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Clinicopathological significance of MMP-2 and TIMP-2 genotypes in gastric cancer

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ABSTRACT

Aims: Single nucleotide polymorphisms in matrix metalloproteinase-2 (MMP-2) −1306 C/T and tissue inhibitor of metalloproteinase-2 (TIMP-2) −418 G/C abolish the Sp-1 binding site and down-regulate expression of these genes. We aim to elucidate the role of MMP-2 and TIMP-2 in clinicopathological manifestations of gastric cancer.

Methods: We enrolled 240 gastric cancer patients and 283 controls. DNA was extracted from peripheral blood leucocytes. MMP-2 and TIMP-2 genotypes were analysed by PCR-direct sequencing and PCR-RFLP method, respectively.

Results: MMP-2 and TIMP-2 genotypes were not associated with gastric cancer development. However, patients with MMP-2 −1306 C/C genotype showed higher risk of lymphatic invasion (odds ratio (OR) = 2.77, $p = 0.01$) and venous invasion (OR = 2.93, $p = 0.012$). TIMP-2 G/G genotype was associated with serosal invasion (OR = 1.89, $p = 0.009$), lymph node metastasis (OR = 2.19, $p = 0.021$), lymphatic invasion (OR = 2.87, $p = 0.016$) and venous invasion (OR = 2.65, $p = 0.033$).

Conclusion: Our results suggest MMP-2 and TIMP-2 genotypes play a crucial role in gastric cancer invasion, but not with development of gastric cancer.

1. Introduction

Tumour invasion is a multi-step process and many of the stages require degradation of the extracellular matrix (ECM). Matrix metalloproteinases (MMPs) are a family of extracellular zinc-dependent neutral endopeptidases collectively capable of degrading essentially all ECM components. MMPs not only play important roles in physiologic ECM remodelling, such as tissue regeneration, wound repair and embryo development, but are also involved in pathological conditions, including rheumatoid arthritis, atherosclerotic plaque rupture and autoimmune blistering disorders of the skin. There is also considerable evidence indicating that some MMPs play important roles in tumour invasion and metastasis.[1–4]

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MMP-2, also know as gelatinase A or 72 kDa collagenase IV, is a member of the MMP family which degrades gelatine and type IV collagen.[5] In contrast to other MMPs, MMP-2 is broadly, often constitutively, expressed by a large number of cell types and overexpressed in a wide variety of human cancers, including gastric, lung, prostate, ovarian and bladder cancers.[6–13] Human MMP-2 promoter has been shown to contain several *cis*-acting regulatory elements. Among them, a −1306 C → T transition interrupts Sp1-binding site and consequently diminishes promoter activity.[14] Transient transfection experiments have shown that MMP-2 expression is ~1.4–2 fold higher with the C allele than with the T allele.[14] The importance of Sp-1 binding activity in MMP-2 expression has also been reported in other MMP-2 promoter deletion or site-directed mutagenesis studies.[15,16] These results suggest that patients with MMP-2 −1306 C/C genotype have higher MMP-2 expression than patients with C/T or T/T genotype. Recently, Miao and colleagues reported that −1306C/T is associated with gastric cardia adenocarcinoma risk.[7] Subjects with the C/C genotype had greater than 3-fold risk for developing gastric cardia adenocarcinoma when compared with those with the variant C/T or T/T genotype.[7]

The activity of MMP-2 is not only regulated by transcriptional regulation, but also by tissue inhibitors of metalloproteinases (TIMPs), which can form complexes either with latent or activated MMPs.[4,17] Among the TIMP family, TIMP-2 is particularly interesting due to its dual functions of regulating MMP-2 activity[18,19] and its controversial effects on tumour progression.[3] TIMP-2 has been reported to be greater than 10-fold more effective than TIMP-1 in the inhibition of MMP-2 activation.[18] On the other hand, TIMP-2 has been found to be required for efficient activation of pro-MMP-2 *in vivo*.[19] A TIMP-2 promoter polymorphism (−418 G → C) has been identified, which is also located in the consensus sequence for the Sp-1 binding site.[20] Although the functional significance of this polymorphism is still unknown, it is reasonable to postulate that it interrupts the Sp-1 binding site and decreases TIMP-2 gene transcription, leading to MMP-2 and TIMP-2 imbalance.[21]

MMP-2 promoter polymorphism has been found to be associated with susceptibility to gastric cancer.[7] However, there have been no studies conducted to elucidate the associations between MMP-2 polymorphism and gastric cancer invasive phenotype and survival. If MMP-2 polymorphism influences susceptibility to gastric cancer, it may also affect tumour progression and patient survival. As for TIMP-2 polymorphism, no studies have been conducted on gastric cancer patients. In the present study, we hypothesised that in gastric cancer, MMP-2 and TIMP-2 polymorphisms not only correlate well with susceptibility, but also with invasive phenotype and survival. A hospital-based case-control study was conducted to access this hypothesis.

