGEmap) and 1 lymph node metastasis. Because some genes share the same SAGE tag, these 14 tags represented 22 genes (Fig. 2 and Table 2). To validate the SAGE data, the expression of 12 known genes was analyzed by quantitative RT-PCR of an additional 46 GC samples and corresponding nonneoplastic mucosa samples. Frequently overexpressed genes were *APOC1* (percentage of samples with overexpression: T/N ratio > 2; 67.4%), *YF13H12* (52.2%), and *CEACAM6* (50.0%; Fig. 3*A*, see also Supplementary Fig. 1). Other genes were less frequently overexpressed. The expression levels of all 12 genes were not associated with T grade (depth of tumor invasion), N grade (degree of lymph node metastasis), or tumor stage.

Comparison of Expression Patterns in Early and Advanced GC. To identify genes involved in tumor progression, we compared tags from early GC (W226T) and advanced GC (W246T). The 10

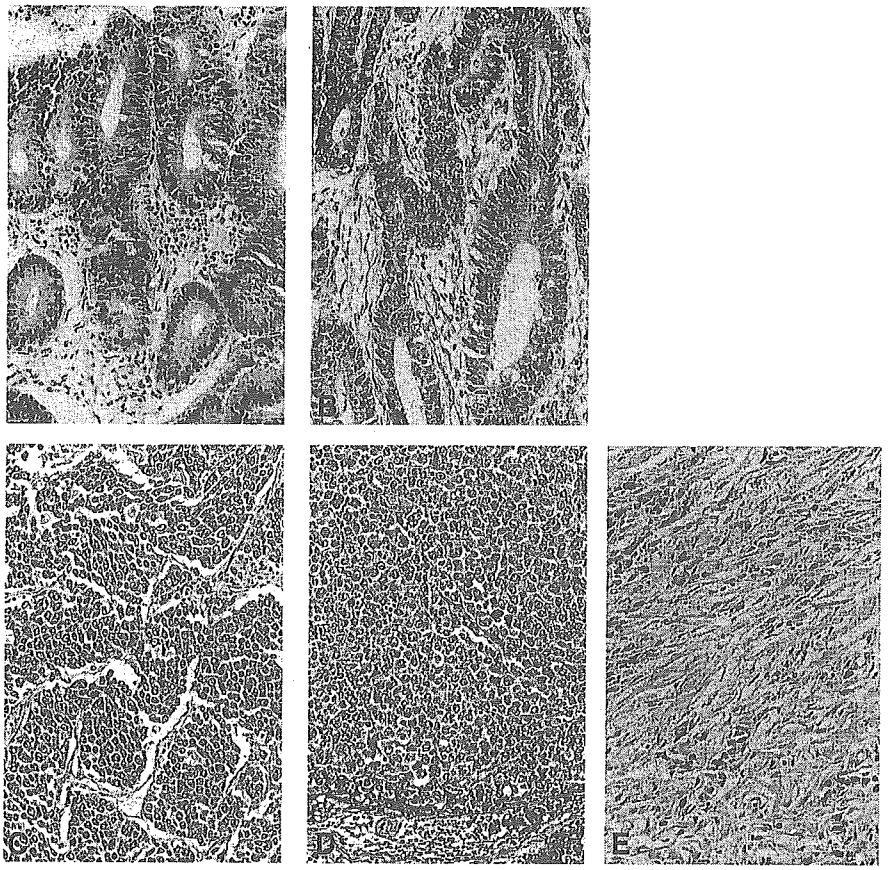

Fig. 1. Histological features of gastric carcinoma (GC) samples analyzed by serial analysis of gene expression. Formalin-fixed, paraffin-embedded sections were stained with H&E. Both W226T (*A*) and W246T (*B*) were to intestinal type GC, and histological features were similar. Both P208T (*C*) and P208L (*D*) were diffuse-adherent type GC. P208L was a lymph-node metastasis of P208T. S219T (*E*) was diffuse-scattered type GC. Obvious histological heterogeneity was not seen in all specimens (original magnification, ×100).

most up-regulated tags are shown in Table 3, and the 10 most down-regulated tags are shown in Supplementary Table 3. Because some genes share the same SAGE tag, these up-regulated 10 tags represented 12 genes (Table 3). To validate the SAGE data, the expression of 9 known genes was analyzed by quantitative RT-PCR of an additional 46 GC samples and corresponding nonneoplastic mucosa samples. Genes frequently overexpressed in GC compared with nonneoplastic mucosa were *COL1A1* (78.3%), *CDH17* (73.9%), *COL1A2* (58.7%), and *FUS* (41.3%; Fig. 3*B*, see also Supplementary Fig. 2). Other genes were less frequently overexpressed. The mRNA expression levels of *CDH17* were associated with T grade ($P = 0.0060$). The mRNA expression levels of *FUS* were associated with N grade ($P = 0.0416$). The mRNA expression levels of *FUS*, *COL1A1*, and *COL1A2* were associated with tumor stage ($P = 0.0414$, $P = 0.0156$, and $P = 0.0395$, respectively; Table 4).

