Associations of Matrix Metalloproteinase-9 Protein Polymorphisms with Lymph Node Metastasis but not Invasion of Gastric Cancer

Yang Tang, Jinwei Zhu, Ling Chen, Linyin Chen, Sheng Zhang, and Jianyin Lin

Abstract

Purpose: Like most cancers, gastric cancer has a complex multistep etiology that involves both environmental and genetic factors. Matrix metalloproteinase-9 (MMP-9) is frequently over-expressed in gastric cancer. We investigated the effect of the genetic differences in MMP-9 coding region on the occurrence and progression of gastric cancer.

Experimental Design: A case-control study was conducted in a population of 74 patients and 100 healthy people in southeast China. Individuals were genotyped for two single nucleotide polymorphisms (SNP) in MMP-9: R279Q and P574R. Genotypic distributions between patient and control groups were compared for correlations with cancer occurrence. Associations between genotypic distributions and several clinicopathologic features were also analyzed using univariate tests, multivariate logistic regression modeling, and stratified analyses.

Results: Significant associations were revealed between both SNPs and lymph node metastasis \( P = 0.012 \) and 0.025; odds ratio (OR), 3.4 and 2.8, respectively. After adjustment using logistic regression for the potential confounding effects of gender, age, and location of the tumors, homozygous MMP-9 279RR and 574PP are more evidently associated with lymph node metastasis with \( \text{OR}_{\text{adjusted}} \) of 5.7 [95% confidence interval (95% CI), 1.80-18.34] and 4.2 (95% CI, 1.37-12.69). The homozygous 279R-574P haplotype showed a stronger association by an \( \text{OR}_{\text{adjusted}} \) of 6.1 (95% CI, 1.92-12.29) and was also associated with the 1-year postoperative mortality (\( \text{OR}_{\text{adjusted}} \) 6.5; 95% CI, 1.18-35.74). Interestingly, our data also suggested that the MMP-9 polymorphisms seem to result in higher risk of lymph node metastasis through a pathway independent of cancer invasion because no positive associations were found between these polymorphisms and cancer invasion (OR, 0.59 < 1). The stratified analyses indicated a synergistic interaction between the MMP-9 polymorphisms and the type of diffuse in affecting lymph node metastasis (OR, 13.4; \( P_{\text{between strata}} = 0.04 \)). Significant association between both SNPs and the overall occurrence of gastric cancer was not observed.

Conclusion: The present study has shown significant associations between the two nonsynonymous MMP-9 polymorphisms with lymph node metastasis in gastric cancer, especially with the diffuse type. The relatively large values of ORs and disassociation with cancer invasion suggest that the genetic differences of MMP-9 protein play an important and specific role in lymph node metastases, and therefore, further investigation of the underlying molecular mechanism is warranted.

Matrix metalloproteinases (MMP) have been well documented to have important functions in cancer invasion and metastasis mediated principally by their activities in degrading extracellular matrix and basement membrane (1). Recent studies also suggested that MMPs have other functions related to cancer development and progression, such as activating growth factors (2, 3). Together with MMP-2, MMP-9 is a gelatinase and belongs to a subfamily of MMP family. It can degrade denatured collagens (gelatins) and type IV collagen, which is the major structural component of the extracellular matrix (4, 5). Higher expression of MMP-9 is involved in the progression of many cancers, including gastric cancer (6–9). Overexpression of MMP-9 can result from regulation at the genetic, epigenetic, or the physiologic levels. A single nucleotide polymorphism (SNP) located in a transcription factor-binding motif in the
MMP-9 promoter (10) has been reported to be associated with the progression of gastric cancer (11), and also other cancers (12–14), presumably by the increase of MMP-9 expression. Gain of 20q12-q13, where MMP-9 is located, has been reported to be one of the most frequent regions with genomic gains in gastric cancer (15–18). Moreover, several reports suggested that the gain at 20q was related to lymph node metastasis of gastric cancer (19, 20). Whereas the progression of gastric cancer is obviously influenced by the quantitative changes in MMP-9 expression, the effects of qualitative changes (variations in amino acid sequence) are largely unknown.

To date, three nonsynonymous SNPs in MMP-9 have been identified in the Chinese population: R279Q, P574R, and R668Q. R279Q and P574R have also been reported to be associated with initiation and progression in lung cancer (21), and R279Q with cancer progression in melanoma (22). R279Q was reported to be correlated with the metastasis of renal cell carcinoma (23). Given these observations, we suspect that R279Q and P574R of MMP-9 may affect the progression of gastric cancer. In the present study, we investigated the effects of these two polymorphisms on the development and progression of gastric cancer and also evaluated their relative strength.