2. Materials and methods

2.1. Study subjects

Between January 1989 and January 1995, blood samples have been prospectively collected from individuals participating in two national projects to investigate the risk factors of gastric cancer in Taiwan. All patient-derived specimens were collected and archived under protocols approved and supported by the National Science Council, Taiwan. A full verbal explanation of the study was given to all participants. They consented to participate on a voluntary basis. Patients with newly diagnosed gastric cancer undergoing gastrectomy in the inpatient unit and outpatient cancer clinics of four major medical centres in Taiwan were enrolled. Inclusion and exclusion criteria were detailed previously.[22,23] Gastric adenocarcinoma was histopathologically confirmed by surgical specimens. No patients in this study had been prescribed chemotherapy or radiotherapy prior to surgery, and there was no evidence of any other malignancy. Patients who died of surgical complications in the first 30 days were excluded. In total, we studied 240 consecutive patients with gastric cancer, for whom complete clinical data and a peripheral blood leukocytes DNA sample were available. Among the 240 gastric cancer patients, 211 received operation and had clinicopathological data available. One hundred and forty-six patients (69.2%) underwent subtotal gastrectomy and 65 patients (30.8%) received total gastrectomy. Lymph node dissection of compartment I was performed in every patient.

Clinicopathological characteristics were studied by independent pathologists. Histopathologic data and the presence and extent of residual tumour were determined according to the guidelines of the UICC.[24,25] A UICC R0 resection was defined as a resection that resulted in complete macroscopic and microscopic tumour removal on intraoperative and histopathologic evaluation. Vascular invasion is defined when metastatic tumour cells reside within the lumen of a vessel where red blood cells are seen and its endothelial cells could be stained positive for CD 31, CD 34 or factor VIII Ag. Lymphatic invasion is defined when malignant cells reside within the lumen of a dilated lymphatic duct where less red blood cells are seen and could not be stained positive more of CD31, and ulex europaeus lectin 1. All patients were Han Chinese, and none had a family history of gastric malignancy.

Control blood samples were obtained from 283 individuals who visited health examination clinics with minimal gastritis or normal appearance of the gastric mucosa on gastroscopic examination. The controls were matched by age (± 3 years) and date of blood collection (± 3 months).

2.2. Laboratory analysis

Genomic DNA was isolated from cryopreserved white blood cells by standard proteinase K digestion and phenol-chloroform method. PCR amplification of the promoter regions of the genes was performed using specifically designed pairs of oligonucleotide primers. The primers used to amplify a 295 bp fragment of the MMP-2 promoter containing the −1306 C/T site and a 176 bp fragment containing the −418 G/C site are listed in Table 1. Amplification was verified on agarose gel (2%) followed directly by sequencing with an automatic sequencer using fluorescent DNA capillary electrophoresis. The success rate in extracting and DNA was 98.77% in cases and 98.26% in controls.

Polymorphism analyses for MMP-2 −1306 C/T and TIMP-2 −418 G/C were performed in duplicate according to modified

Table 1 – Primer sequences and −1306 C/T MMP-2 and −418 G/C TIMP-2 promoter polymorphism detection methods

Genes	Primers	PCR Conditions	Detection methods
MMP2 (−1306)	5′-CTGACCCCCAGTCCTATCTGCC-3′ 5′-TGTTGGGAACGCCTGACTTCAG-3′	95 °C 30 s 62 °C 30 s 72 °C 30 s 35 cycles	Direct sequencing with primer 5′-CTGACCCCCAGTCCTATCTGCC-3′
TIMP2 (−418)	5′-GGATCCTGTCAGTTTCTCAA-3′ 5′-TTTCCCCTTCAGCTCGACTCT-3′	95 °C 30 s 60 °C 30 s 72 °C 30 s 35 cycles	*Hga*I digestion, 2.5% agarose gel electrophoresis

protocols based on previously reported assays.[7,9,26] Briefly, MMP-2 genotypes were determined by direct DNA sequencing. TIMP-2 genotypes were determined by restriction fragment length polymorphism using *Hga*I (New England Biolabs, Beverly, MA) and separated on a 2.5% agarose w/v gel. The forward primers were used as sequencing primers. All laboratory assays were conducted and interpreted blindly without the knowledge of case or control status. PCR conditions and detection methods are summarized in Table 1. *H. pylori* status was determined by serum samples using standard enzyme-linked immunosorbent assay.[27]

2.3. Statistical analysis

The demographic characteristics of patients and controls were compared using the chi-square test and Student's *t* test. According to previously reported proportions of MMP-2 and TIMP-2 polymorphisms in Chinese populations[7,28] and a 1:1 control-to-case ratio, the statistical power for detecting an odds ratio (OR) of 2.0 was more than 80% on the basis of 200 cases and 200 controls at the $\alpha = 0.05$ level of significance.

Logistic regression analyses were used to evaluate the associations between promoter polymorphisms and development and clinicopathological features of gastric cancer patients. Because the MMP-2 T/T and TIMP-2 C/C variants were extremely rare, these genotypes were respectively combined with the MMP-2 C/T and TIMP-2 C/G genotypes on logistic regression analysis. For MMP-2 −1306 C/T polymorphism, variant genotypes (C/T + T/T) were used as the reference groups. For TIMP-2 −418 G/C polymorphism, variant genotypes (C/G + C/C) were used as the reference groups. ORs were presented with 95% confidence intervals (95% CIs) and adjusted for age and sex.