Comparison of Expression Patterns in Primary GC and Associated Lymph Node Metastasis. To identify genes involved in tumor metastasis, we compared tags from primary GC (P208T) and its lymph node metastasis (P208L). The 10 most up-regulated tags are shown in Table 5, and the 10 most down-regulated tags are shown in Supplementary Table 4. The up-regulated tags represented 12 genes (Table 5). To validate the SAGE data, the expression of 5 known genes was analyzed by quantitative RT-PCR of an additional 46 GC samples and their lymph node metastases in 9 samples. A frequently overexpressed gene in lymph node metastasis compared with primary GC was not found (Fig. 3*C*, see also Supplementary Fig. 3). *APOE* mRNA expression in lymph node metastasis tended to be higher than that in primary GC. Other genes were less frequently overexpressed. Genes frequently overexpressed in GC compared with nonneoplastic

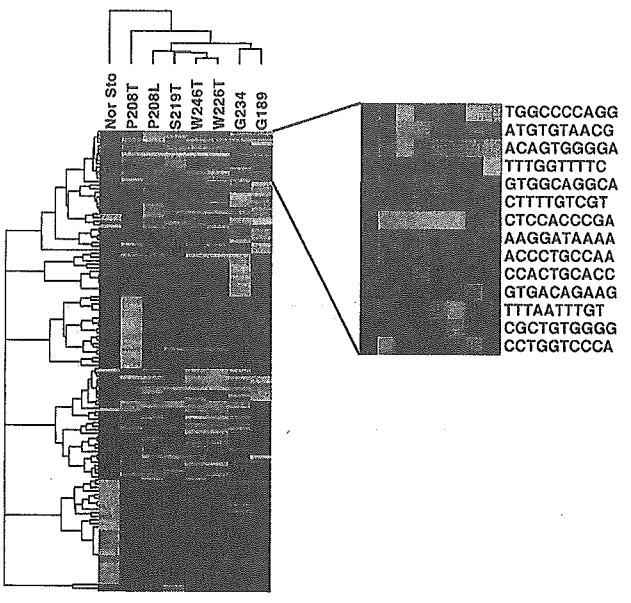

Fig. 2. Cluster analysis of 128 tags form eight serial analysis of gene expression (SAGE) libraries and dendrogram showing similarities in expression patterns among libraries. Tags were selected as described in the "Materials and Methods." *Brackets* indicate the cluster of tags commonly up-regulated in gastric carcinoma, which is expanded in size on the right for visualization. On the dendrogram, two intestinal type gastric carcinoma samples cluster together, indicating their high degree of similarity. Each row represents a tag, whereas each column corresponds to a SAGE library sample. The absolute abundance of the SAGE tag in the library (SAGE tag number) correlates with the intensity of the red color (*black*, not present; *intense red*, highly abundant).

Table 2 *Genes and tags commonly up-regulated in gastric carcinoma obtained by serial analysis of gene expression*

Tag sequence	UniGene ID	Symbol	Description
TGGCCCCAGG	Hs.268571	*APOC1*	Apolipoprotein C-I
ATGTGTAACG	Hs.81256	*S100A4*	S100 calcium binding protein A4 (calcium protein, Calvasculin, metastasin, murine placental homologue)
	Hs.173611	*NDUFS2*	NADH dehydrogenase (ubiquinone) Fe-S protein 2, 49 kDa (NADH-coenzyme Q reductase)
ACAGTGGGGA	Hs.278270	*TEBP*	Unactive progesterone receptor, 23 kDa
	Hs.288443		Homosapiens transcribed sequences
	Hs.355693		Homosapiens transcribed sequence with strong similarity to protein pir:A56211 (H. sapiens) A56211 progesterone receptor-related protein P23-human
TTTGGTTTTC	Hs.179573	*COL1A2*	Collagen, type I, α2
	Hs.21431	*SUFU*	Suppressor of fused homologue (Drosophila)
GTGGCAGGCA	Hs.47334	*SYAP1*	Synapse associated protein 1, SAP47 homologue (Drosophila)
	Hs.13255	*KIAA0930*	KIAA0930 protein
CTTTTGTCGT	Hs.19597	*KIAA1694*	KIAA1694 protein
CTCCACCCGA	Hs.82961	*TFF3*	Trefoil factor 3 (intestinal)
AAGGATAAAA	Hs.73848	*CEACAM6*	Carcinoembryonic antigen-related cell adhesion molecule 6 (nonspecific cross reacting antigen)
ACCCTGCCAA	Hs.405871	*FLJ20249*	Hypothetical protein FLJ20249
CCACTGCACC	Hs.6853	*FLJ22167*	Hypothetical protein FLJ22167
	Hs.146844		na similar to hypothetical protein FLJ10891
GTGACAGAAG	Hs.356129	*EIF4A1*	Eukaryotic translation initiation factor 4A, isoform 1
TTTAATTTGT	Hs.182793	*GOLPH2*	Golgi phosphoprotein 2
	Hs.220689	*G3BP*	Ras-GTPase-activating protein SH3-domain-binding protein
CGCTGTGGGG	Hs.7486	*YF13H12*	Protein expressed in thyroid
CCTGGTCCCA	Hs.23881	*KRT7*	Keratin 7
	Hs.167679	*SH3BP2*	SH3-domain binding protein 2
TGGAAATGAC	Hs.172928	*COL1A1*	Collagen, type I, α1
	Hs.20506	*LOC284371*	Hypothetical protein LOC284371

mucosa were *APOE* (50.0%) and *S100A11* (41.3%; Fig. 3*D*). The mRNA expression levels of *APOE* were associated with T grade ($P = 0.0139$), N grade ($P = 0.0006$), and tumor stage ($P = 0.0125$; Table 4).