Materials and Methods

Study population. The study population included 74 patients with gastric carcinoma and 100 cancer-free controls. All subjects were genetically unrelated ethnic Han Chinese from Fuzhou City in Fujian Province and surrounding regions. The case population was drawn from archived patients, who were diagnosed with primary incident gastric cancer and were recruited between 1993 to 1995 at the First Affiliated Hospital of Fujian Medical University, Fuzhou, Fujian. All were histopathologically confirmed gastric cancer cases, underwent surgical resection, and had detailed clinicopathologic data based on the postoperative histopathologic examination. Short-term or long-term follow-up postoperative survival information is available for 58 patients. One hundred cancer-free controls were chosen randomly from local residents, who underwent a routine health check, had no history of cancer, and were free of any known major diseases. The age distribution of the two populations was similar. However, selection of the controls was done regardless of gender because the effects of gender on the genotypic distribution of autosomal alleles can be logically ignored in this study. To avoid the introduction of a bias, the population in the study was assembled before any genotyping was done and did not change during the course of study. The individuals selected for the study provided informed consent, and the study was approved by the Institutional Review Board of Fujian Medical University. Gastric cancer was classified histologically according to the criteria of Lauren (24). Gastric cancer patients were also grouped according to tumor-node-metastasis classification based on postoperative histopathologic evaluation (25, 26).

MMP-9 genotyping. For control cases, a 5-mL venous blood sample was collected from each subject. Genomic DNA was extracted from the cell pellet in whole blood using the Blood Genome DNA Extraction kit (TaKaRa) and stored at -20°C until genotypic analysis was done. Gastric cancer patient DNA was isolated from paraffin-embedded normal stomach tissue adjacent to the tumor (distance >5 cm). DNA isolation was done using the conventional proteinase K-phenol/chloroform-ethanol method. DNA concentration was determined by the ratio A260/A280.

The genotype of MMP-9 R279Q (SNP rs17576) was detected using the PCR-RFLP assay. The primers were 5′-GTGCTGTCTCCGCTTCTC-3′ (forward) and 5′-GGAGTAGGATTGGCTTGG-3′ (reverse), which amplified a 124-bp fragment containing the R279Q polymorphic site. Each 25 μL PCR mixture consisted of 50 ng of genomic DNA, with 0.4 μmol/L of both primers, 200 μmol/L deoxynucleotides triphosphates, 1 unit Taq DNA polymerase (TaKaRa), and 1× PCR buffer (50 mMol/L KCl, 10 mmol/L Tris-HCl, 0.1% Triton X-100, 1.5 mmol/L MgCl2). The PCR product was then digested overnight at 37°C with 2 units of the restriction enzymeMspI (TaKaRa) and separated on a 4% agarose gel. The 279R (A) allele has no restriction site and the 279Q (G) allele has one restriction site and produces two fragments of 58 and 66 bp (Fig. 1A).

The MMP-9 P574R (rs2250889) was detected using a primer-introduced restriction analysis-PCR assay (21). The forward primer included a mismatched T to replace G at -2 bp from the polymorphic site to create a BamHI restriction site. The primers used to detect this polymorphism were 5′-GCTGGACCTGGTCTTGAGATC-3′ (forward) and 5′-CGTTTCTGTGGGAATC-3′ (reverse). The PCR products were then digested by BamHI. The 574R allele (G) produces a single 137 bp PCR product and the 574P allele (C) produces two fragments of 118 and 19 bp after digestion (Fig. 1B).

Genotyping was done in a blinded fashion and the patient and normal samples were assayed in batches with a negative control.
Table 1. Associations of MMP-9 polymorphisms with the occurrence of gastric cancer

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>MMP-9 R279Q QQ + RQ, n (%)</th>
<th>RR, n (%)</th>
<th>P</th>
<th>MMP-9 P574R RR + PR, n (%)</th>
<th>PP, n (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disease status</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (n = 100)</td>
<td>5 + 37 (42)</td>
<td>58 (58)</td>
<td>0.49*</td>
<td>4 + 32 (46)</td>
<td>64 (64)</td>
<td></td>
</tr>
<tr>
<td>Gastric cancer (n = 74)</td>
<td>3 + 32 (47)</td>
<td>39 (53)</td>
<td></td>
<td>3 + 29 (43)</td>
<td>42 (57)</td>
<td>0.33*</td>
</tr>
<tr>
<td>With lymph node metastasis (n = 45)</td>
<td>16 (36)</td>
<td>29 (64)</td>
<td>0.46*</td>
<td>15 (33)</td>
<td>30 (67)</td>
<td>0.32*</td>
</tr>
<tr>
<td>Without lymph node metastasis (n = 29)</td>
<td>19 (54)</td>
<td>10 (26)</td>
<td>0.004*</td>
<td>17 (53)</td>
<td>12 (29)</td>
<td>0.01*</td>
</tr>
</tbody>
</table>

*By \( \chi^2 \) test, compared with control.