Overall survival was calculated from the first day of diagnosis until death or the last follow-up visit. Other competitive mortality causes other than gastric cancer were treated as censor. Life-table estimation was performed according to the method of Kaplan and Meier. Univariate comparison of survival curves was performed with the use of the two-tailed log rank test. To determine whether the MMP-2 and TIMP-2 genotypes were independent prognostic factors for survival, hazard ratios and 95% confidence intervals were calculated using the Cox proportional hazards model. Variables in the model included age, sex, *H. pylori* seropositivity, Lauren's classification, residual tumour status, depth of tumour invasion, lymph node metastasis, lymphatic invasion, venous invasion, MMP-2 −1306 C/T polymorphism and TIMP-2 −418 G/C polymorphism. Assessment of goodness-of-fit of the models with step-down method has been used to analyse the independent prognostic factors. Statistical analyses were conducted independently by statisticians in Taichung Veterans General Hospital. Samples from cases and controls have been included in each batch analysis. All *p* values were two-tailed with statistical significance indicated by a value of $p < 0.05$. All data were analysed via the SPSS program for Windows 11.0 (SPSS Inc., Chicago, Illinois, USA).

3. Results

3.1. Associations between genotypes and occurrence of gastric cancer

Demographic characteristics of the study population and controls are summarised in Table 2. Control subjects were younger than gastric cancer cases (59 versus 61 years, $p < 0.05$), although we have tried to enroll controls matched according to age (± 3 years). Gastric cancer patients had higher prevalence of *H. pylori* infection when compared with control subjects (63.3% versus 52.7%, $p = 0.016$). There was no difference in the distributions of gender. The prevalences of current smoker and previous smoker among gastric cancer patients were higher than control subjects ($p < 0.001$).

The genotype distributions of MMP-2 and TIMP-2 in cases and controls and their associations with gastric cancer development are summarised in Table 3. The prevalence of MMP-2 C/C genotype in gastric cancer cases was 79.6% (95% CI: 73.9–84.5%), which was not significantly different from that of controls (78.1%, 95% CI: 72.8–82.8%). As for TIMP-2 polymorphism, the prevalences of G/G genotype were 81.7% (95% CI: 76.2–86.4%) and 78.8% (95% CI: 73.6–83.4%) in gastric cancer cases and controls, respectively ($p = 0.42$). The allelic frequencies of the MMP-2 −1306T and −1306C were 11.8% and 88.2% for cases, compared with 11.3% and 88.7% for controls ($p = 0.79$). The allelic frequencies for TIMP-2 −418C and −418G were 10.4% and 89.6% for cases, compared with 12.9% and 87.1% for controls ($p = 0.21$). There was no statistically significant difference in the overall risk of developing gastric cancer among the MMP-2 and TIMP-2 genotypes.

We have tried to analyse the combined effect between MMP-2 and TIMP-2 genotypes on the risk of gastric cancer (Table 3). However, there was no combined effect found between MMP-2 and TIMP-2 genotypes.

Table 2 – Demographic data of gastric cancer patients and controls

	Cases (n = 240)	Controls (n = 283)	P values
Age			
Range (years)	26–88	22–86	
Mean (years)	61.12 ± 14.72	58.96 ± 13.08	< 0.05
Gender			
Male (n = 313)	143 (59.6%)	170 (60.0%)	
Female (n = 210)	97 (40.4%)	113 (40.0%)	0.91
H. pylori			
Seronegative (n = 222)	88 (36.7%)	134 (47.3%)	
Seropositive (n = 301)	152 (63.3%)	149 (52.7%)	0.016
Smoking			
Never smoking	139 (57.9)	168 (59.4)	<0.001
Current smoker	62 (25.8)	40 (14.1)	
Previous smoker	20 (8.3)	8 (2.8)	
Data not available	19 (7.9)	67 (23.7)	

Table 3 – MMP-2 and TIMP-2 genotypes in cases and controls and their association with gastric cancer risk

	Cases (n = 240)	Controls (n = 283)	OR (95%CI)[a]	
			Crude	Adjusted[b]
MMP-2 (–1306)				
T/T	8 (3.3%)	2 (0.7%)		
C/T	41 (17.0%)	60 (21.2%)		
C/C	191 (79.6%)	221 (78.1%)	1.09 (0.72–1.67)	1.27 (0.81–1.98) P = 0.30
T allele	57 (11.9%)	64 (11.3%)		
C allele	423 (88.1%)	502 (88.7%)		P = 0.78
TIMP-2 (–418)				
C/C	6 (2.5%)	13 (4.6%)		
C/G	38 (15.8%)	47 (16.6%)		
G/G	196 (81.7%)	223 (78.8%)	1.20 (0.78–1.85)	1.20 (0.77–1.90) P = 0.42
C allele	50 (10.4%)	73 (12.9%)		
G allele	430 (89.6%)	493 (87.1%)		P = 0.21
Combined effect				
MMP-2 C/T or T/T + TIMP-2 C/C or C/G	10 (4.2%)	15 (5.3%)		P = 0.84
MMP-2 C/T or T/T + TIMP-2 G/G	39 (16.3%)	47 (16.6%)		
MMP-2 C/C + TIMP-2 C/C or C/G	34 (14.2%)	45 (15.9%)		
MMP-2 C/C + TIMP-2 G/G	157 (65.4%)	176 (62.2%)		

a Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated by logistic regression, with MMP-2 variant genotypes (C/T + T/T) and TIMP-2 variant genotypes (C/G + C/C) as the reference groups.
b Adjusted for age and sex with logistic regression model.

3.2. *Associations between genotypes and clinicopathological characteristics*

Among the 240 gastric cancer cases, 211 who received operation with complete staging were recruited to analyse the genotype distributions of MMP-2 and TIMP-2 and their associations with clinicopathological characters of gastric cancer (Tables 4 and 5). MMP-2 genotypes were not associated with age, gender, *H. pylori* seropositivity, Lauren's classification, residual tumour status, depth of invasion or lymph node metastasis. However, cases with MMP-2 –1306 C/C genotype had significantly higher risk of lymphatic invasion (adjusted OR = 2.77, p = 0.01) and venous invasion (adjusted OR = 2.93, p = 0.012) than cases with C/T or T/T genotype (Table 4). TIMP-2 genotypes were not associated with age, gender, *H. pylori* seropositivity, Lauren's classification, or residual tumour status. However, cases carrying TIMP-2 –418 G/G genotype had significantly higher risks of serosal invasion (adjusted OR = 1.89, p = 0.009), adjacent structure invasion (adjusted OR = 1.62, p = 0.014), more than six lymph nodes metastasis (adjusted OR = 2.19, p = 0.021), lymphatic invasion (adjusted OR = 2.87, p = 0.016) and venous invasion (adjusted OR = 2.65, p = 0.033) than those carrying C/G or C/C genotype (Table 5).

Table 4 – Associations between −1306 C/T polymorphism in MMP-2 promoter and clinicopathological features of gastric cancer (data from 211 operated cases)

	Genotypes (%)			Adjusted OR (95% CI)[a]	P value
	TT	CT	CC		
Age (years)					
<65 (*n* = 103)	4 (3.9)	13 (12.7)	86 (83.3)	1	
⩾65 (*n* = 108)	3 (2.8)	20 (18.3)	85 (78.9)	0.73 (0.37–1.46)	*P* = 0.376
Gender					
Female (*n* = 86)	5 (5.8)	13 (15.1)	68 (79.1)	1	
Male (*n* = 125)	2 (1.6)	20 (16.0)	103 (82.4)	1.24 (0.62–2.48)	*P* = 0.545
H. pylori					
Seronegative (*n* = 74)	4 (5.4)	10 (13.5)	60 (81.1)	1	
Seropositive (*n* = 137)	3 (2.2)	23 (16.8)	111 (81.0)	1.00 (0.48–2.06)	*P* = 1.000
Lauren's classification					
Intestinal (*n* = 85)	3 (3.5)	17 (20.0)	65 (76.5)	1	
Diffuse (*n* = 126)	4 (3.2)	16 (12.7)	106 (84.1)	1.74 (0.82–3.67)	*P* = 0.148
R category					
R0 resection (*n* = 148)	5 (3.4)	24 (16.2)	119 (80.4)	1	
R1/2 resection (*n* = 63)	2 (3.2)	9 (14.3)	52 (82.5)	1.15 (0.54–2.48)	*P* = 0.718
pT category (UICC)					
pT1 (*n* = 38)	0 (0)	9 (23.7)	29 (76.3)	1	
pT2 (*n* = 35)	1 (2.9)	7 (20.0)	27 (77.1)	1.05 (0.35–3.11)	*P* = 0.933
pT3 (*n* = 87)	4 (4.6)	10 (11.5)	73 (83.9)	1.27 (0.79–2.04)	*P* = 0.266
pT4 (*n* = 49)	2 (3.9)	7 (13.7)	42 (82.4)	1.13 (0.80–1.60)	*P* = 0.484
pN category (UICC)					
pN0 (*n* = 56)	1 (1.8)	10 (17.9)	45 (80.4)	1	
pN1 (*n* = 93)	4 (4.3)	19 (20.4)	70 (75.3)	0.74 (0.33–1.67)	*P* = 0.474
pN2, N3 (*n* = 62)	2 (3.2)	4 (6.5)	56 (90.3)	1.51 (0.89–2.58)	*P* = 0.131
Lymphatic invasion					
Negative (*n* = 118)	4 (3.4)	26 (22.0)	88 (74.6)	1	
Positive (*n* = 93)	3 (3.2)	7 (7.5)	83 (89.2)	2.77 (1.27–6.04)	*P* = 0.010
Venous invasion					
Negative (*n* = 129)	5 (3.9)	27 (21.3)	97 (75.2)	1	
Positive (*n* = 82)	2 (2.4)	6 (7.3)	74 (90.2)	2.93 (1.27–6.78)	*P* = 0.012

a Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated by logistic regression, with MMP-2 variant genotypes (C/T + T/T) as the reference groups, adjusted for age and sex.