REGIV Overexpression in GC. Among the 20 up-regulated tags in each GC sample (Supplementary Table 2), we focused on *REGIV* because *REGIV* expression was narrowly restricted by Virtual Northern analysis by SAGEmap (Fig. 4*A*). Besides GCs, *REGIV* was detected at low levels in only eight libraries, including one colon cancer and two normal colon libraries. Quantitative RT-PCR analysis showed overexpression of the *REGIV* gene in 22 samples of the 46 GC samples (47.8%; Fig. 4*B*). When we focused on *REGIV* gene expression in GC, high levels of *REGIV* expression (>100, arbitrary units) were found in 14 of 46 samples (30.4%; Fig. 4*C*). Among various normal tissues obtained from an autopsy, obvious *REGIV* expression was found in noncancerous stomach, duodenum, ileum, colon, and pancreas, as reported elsewhere (22). However, the levels of *REGIV* expression were low (<60 arbitrary units).

Analysis of the amino acid sequence of the RegIV protein suggests that it may be secreted. To investigate whether RegIV is a secreted protein, we performed Western blot analysis of cell extracts and culture media of MKN-28 cells transiently transfected with pcDNA 3.1 or pcDNA-RegIV-V5. With an anti-V5 antibody, we detected an approximate M_r 20,000 band corresponding to V5-tagged RegIV protein in cell extracts and culture media from RegIV-V5-expressing MKN-28 cells but not in control cells (Fig. 4*D*).

DISCUSSION

To identify potential molecular markers for GC and to better understand the development of GC at the molecular level, we performed SAGE on 5 GC samples from 4 patients that showed distinct histological types and tumor stages. We analyzed with respect to (*a*) commonly up-regulated genes in GC compared with normal stomach, (*b*) up-regulated genes in advanced compared with early GC (genes potentially involved in tumor progression), (*c*) up-regulated genes in GC lymph node metastasis compared with primary GC (genes potentially involved in tumor metastasis), and (*d*) genes specifically ex-

Table 3 *The 10 most up-regulated tags in advanced gastric carcinoma in comparison with early gastric carcinoma*

Tag sequence	Tags/million W226T	Tags/million W246T	UniGene ID	Symbol	Description
TCCCCGTAAA	22[a] (1)[b]	559 (18)			No match
TCCCGTACAT	0 (0)	279 (9)			No match
AAAAGAGTGG	0 (0)	217 (7)	Hs.89436	*CDH17*	Cadherin 17, LI cadherin (liver-intestine)
			Hs.99969	*FUS*	Fusion, derived from t(12;16) malignant liposarcoma
CCAGAGAACT	0 (0)	217 (7)	Hs.356442	*PRO1073*	PRO1073 protein
AACCTCCCCA	0 (0)	186 (6)	Hs.137396		Sapiens cDNA FLJ36926 fis, clone BRACE2005196
			Hs.232092		Sapiens cDNA FLJ30146 fis, clone BRACE2000256
AATACTTTTG	0 (0)	186 (6)	Hs.356427	*PAI-RBP1*	PAI-1 mRNA-binding protein
			Hs.179573	*COL1A2*	Collagen, type I, α2
TCCTATTAAG	22 (1)	372 (12)			No match
TGGAAATGAC	0 (0)	186 (6)	Hs.172928	*COL1A1*	Collagen, type I, α1
			Hs.193076	*GRAP2*	GRB2-related adaptor protein 2
TCCCCGTACA	227 (10)	3325 (107)	Hs.2730	*HNRPL*	Heterogeneous nuclear ribonucleoprotein L
			Hs.151734	*NUTF2*	Nuclear transport factor 2
AAGTGAAACA	0 (0)	155 (5)	Hs.93659	*ERP70*	Protein disulfide isomerase-related protein (calcium-binding protein, intestinal-related)

[a] The absolute tag counts are normalized to 1,000,000 total tags/sample.
[b] Number in parentheses indicates the absolute tag counts.

Table 4 *Association between clinicopathological features and mRNA expression levels of genes involved in tumor progression and metastasis obtained by serial analysis of gene expression*