The allelic frequencies were calculated and their genotypic distributions were tested for Hardy-Weinberg equilibrium and showed no significant deviation \( (P > 0.75 \) in both groups). The control and patient groups were approximately age matched, but not gender matched; however, the effects of gender on the genotypic distribution of the autosomal MMP-9 should be negligible. Comparisons between the case and control group showed no significant differences in the genotypic distributions \( (P = 0.49 \) and 0.33; Table 1, row 2), indicating that these two polymorphisms are not associated with the occurrence of gastric cancer. The comparisons between the patients with lymph node metastasis and the controls also rendered no associations (Table 1, row 3). Interestingly, when compared with the control, homozygous 279R and 574P were negatively associated with the occurrence of gastric cancer without lymph node metastasis \( (P = 0.004 \) and 0.01; OR, 2.62 and 1.97; Table 1, row 4).

Associations of MMP-9 SNPs with clinicopathologic features of gastric cancer. The overall clinicopathologic features of the patient group are summarized in Supplementary Table S1 (column 1). No major aberrations among these features in this group were observed in comparison with other patients with gastric cancer recruited in this hospital.

The associations of the genotypic distribution of MMP-9 SNPs with clinicopathologic features were analyzed using univariate analysis (Supplementary Table S1). Significant or the relevant results of the analyses were summarized in Table 2. The homozygous 279R and 574P genotype showed significant association only with lymph node metastasis \( (P = 0.012 \) and 0.025; OR, 3.4 and 2.8; 95% CI, 1.29-9.17 and 1.08-7.43; Table 2). Double homozygosity for both susceptible alleles \( (279R \& 574P) \) also showed a significant association \( (OR = 4.03; 95\% CI, 1.49-10.90; Table 2) \). It is important to point out that those SNPs not only were not associated with the increased risk of advanced invasion (depth of invasion reaching the serosa or beyond) but showed a trend toward negative correlation \( (OR = 0.59 \) and 0.66; \( P = 0.25 \) and 0.38; Table 2). Comparisons after detailed categorization of the invasion status gave a similar result.

Multivariate analyses of associations of MMP-9 polymorphisms with lymph node metastasis of gastric cancer using logistic regression modeling. To more accurately evaluate the strength of association and eliminate the distortion caused by confounding effects, it was necessary to do a multivariate analysis. Analyses of the association between clinicopathologic features (including several demographic characteristics) and lymph node metastasis could help identify potential confounding factors. Univariate analyses were done by \( \chi^2 \) or \( t \) test (Supplementary Table S2). The results showed that age, gender of the patients, differentiation status, and Lauren’s classification of the cancer showed no statistically significant associations and therefore were not major confounding factors. However, the small \( P \) values indicated that Lauren’s classification (and differentiation status) of the cancer might be minor confounding factors due to small associations \( (P = 0.21 \) and 0.20; Supplementary Table S2). Location, size of the tumor cancer, and depth of the invasion had significant association \( (P = 0.022\).
Table 2. Associations of MMP-9 polymorphisms with several clinicopathologic features of gastric cancer