3.3. *Associations between genotype and survival*

The Kaplan–Meier survival curves for the different MMP-2 and TIMP-2 genotypes are shown in Fig. 1. The survival rate for cases with MMP-2 −1306 C/C genotype did not differ significantly from the survival rates of those with −1306 C/T or T/T genotype ($p = 0.60$). The survival rate for cases with TIMP-2 −418G/G genotype did not differ significantly from the survival rate of those with −418 C/G or C/C genotype. To investigate the potential interactions between MMP-2 and TIMP-2 genotypes, we conducted survival analysis for combined genotypes. However, we did not find a difference among these combinations.

To adjust these curves for any other factors that may have influenced survival, we used the Cox proportional hazards model with the step-down method to analyse the following covariates: age, gender, *H. pylori* seropositivity, Lauren's classification, residual tumour status, depth of tumour invasion, lymph node metastasis, lymphatic invasion, venous invasion, lymph node metastasis, MMP-2 and TIMP-2 genotypes. On univariate analysis, we found that R1/2 residual tumour status, serosal invasion, lymph node metastasis, lymphatic invasion, and venous invasion were of prognostic significance for poor overall survival. MMP-2 and TIMP-2 genotypes were not predictive factors for survival (Table 6). On multivariate analysis, only serosal invasion (adjusted HR = 3.48, $p < 0.001$) and lymph node metastasis (adjusted HR = 2.20, $p = 0.014$) were independent risk factors for poor overall survival.

4. Discussion

In the present study, we found that MMP-2 and TIMP-2 genotypes play a crucial role in gastric cancer invasion and progression, but not with development of gastric cancer. To the best of our knowledge, this is the first study to investigate whether gastric cancer invasive phenotypes and survival are associated with the MMP-2 −1306 C/T and TIMP-2 −418 G/C polymorphisms.

Carcinogenesis is a multicellular and multistage process in which breakdown of the microenvironment is required for

Table 5 – Associations between –418 G/C polymorphism in TIMP-2 promoter and clinicopathological features of gastric cancer (data from 211 operated cases)

	Genotypes(%)			Adjusted OR (95% CI)[a]	P value
	CC	CG	GG		
Age (years)					
<65 (*n* = 103)	3 (2.9)	13 (12.6)	87 (84.5)	1	
⩾65 (*n* = 108)	2 (1.9)	15 (13.9)	91 (84.3)	0.98 (0.47–2.07)	*P* = 0.967
Gender					
Female (*n* = 86)	3 (3.5)	14 (16.3)	69 (80.2)	1	
Male (*n* = 125)	2 (1.6)	14 (11.2)	109 (87.2)	1.68 (0.80–3.54)	*P* = 0.174
H. pylori					
Seronegative (*n* = 74)	1 (1.4)	9 (12.2)	64 (86.5)	1	
Seropositive (*n* = 137)	4 (2.9)	19 (13.9)	114 (83.2)	0.79 (0.35–1.77)	*P* = 0.565
Lauren's classification					
Intestinal (*n* = 85)	1 (1.2)	11 (12.9)	73 (85.9)	1	
Diffuse (*n* = 126)	4 (3.2)	17 (13.5)	105 (83.3)	0.92 (0.40–2.11)	*P* = 0.849
R category					
R0 resection (*n* = 148)	2 (1.4)	20 (13.5)	126 (85.1)	1	
R1/2 resection (*n* = 63)	3 (4.8)	8 (12.7)	52 (82.5)	0.83 (0.37–1.82)	*P* = 0.635
pT category (UICC)					
pT1 (*n* = 38)	1 (2.6)	11 (28.9)	26 (68.4)	1	
pT2 (*n* = 35)	1 (2.9)	5 (14.3)	29 (82.9)	2.23 (0.73–6.79)	*P* = 0.158
pT3 (*n* = 87)	1 (1.1)	9 (10.3)	77 (88.5)	1.89 (1.17–3.03)	*P* = 0.009
pT4 (*n* = 49)	2 (3.9)	3 (5.9)	46 (90.2)	1.62 (1.10–2.38)	*P* = 0.014
pN category (UICC)					
pN0 (*n* = 56)	1 (1.8)	10 (17.9)	45 (80.4)	1	
pN1 (*n* = 93)	3 (3.2)	16 (17.2)	74 (79.6)	0.95 (0.42–2.18)	*P* = 0.908
pN2, N3 (*n* = 62)	1 (1.6)	2 (3.2)	59 (95.2)	2.19 (1.13–4.27)	*P* = 0.021
Lymphatic invasion					
Negative (*n* = 118)	3 (2.5)	22 (18.6)	93 (78.8)	1	
Positive (*n* = 93)	2 (2.2)	6 (6.5)	85 (91.4)	2.87 (1.22–6.76)	*P* = 0.016
Venous invasion					
Negative (*n* = 129)	4 (3.1)	22 (17.1)	103 (79.8)	1	
Positive (*n* = 82)	1 (1.2)	6 (7.3)	75 (91.5)	2.65 (1.08–6.49)	*P* = 0.033

a Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated by logistic regression, with TIMP-2 variant genotypes (C/G + C/C) as the reference groups, adjusted for age and sex.