Gene name		Case number	mRNA expression level Mean ± SE	P^a
CDH17				
T grade[b]	1/2	20	0.065 ± 0.022	0.0060
	3/4	26	0.275 ± 0.068	
N grade[b]	0	11	0.127 ± 0.054	0.8367
	1/2/3	35	0.201 ± 0.053	
Stage[b]	I/II	18	0.102 ± 0.036	0.2035
	III/IV	28	0.235 ± 0.064	
FUS				
T grade	1/2	20	0.050 ± 0.017	0.5714
	3/4	26	0.062 ± 0.014	
N grade	0	11	0.027 ± 0.016	0.0416
	1/2/3	35	0.066 ± 0.013	
Stage	I/II	18	0.033 ± 0.012	0.0414
	III/IV	28	0.072 ± 0.015	
COL1A1				
T grade	1/2	20	6.84 ± 1.34	0.1407
	3/4	26	20.66 ± 8.14	
N grade	0	11	5.57 ± 1.67	0.1048
	1/2/3	35	17.50 ± 6.11	
Stage	I/II	18	5.32 ± 1.14	0.0156
	III/IV	28	20.64 ± 7.54	
COL1A2				
T grade	1/2	20	10.91 ± 1.86	0.1377
	3/4	26	27.90 ± 9.14	
N grade	0	11	10.45 ± 2.30	0.2572
	1/2/3	35	23.67 ± 6.91	
Stage	I/II	18	9.45 ± 1.54	0.0395
	III/IV	28	27.62 ± 8.49	
APOE				
T grade	1/2	20	6.17 ± 2.90	0.0139
	3/4	26	11.29 ± 2.98	
N grade	0	11	1.36 ± 0.22	0.0006
	1/2/3	35	11.49 ± 2.66	
Stage	I/II	18	6.33 ± 3.22	0.0125
	III/IV	28	10.83 ± 2.78	

[a] Mann-Whitney *U* test.
[b] Tumor staging of gastric carcinoma were done according to the Tumor-Node-Metastasis Stage Grouping (15).

pressed in GC. Quantitative RT-PCR analysis of 27 selected genes showed that *COL1A1*, *CDH17*, *APOC1*, *COL1A2*, *YF13H12*, *CEACAM6*, *APOE*, *REGIV*, *FUS*, and *S100A11* were overexpressed in 40–80% of the 46 GC samples analyzed. Among them, *TFF3*, *REGIV*, and *S100 calcium-binding proteins* have been reported to be commonly up-regulated in GC by other SAGE studies (10, 12).

Among the 27 selected genes, only *COL1A1* and *CDH17* were overexpressed in >70% of the 46 GC samples. *COL1A1* was most frequently overexpressed, and *COL1A2* was also frequently overexpressed as determined by quantitative RT-PCR. Although *COL1A1* expression has been demonstrated in tumor cells and tumor-associated stromal cells in multiple cancers (23, 24), *COL1A1* and *COL1A2* have been reported to be elevated in tumor endothelium as compared with normal endothelium (25), suggesting that they play an important role in angiogenesis and the formation of desmoplasia in GC. In fact, we found a significant association between tumor stage and mRNA expression level for both genes. *CDH17* is a structurally unique member of the cadherin superfamily and is expressed in intestinal epithelial cells (26) and in intestinal metaplasia of the stomach (27). Although overexpression of *CDH17* has been reported in intestinal type GC (27), the association between *CDH17* and tumor invasion has not been examined. In the present study, we showed that the high level of *CDH17* expression was associated with advanced T grade, indicating that *CDH17* is a candidate marker gene for tumor progression. However, a recent study of pancreatic cancer reported that high CDH17 expression correlates with good survival (28). Thus, the significance of the association of high *CDH17* expression and advanced tumor invasion remains unclear. Organ specificity of CDH17 expression may be involved in tumor invasion and progression.

Frequently overexpressed genes in this study included 2 apolipoproteins. *APOC1* was commonly up-regulated in GC, and *APOE* was a candidate marker for tumor metastasis. Although the expression status of these genes has not been previously examined in GC, it has been reported in certain cancers. *APOC1* gene expression localizes to tumor-associated macrophages in breast carcinoma (24). In colorectal carcinoma, intense apolipoprotein E expression has been identified in macrophages surrounding the tumor area (29), suggesting that overexpression of these 2 apolipoproteins occur in tumor-associated macrophages. Macrophages appear to play a pivotal role in tumor angiogenesis, and in our previous observation, macrophage infiltration is significantly associated with tumor vessel density in GC (30). In addition, we found that a high level of *APOE* expression was associated with advanced T grade, N grade, and stage. Apolipoprotein E produced by tumor-associated macrophages may play an important role in tumor progression. Because *APOE* mRNA expression in lymph node metastasis tended to be higher than that in primary GC, *APOE* expression may be up-regulated in GC cells. In prostate cancer, apolipoprotein E expression was identified in cancer cells and correlated directly with Gleason grade (31). Whether GC cells or tumor-associated macrophages express apolipoprotein E remains unclear. Immunohistochemical analysis will be required to answer this question.

S100 calcium-binding proteins (*S100A4*, *S100A9*, *S100A10*, and *S100A11*) were among the 20 up-regulated genes. *S100A4* is commonly up-regulated in GC. In fact, S100A4 expression was detected in 51 of 92 primary GC samples (55%; Ref. 32). Previous SAGE analysis of moderately differentiated GC indicated that 5 calcium-binding proteins (*S100A2*, *S100A7*, *S100A8*, *S100A9*, and *S100A10*) are overexpressed (10). *S100A11* is potentially involved in tumor metastasis. However, no obvious up-regulation of *S100A11* was identified in lymph node metastasis of GC. *S100A11* may be important for stomach carcinogenesis, and overexpression of S100 calcium-binding proteins may be a common alteration in GC.