<table>
<thead>
<tr>
<th>Genotype</th>
<th>MMP-9 R279Q</th>
<th></th>
<th>MMP-9 P574R</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>QQ + RQ, n (%)</td>
<td>RR, n (%)</td>
<td>P</td>
<td>RR + PR, n (%)</td>
</tr>
<tr>
<td>Invasion*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Within serosa (n = 39)</td>
<td>16 (46)</td>
<td>23 (59)</td>
<td>0.25 †</td>
<td>15 (47)</td>
</tr>
<tr>
<td>Serosa and beyond (n = 45)</td>
<td>19 (54)</td>
<td>16 (41)</td>
<td>OR, 0.59</td>
<td>17 (53)</td>
</tr>
<tr>
<td>Total (n = 74)</td>
<td>35 (100)</td>
<td>39 (100)</td>
<td></td>
<td>32 (100)</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No metastasis (n = 29)</td>
<td>19 (54)</td>
<td>10 (26)</td>
<td>0.012 ‡</td>
<td>17 (53)</td>
</tr>
<tr>
<td>Metastasis (n = 45)</td>
<td>16 (46)</td>
<td>29 (74)</td>
<td>0.095 ‡</td>
<td>23 (88)</td>
</tr>
<tr>
<td>Total (n = 74)</td>
<td>35 (100)</td>
<td>39 (100)</td>
<td></td>
<td>32 (100)</td>
</tr>
<tr>
<td>1-y Postoperative mortality</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Survived (n = 43)</td>
<td>25 (89)</td>
<td>18 (69)</td>
<td>0.008 ‡</td>
<td>23 (88)</td>
</tr>
<tr>
<td>Deceased (n = 11)</td>
<td>3 (11)</td>
<td>8 (31)</td>
<td></td>
<td>3 (12)</td>
</tr>
<tr>
<td>Total (n = 54)</td>
<td>28 (100)</td>
<td>26 (100)</td>
<td></td>
<td>26 (100)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Haplotype of polymorphisms</th>
<th>MMP-9 279R-574P</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-double homozygotes, n (%)</td>
<td>Double homozygotes, n (%)</td>
<td>P</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No metastasis (n = 29)</td>
<td>20 (55)</td>
<td>9 (24)</td>
<td>OR, 4.03; 95% CI, 1.49-10.90</td>
</tr>
<tr>
<td>Metastasis (n = 45)</td>
<td>16 (44)</td>
<td>29 (76)</td>
<td></td>
</tr>
<tr>
<td>Total (n = 74)</td>
<td>36 (100)</td>
<td>38 (100)</td>
<td></td>
</tr>
<tr>
<td>1-y Postoperative mortality</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Survived (n = 43)</td>
<td>26 (90)</td>
<td>17 (68)</td>
<td>OR, 4.08; 95% CI, 0.95-17.58</td>
</tr>
<tr>
<td>Deceased (n = 11)</td>
<td>3 (10)</td>
<td>8 (32)</td>
<td></td>
</tr>
<tr>
<td>Total (n = 54)</td>
<td>28 (100)</td>
<td>26 (100)</td>
<td></td>
</tr>
</tbody>
</table>

*Within serosa: depth of the invasion includes submucosa, mucosa, and muscularis propria; serosa and beyond: depth of the invasion includes serosa and over serosa.
† By χ² test between subgroups.
‡ By Fisher’s exact test.

0.008, and 0.006; Supplementary Table S2) and were deemed likely to be significant confounders. There was a strong association between tumor-node-metastasis and lymph node metastasis; however, tumor-node-metastasis grade was partially determined by the extent of lymph node metastasis and therefore was not an independent variable and thus not considered as a confounding factor. Moreover, in multivariate analyses by logistic regression modeling, the variables need to be independent from each other. However, Lauren’s classification, location of the gastric cancer, depth of the invasion, and tumor size were significantly associated with each other to a certain extent (P < 0.05; data not shown). They could not be included in multivariate analyses at the same time. Gender and age have clearly no association with either MMP-9 SNPs or lymph node metastasis (Supplementary Tables S1 and S2), thus probably were not significant confounding factors. However, because they are conventional good independent factors, we included them in all logistic regression modeling to adjust for any small confounding effects.

The results of the multivariate logistic regression with these factors are summarized in Supplementary Table S3. The results indicated that, after adjustment for genotype, patients’ gender and age, and Lauren’s classification, location, size of the tumor, or depth of the invasion, the homozygous 279R-574P was associated with lymph node metastasis (in respective analyses, Supplementary Table S3, top). For example, after adjustment for gender, age, and location of the tumor, ORadjusted was 6.09 (95% CI, 1.92-19.29; Supplementary Table S3, top, analysis E). It is interesting to note that the adjustment for depth of cancer invasion revealed the strongest association between MMP-9 and lymph node metastasis (ORadjusted 7.4; 95% CI, 2.13-35.74; Supplementary Table S3, top, analysis G). We believe that the small negative association of cancer invasion with MMP-9 SNPs might cause this phenomenon.