conversion of normal tissue to tumour.[29] We hypothesised that MMP-2 and TIMP-2 promoter polymorphisms alter the stomach microenvironment and are involved in the process of carcinogenesis. However, the allelic frequencies of gastric cancer patients were not different from those of controls. In 2003, Miao and colleagues reported that MMP-2 C/C genotype is associated with a 3.36-fold risk for gastric cardia adenocarcinoma.[7] In contrast to Miao and colleagues' study, we did not find an association between MMP-2 polymorphism and development to gastric cancer. Miao et al. recruited only gastric cardia adenocarcinoma patients and found that 87.6% of them had C/C genotype compared with 68.7% of controls.[7] Our present study included all gastric adenocarcinomas, including 34 gastric cardia adenocarcinoma cases (14.0%). Among our study subjects, C/C genotype was present in 79.6% of gastric cancer patients and 78.1% of controls. We found a higher prevalence of C/C genotype (78.1%) in healthy Taiwanese Chinese than in the healthy northern Chinese population of Miao and colleagues' study (68.7%). Xu and colleagues reported a C/C genotype prevalence of 73% among a healthy southern Chinese population,[30] which was closer to our observed prevalence. In the subgroup of gastric cardia cancer patients, the prevalence of C/C genotype (75.8%) was not significantly different from that of controls. Ethnic difference may account for the discrepancy between our results and those of Miao and colleagues. In a recent published study, Kubben and colleagues reported a lower C/C genotype prevalence among Netherlands gastric cancer patients (63.3%) and controls (60.4%).[31] In consistent with our results, Kubben and colleagues did not find an association between MMP-2 polymorphism and development to gastric cancer.[31]

As for TIMP-2 polymorphism, we found that 81.7% of gastric cancer patients had G/G genotype and 78.8% of controls had G/G genotype. In a study of a northern Chinese population, TIMP-2 G/G genotype prevalences were 69.5% among breast cancer patients and 63.3% among controls.[9] Another study among a Japanese population reported prevalences of 61.3% among chronic obstructive pulmonary disease (COPD) patients and 82.5% among controls.[21] Our controls had a higher prevalence of G/G genotype than the northern Chinese