CEACAM6 is a member of the immunoglobulin superfamily (33) and functions as an intercellular adhesion protein (34). *CEACAM6* overexpression independently predicts poor overall survival and disease-free survival in colorectal carcinoma (35). In GC, although frequent overexpression of *CEACAM6* was identified in the present study, we found no association between the expression levels of *CEACAM6* and clinicopathological features.

Overexpression of 2 genes related to wound-healing was identified in the present study. *TFF3* functions in the maintenance and repair of the intestinal mucosa (36). *TFF3* was commonly up-regulated in GC, and overexpression of *TFF3* in GC has been reported previously (37). *REGIV* was a candidate gene specifically expressed in GC. *REGIV* is a member of the *Reg* gene family, which includes 3 other genes (22). *REGIV* expression is restricted to the gastrointestinal tract and pancreas and is up-regulated in response to mucosal injury in active Crohn's disease and ulcerative colitis (22). It has been reported that *REGIV* expression is increased in most colorectal cancers compared with normal tissues (38). Although overexpression of *REGIV* has been reported by conventional RT-PCR in 6 GC samples (12), the specificity of *REGIV* expression has not been investigated. In our study, Virtual Northern and quantitative RT-PCR analysis showed *REGIV* expression to be narrowly restricted. We performed additional quantitative RT-PCR analysis of 10 colorectal cancers, 10 lung cancers, and 10 breast cancers (data not shown). Although *REGIV* expression was identified in all 10 colorectal cancers, the levels of *REGIV* expression were <100 arbitrary units. We also confirmed that the expression levels of *REGIV* in all 10 colorectal cancers were higher than those in normal colon. No *REGIV* expression was identified in lung or breast cancers. These results are consistent with the Virtual

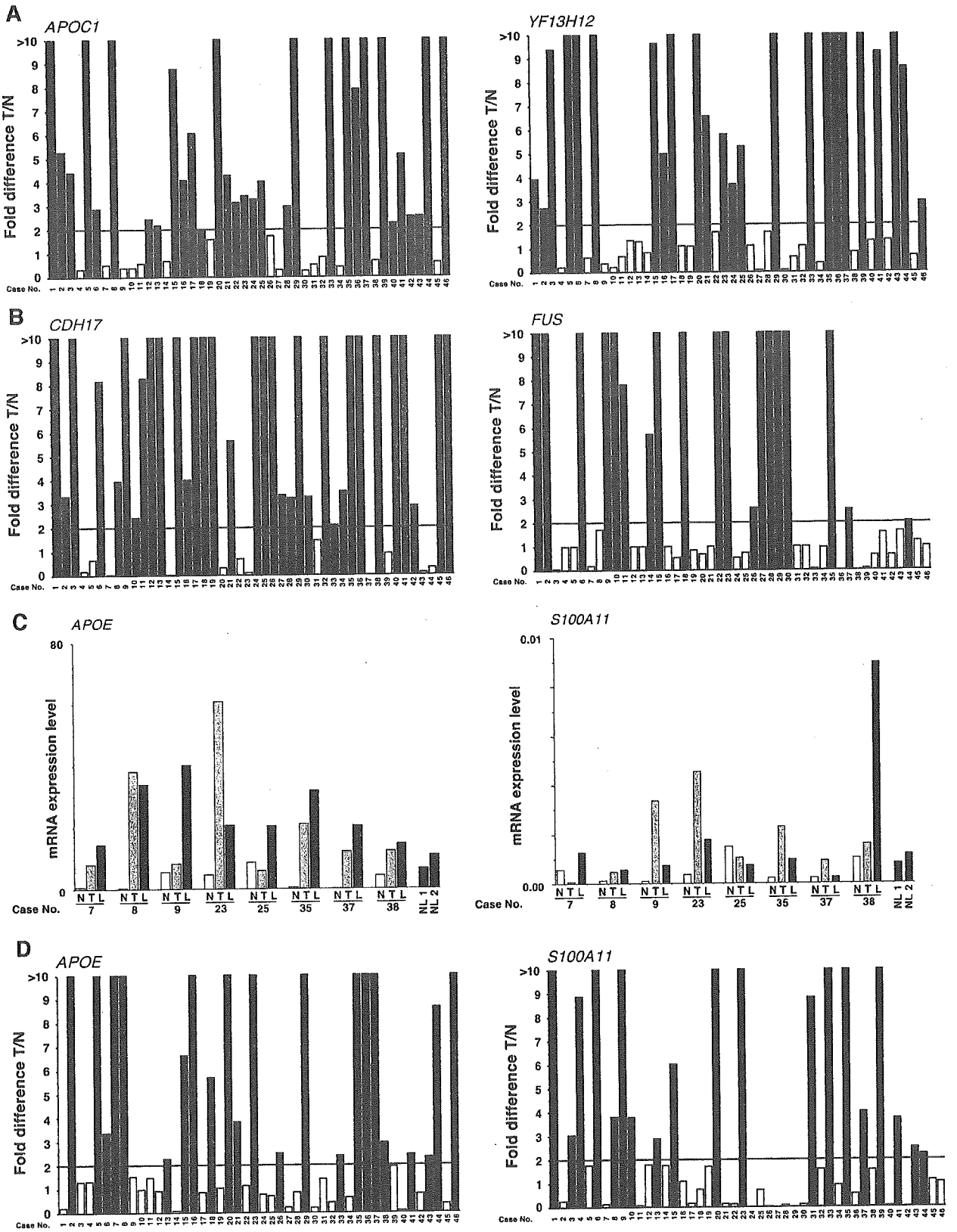

Fig. 3. Validation of serial analysis of gene expression (SAGE) data by quantitative reverse transcription-PCR (RT-PCR). Fold change indicates the ratio of target gene mRNA level in gastric carcinoma (GC) to that in corresponding nonneoplastic mucosa. *A*, quantitative RT-PCR analysis of genes commonly up-regulated according to SAGE analysis. Of the 46

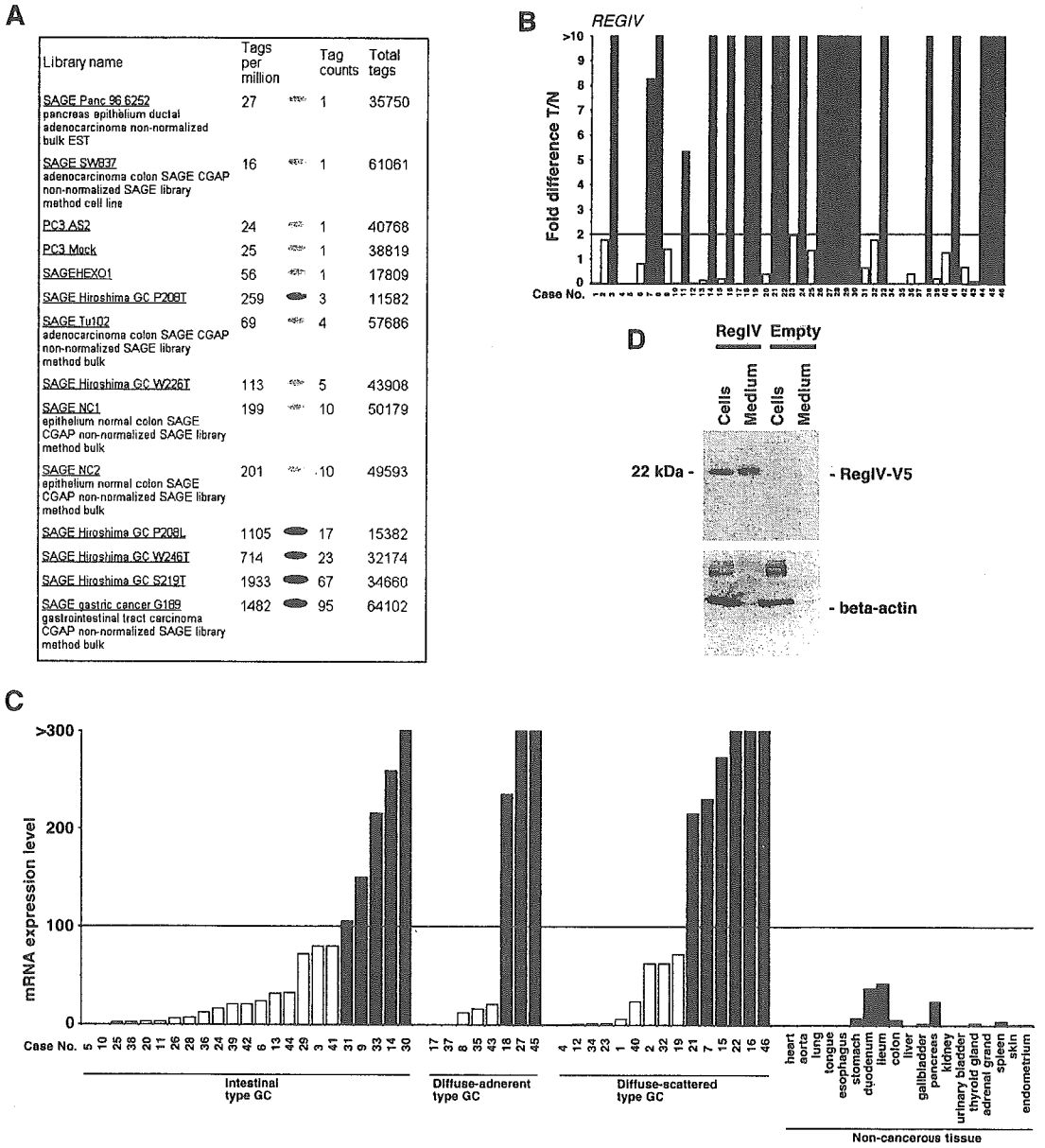

Fig. 4. *A*, Virtual Northern analysis shows *REGIV* expression to be narrowly restricted. Poorly differentiated gastric cancer (GC G189) showed strong expression of *REGIV*, whereas moderately differentiated GC (GC G234) did not. In our SAGE analysis, *REGIV* expression was detected as follows: 1933 in S219T; 714 in W246T; 1105 in P208L; 113 in W226T; and 259 in P208T. *B*, quantitative RT-PCR analysis of *REGIV* in primary GC and corresponding nonneoplastic mucosa. Fold change indicates the ratio of *REGIV* mRNA level in GC to that in corresponding nonneoplastic mucosa. Of the 46 GC samples, overexpression ($T/n > 2$) of *REGIV* was identified in 22 (47.8%). *C*, quantitative RT-PCR analysis of *REGIV* in primary GC samples and various noncancerous tissues. In GC, a high level of *REGIV* expression (>100 arbitrary units) was identified in samples 31, 9, 33, 14, 30, 18, 27, 45, 21, 7, 15, 22, 16, and 46. In various noncancerous tissues, a high level of *REGIV* expression was not identified. *REGIV* expression was found in noncancerous stomach, duodenum, ileum, colon, and pancreas. The units are arbitrary, and we calculated *REGIV* mRNA expression by standardization to 1.0 μg of total RNA from HSC-39 as 1.0. *D*, anti-V5 Western blot assay of V5 epitope- tagged RegIV protein. Cells and media from MKN-28 cells transfected with pcDNARegIV-V5 (RegIV) or pcDNA 3.1 (empty) constructs were lysed, resolved by SDS-PAGE, and immunoblotted with monoclonal mouse anti-V5 antibody. We confirmed by anti-β-actin Western blot that contamination of cells in culture medium was minimal.

Northern analysis. Furthermore, we showed that the RegIV protein is secreted, suggesting that RegIV may serve as a serum tumor marker. The number of samples we studied was small, and serum RegIV protein levels have not been examined. Additional investigation will clarify whether the RegIV protein can serve as a serum tumor marker. The role of *REGIV* gene overexpression in stomach carcinogenesis

GC samples, overexpression ($T/n > 2$) was detected at the following frequencies: 31 (67.4%) for *APOC1* and 24 (52.2%) for *YF13H12*. *B*, quantitative RT-PCR analysis of genes potentially involved in tumor progression according to SAGE analysis. Of the 46 GC samples, overexpression ($T/n > 2$) was detected at the following frequencies: 34 (73.9%) for *CDH17* and 19 (41.3%) for *FUS*. *C* and *D*, quantitative RT-PCR analysis of genes potentially involved in tumor metastasis according to SAGE analysis. *C*, mRNA expression levels of indicated genes in nonneoplastic mucosa, tumor, and lymph node metastasis. The units are arbitrary, and we calculated the target mRNA expression level by standardization to 1.0 μg of total RNA from HSC-39 as 1.0. T, tumor; n = nonneoplastic mucosa; L, lymph node metastasis; NL, normal lymph node from autopsy. *D*, mRNA expression levels of indicated genes in 46 GC samples. Of the 46 GC samples, overexpression ($T/n > 2$) was detected at the following frequencies: 23 (50%) for *APOE* and 19 (41.3%) for *S100A11*.