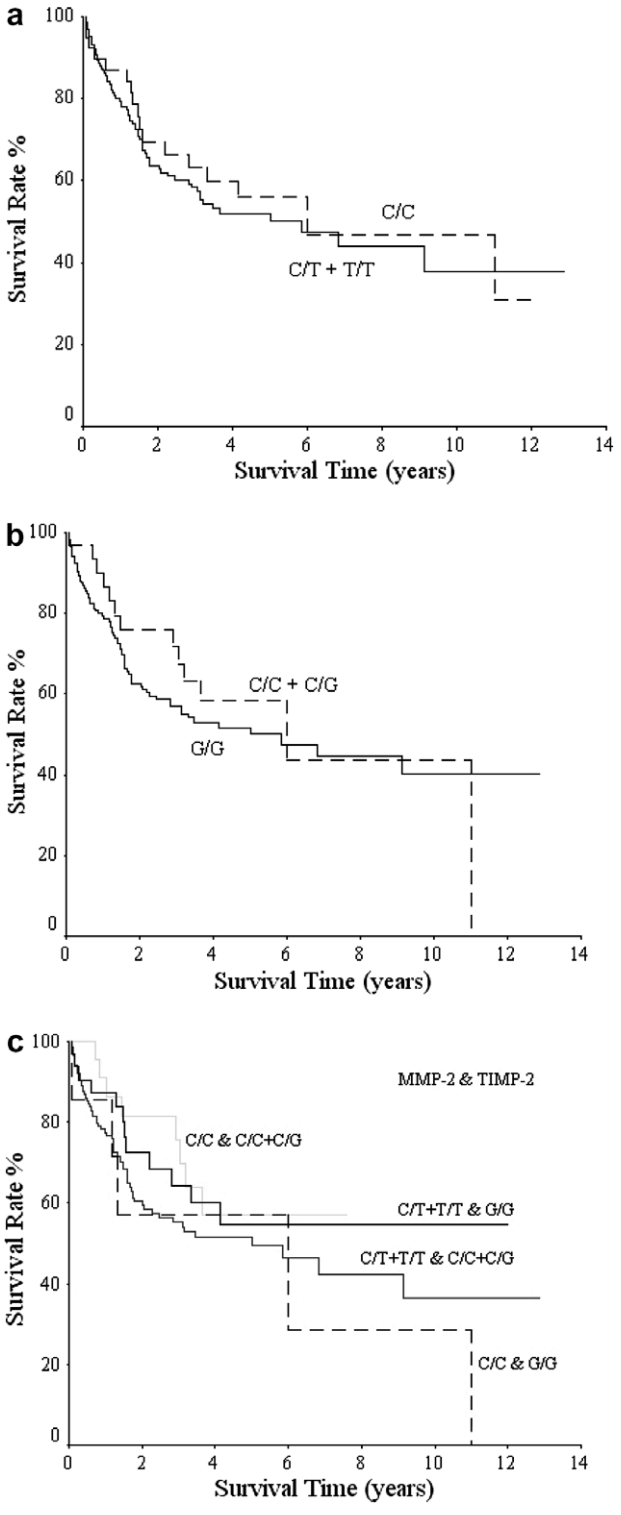

Fig. 1 – Survival curves for patients with different MMP-2 and TIMP-2 genotypes. (a) The survival rates for those with −1306 C/C was not different from the survival rate for those with −1306 C/T or T/T ($p = 0.60$). (b) The survival rates for those with −418 G/G was not different from the survival rate for those with −418 C/G or C/C ($p = 0.52$). Comparison of survival curves was performed with the use of the two-tailed log rank test. (c) The survival rates of the combined MMP-2 and TIMP-2 genotypes were not different ($p = 0.41$).

population, but a lower prevalence than the Japanese population. In contrast to breast cancer and COPD study results,[9,21] there was no evidence of association between gastric cancer development and TIMP-2 polymorphism in our present study. In the Netherlands study, TIMP-2 G/G genotype prevalences were 98.7% among gastric cancer patients and 99.4% among controls.[31] In consistence with our results, Kubben's study did not find association between TIMP-2 genotype and gastric cancer development.

One of the interesting findings of this study was that MMP-2 −1036 C → T polymorphism is associated with lymphatic invasion and venous invasion, but not with survival. The association between MMP-2 polymorphism and invasive phenotypes of gastric cancer is consistent with the biological function of MMP-2. *In vitro* studies have demonstrated that −1306 C → T transition interrupts Sp1-binding site and consequently diminishes promoter activity.[14] Deletion or site-directed mutagenic analysis of MMP-2 promoter demonstrates that Sp-1 site is critical for constitutive activation of MMP-2.[15] Furthermore, MMP-2 can be inhibited by non-steroidal anti-inflammatory drugs through ERK/Sp1-mediated transcription pathway.[16] These *in vitro* observations suggest that patients with MMP-2 −1306 C/C genotype have higher MMP-2 expression and more dismal outcomes. However, clinical study results have been controversial. Nomura and colleagues first reported that proMMP-2 was activated by membrane-type MMP (MT-MMP).[32] MT-MMP expression was found closely associated with gastric cancer invasive type, nodal involvement, lymphatic invasion, vessel invasion, and peritoneal dissemination.[33] Sier and colleagues reported that tissue MMP-2 protein level does not correlate well with histological Lauren's classification or TNM staging.[34] However, higher tissue MMP-2 level is an independent risk factor for poor survival.[34] In contrast to Sier and colleagues' study, Monig and colleagues found the intensity of MMP-2 staining in tumour cells correlated significantly with depth of tumour infiltration, lymph node metastasis, and distant metastasis.[35] A recent study reported that higher tissue MMP-2 mRNA expression is associated with lymph node metastasis and Lauren's classification, but not with stage or survival.[36] Wu and colleagues' study confirmed that MMP-2 mRNA expression has significant correlation with gastric cancer invasion and lymph node metastasis and suggested that MMP-2 may participate in the development of lymph node micrometastasis.[37] Kubben and colleagues reported that gastric cancer patients with MMP-2 −1306 C/C genotype had significantly higher MMP-2 protein expression in tumour tissues; however, MMP-2 genotype was not associated with patients' survival.[31] In the present study, we found that MMP-2 C/C genotype is associated with higher risks of lymphatic invasion and venous invasion, but not with survival. In addition, Cox multivariate proportional hazards analyses indicated that only serosal invasion and lymph node metastasis were independent prognostic factors for poor survival whereas lymphatic invasion and venous invasion were not.