Table 5 *The 10 most up-regulated tags in lymph node metastasis of gastric carcinoma in comparison with primary gastric carcinoma*

Tag sequence	Tags per million		UniGene ID	Symbol	Description
	P208T	P208L			
ATCGGGCCCG	0[a] (0)[b]	1105 (17)	Hs.274411	*SCAND1*	SCAN domain containing 1
TATGAGGGTA	0 (0)	975 (15)	Hs.24950	*RGS5*	Regulator of G-protein signalling 5
CAGGCCCCAC	0 (0)	780 (12)	Hs.417004	*S100A11*	S100 calcium binding protein A11 (calgizzarin)
			Hs.145696	*RNPC2*	RNA-binding region (RNP1, RRM) containing 2
CGACCCCACG	0 (0)	780 (12)	Hs.169401	*APOE*	Apolipoprotein E
GCCCAGGTCA	86 (1)	1560 (24)	Hs.10499	*FLJ10815*	Hypothetical protein FLJ10815
TTAACCCCTC	86 (1)	1430 (22)	Hs.78224	*RNASE1*	Ribonuclease, RNase A family, 1 (pancreatic)
			Hs.393660	*H3F3B*	H3 histone, family 3B (H3.3B)
CAAGCAGGAC	0 (0)	650 (10)	Hs.424551	*P24B*	Integral type I protein
TAGAAAGGCA	0 (0)	650 (10)	Hs.457718		na LOC151103
CTCGCGCTGG	0 (0)	585 (9)	Hs.25640	*CLDN3*	Claudin 3
GCTGCTCCCT	0 (0)	585 (9)	Hs.343579	*MRPL14*	Mitochondrial ribosomal protein L14

[a] The absolute tag counts are normalized to 1,000,000 total tags per sample.
[b] Number in parentheses indicates the absolute tag counts.

remains unclear. A possible involvement of *REGIV* in drug (5-fluorouracil or methotrexate) resistance was reported recently (38). Thus, *REGIV* may inhibit apoptosis and may participate in tumor cell growth.

We found that *FUS* and *YF13H12* were overexpressed in GC. *FUS* was first identified as the 5′-part of a fusion gene with *CHOP* in myxoid liposarcomas with the translocation t(12;16)(q13;p11), and FUS protein was found to bind to RNA (39). No studies have analyzed *FUS* expression in human cancers, including GC. However, it has been shown that expression of the *FUS* domain restores liposarcoma development in *CHOP*-transgenic mice (40), suggesting that gain-of-function mutation of both *FUS* and *CHOP* is important. In the present study, *FUS* was a candidate marker for tumor progression, and we showed that a high level of *FUS* expression was associated with advanced N grade and stage. We also found *YF13H12* gene overexpression in GC. However, YF13H12 function remains unclear, and there are no reports on *YF13H12* gene expression. Additional studies will elucidate the biological role of *FUS* and *YF13H12* protein in GC.