Another interesting finding is an association between TIMP-2 −418 G/C polymorphism and invasive phenotypes of gastric cancer. TIMP-2 G/G genotype carried significantly higher risks of serosal invasion, lymph node metastasis, lymphatic invasion and venous invasion than C/G or C/C

Table 6 – Cox proportional hazard model analysis: univariate analysis

	Hazard ratios[a]	95% CI[a]	P values
Age			
⩾65 versus < 65	1.15	(0.75–1.75)	0.52
Gender			
Male versus female	1.19	(0.77–1.84)	0.43
H. pylori			
Seropositive versus seronegative	0.92	(0.59–1.42)	0.70
Lauren's classification			
Diffuse versus intestinal type	1.22	(0.79–1.90)	0.37
R category			
R1/2 versus R0 resection	5.08	(3.22–8.01)	<0.001
Depth of invasion			
Serosa versus non-serosa invasion	4.27	(2.45–7.43)	<0.001
Lymph node metastasis			
Yes versus no	3.23	(1.75–5.96)	<0.001
Lymphatic invasion			
Yes versus no	1.93	(1.26–2.95)	0.002
Venous invasion			
Yes versus no	2.09	(1.36–3.22)	0.001
MMP-2			
C/C versus C/T + T/T	1.16	(0.68–1.97)	0.60
TIMP-2			
G/G versus C/G + C/C	1.21	(0.67–2.19)	0.53

a Hazard ratios (HRs) and 95% confidence intervals (CIs) were calculated using the Cox proportional hazards model.

genotype. The functional significance of TIMP-2 −418 G → C substitution is currently unknown, but it may lead to decreased TIMP-2 transcription due to interruption of Sp-1 binding site.[21] It is reasonable to hypothesise that patients carrying TIMP-2 −418 G/G genotype have higher TIMP-2 expression levels. As an inhibitor of MMP-2, one may infer that TIMP-2 G/G genotype is negatively related to invasive phenotypes. In contrast, we found that TIMP-2 G/G genotype is positively correlated with invasive phenotypes. Accumulating evidence suggests that TIMP-2 acts as a multifunctional molecule.[38] While TIMP-2 is a potent inhibitor of MMP-2, TIMP-2 is an adaptor molecule required for pro-MMP-2 activation at cell surface.[19] In addition to its MMP-2 regulating effects, TIMP-2 promotes cell growth and regulates apoptosis in an MMP-2 independent pathway.[38,39] In fact, several clinical studies have found that high levels of TIMP-2 correlate well with adverse prognosis, such as in cervical cancer[40] and breast cancer.[41] Our results parallel these observations and suggest that higher TIMP-2 expression (G/G genotype carrying patients) correlates well with invasive phenotype. Although TIMP-2 G/G genotype was associated with invasive phenotypes of gastric cancer, including serosal invasion and lymph metastasis, independent prognostic factors for poor survival, this genotype did not correlate well with survival. On survival analysis, patients with TIMP-2 G/G genotype tended to have poor survival rate in the first years following diagnosis; however, the overall survival was not different from those with G/C or C/C genotype. The possible explanation is that overall survival is determined not only by gastric cancer disease activity, but also by underlying diseases and other competitive mortality causes. Disease-free survival analysis may be more suitable for representing the association between TIMP-2 genotype and invasive phenotypes of gastric cancer. Our observation was consistent with the recent study by Kubben and colleagues.[31]

In order to fully appreciate the functional implications of the gene polymorphisms, an assessment of MMP-2 and TIMP-2 levels in gastric cancer would be very informative. Kubben and colleagues reported that gastric cancer patients with MMP-2 C/C genotype has significantly higher MMP-2 protein level in tumour tissues, but similar MMP-2 protein level in normal mucosa tissues. TIMP-2 genotypes are not correlated with protein levels in tumour tissues and normal mucosa tissues. However, there is only one subject studied in the TIMP-2 G/C genotype group. More studies to confirm these observations will be helpful to understand the clinical implications of these gene polymorphisms.

There are several limitations to our study. First, selection bias could not be completely excluded in this hospital-based case-control study, although we tried to enroll cases from three different hospitals in three different regions of Taiwan. Second, the ORs may be imprecise due to limited power or chance findings. Third, the sample size of the present study is relatively small for detecting the moderately increased risk of the cancer associated with the rare MMP-2 T and TIMP-2 C alleles, even though the statistical power of this study has been prior estimated. Fourth, it is important to note that *in vitro* expressions of MMP-2 and TIMP-2 are regulated by

complicated mechanisms in the tumour-stroma microenvironment, and subjects carrying the same genotype may show different MMP-2 and TIMP-2 expressions in the microenvironment. Fifth, although the function of MMP-2 –1306 C/T polymorphism has been investigated, the function of TIMP-2 –418 G/C polymorphism is still unknown. More detailed *in vitro* and *in vivo* studies are needed to explain how genetic heterogeneity in the TIMP-2 promoter modifies gastric cancer development and outcomes. Sixth, although the MMP-2 and TIMP-2 genotypes identified in our study are important in predicting invasive phenotypes of gastric cancer, they only account for a small percentage of genotypes among gastric cancer patients. Other genes not investigated in our current study may act alone or in concert with those studied here, such as MMP-2 –735 C/T polymorphism.[42] Finally, we did not have data for disease-free survival, which would have been valuable for representing the association between MMP-2 and TIMP-2 polymorphisms and invasive phenotypes of gastric cancer.

In conclusion, our study provides the first evidence that the MMP-2 –1306C/T and TIMP-2 –418G/C polymorphisms are associated with invasion and progression of gastric cancer, but not with gastric cancer development. Additional studies, especially among different ethnic populations, are necessary to confirm these observations.

Conflict of interest statement

None declared.

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