Although we found several genes to be overexpressed in GC by SAGE, there were some exceptions of genes overexpressed by SAGE but not by quantitative RT-PCR. It is possible that inconsistent results between SAGE and quantitative RT-PCR represent more than one gene. For example, TTTAATTTGT, represented in both *GOLPH2* and *G3BP*, is commonly up-regulated in GC; however, the expression levels of both *GOLPH2* and *G3BP* were not frequently up-regulated by quantitative RT-PCR. Whether discrepancies between SAGE and quantitative RT-PCR are attributable to differences in methodology remains to be determined. Some GC samples that we analyzed showed overexpression of both *GOLPH2* and *G3BP* by quantitative RT-PCR. Recent evidence indicates that G3BP may serve as an important downstream effector of Ras signaling, and G3BP has been shown to be overexpressed in cancers of the colon, thyroid, breast, and head and neck (41). Thus, genes not frequently overexpressed may play an important role in restricted cases of GC.

Interestingly, among the 20 up-regulated tags in each GC sample, the 2 intestinal-type samples showed distinct tumor stages but showed many of the same tags. Cluster analysis showed that the two intestinal-type GC libraries were the most similar to each other. These results lead us to speculate that morphological phenotype reflects the gene expression profile. Our present results may be due to the selection of samples that represented similar histological features among many variations of intestinal type GC. Additional studies should investigate gene expression profile with respect to morphology. Comparison of expression patterns of W226T and W246T will provide a list of genes involved in tumor progression without the potential bias of histology. Our cluster analysis also showed that the gene expression pattern of SAGE gastric cancer-G234, which is a moderately differentiated tumor and is categorized as an intestinal type GC, was not similar to that of our 2 intestinal type GC samples but is similar to that of SAGE gastric cancer-G189, which is a poorly differentiated tumor and is categorized as a diffuse type GC. The gene expression patterns of GC in Japan may differ from those in the United States. Because we analyzed a limited number of GC samples, additional experiments are needed.

In conclusion, our present SAGE data provide a list of genes potentially involved in invasion, metastasis, and carcinogenesis of GC. We identified several genes by quantitative RT-PCR that have not previously been implicated in GC. Among these, a high level of *REGIV* expression was detected in GC, and expression of *REGIV* was narrowly restricted. Because the RegIV protein is secreted, it may serve as a biomarker for diagnosis of GC.

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ORIGINAL PAPER

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

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

Keywords SNP · MMP-1 · Gastric cancer

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Introduction

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

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

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

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

Materials and methods

Samples

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

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

DNA extraction and PCR-RFLP analysis

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

Sequencing analysis of PCR products

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

Statistical analysis

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

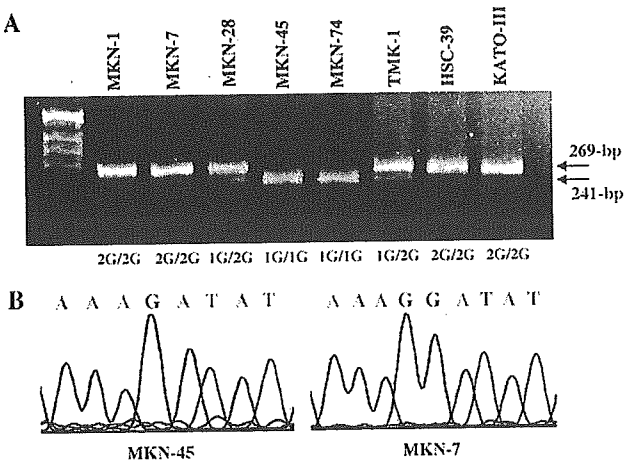

Fig. 1 A Representative PCR-RFLP analysis to confirm the variants in eight human gastric cancer cell lines. Ethidium bromide-stained 2.5% NuSieve 3:1 agarose gel used for genotyping. The target products (269-bp) in the *MMP-1* gene promoter was PCR amplified and digested with *Alu*I, which cleaved the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

Results

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

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

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

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

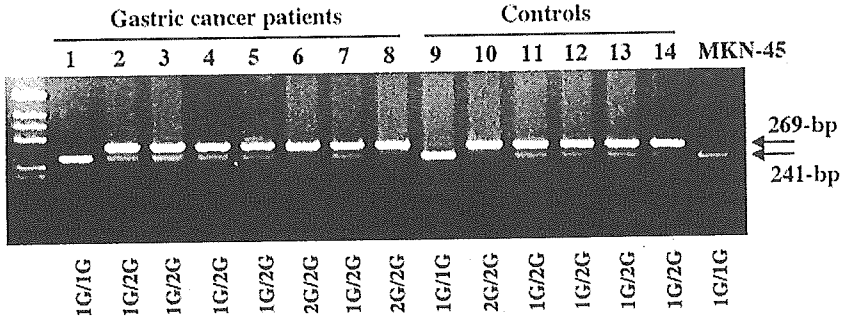

Table 1 *MMP-1*genotype distribution of the study subjects. .*CI* confidence interval

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

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

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

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

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