Homzygosity for both susceptible alleles seems to have a stronger association with lymph node metastasis. Because R279Q and P574R SNP loci are only 2.811 kb apart, it is possible that they are in a single block of disequilibrium. However, from the Ensemble’s Haploview, there are no data for the linkage disequilibrium between R279Q (rs17576) and P574R (rs2250889). We examined the linkage disequilibrium in our control and case population using the software Arlequin and calculated the r² and D’ (0.83 and 0.96 in the control population; 0.81 and 1.0 for the case population). The results indicated that the two alleles are in great linkage disequilibrium. The statistical association shown above only showed the
association of the genotype of a SNP locus to a phenotype and does not necessarily indicate an association in the causative biological function. To further explore the functional cooperation of those two SNPs, we compared the association of genotypic distributions of haplotypes derived from those two loci with lymph node metastasis with the single-locus model. We noticed that, in comparison with the homozygous 279R, the homozygous 279R-574P haplotype was more closely associated than with lymph node metastasis (OR, 4.0 versus 3.4; Table 2) in univariate analysis and in the multivariate analysis adjusted for gender, age, and location of the tumor (e.g., ORadjusted = 6.09 versus 5.74; Supplementary Table S3, top, analysis A versus E). However, the increase of ORs was not significantly dramatic to conclude that there was a functional cooperation between those two SNPs.

Interestingly, when the association in terms of haplotype frequency distribution (estimated by Arlequin software) was analyzed, the differences between patients and controls were not statistically significant (P = 0.57; data not shown). There is a borderline significant difference in the haplotype frequency distribution between nonmetastasis and metastasis cases (P = 0.068). The analysis by haplotype frequency seems to be less sensitive than the two-locus genotype analysis.

Multivariate analyses of associations of MMP-9 polymorphisms with lymph node metastasis of gastric cancer using stratified analyses. For confirmation of the results from logistic regression modeling and analyzing the interaction between factors, we also applied stratification to analyze the association of SNPs with lymph node metastasis using other clinicopathologic features as stratification factors. Not only can stratified analyses analyze the interactions but also can sometimes be more sensitive in examining confounder effects than the multivariate analysis using logistic regression. Due to the small numbers of cases in the subgroups, most of the analyses did not give meaningful or more informative results, including those using gender, age, location, and size of the tumor as stratification factor (data not shown). The results of meaningful stratified analyses are listed in Supplementary Table S4. Using cancer invasion as the stratification factor revealed more evident association between MMP-9 SNPs and lymph node metastasis in both strata of patients without any adjustments (OR, 5.69 and 8.75; Supplementary Table S4). The difference in ORs between strata was not significantly different (P = 0.54; Supplementary Table S4), indicating that the associations are independent of cancer invasion. Interestingly, when using the Lauren's classification as the stratification factor, the association between MMP-9 SNP and lymph node metastasis was different between two strata, and there is a synergistic effect of MMP-9 SNPs and the diffuse type of gastric cancer in affecting lymph node metastasis (OR, 13.36; P = 0.002; Supplementary Table S4), whereas in cases of intestinal/mixed types of Lauren classification, the effect of MMP-9 SNPs on lymph node metastasis was barely detectable (OR, 1.71; P = 0.51). There was a significant difference in the effects of MMP-9 SNPs between two strata (P = 0.04; Supplementary Table S4).

Association of MMP-9 polymorphisms with 1-year postoperative mortality of gastric cancer patients. It is widely accepted that lymph node metastasis is a good prognostic predictor. In our study population, Kaplan-Meier analysis of postoperative survival showed a correlation between lymph node metastasis and higher overall mortality (P = 0.003, log-rank test). Lymph node metastasis is also associated with 1-year postoperative mortality (P = 0.002; Supplementary Table S2). Because MMP-9 polymorphism is associated with metastasis, it is reasonable to infer that MMP-9 polymorphism will also be associated with the slope of survival curves. To examine how well MMP-9 SNPs can serve as a prognostic indicator, we analyzed the relationship of MMP-9 SNPs with the patients’ survival time using the Kaplan-Meier method. The difference in the survival curves between patients of two genotypes (homozygous 279R-574P and nonhomozygous 279R-574P) is not as prominent as using lymph node metastasis as the prognostic indicator (P = 0.14 versus 0.003, log-rank test; Fig. 2). The difference of the survival curves resided mainly in the early phase. After logistic regression modeling to analyze the MMP-9 SNPs (homozygous 279R-574P versus nonhomozygous) with 1-year postoperative mortality, we obtained significant P values for most of the multivariate analyses (Padjusted < 0.05; Supplementary Table S3, bottom, analysis C-G). Not surprisingly, depth of the invasion showed a strong association with 1-year mortality (ORadjusted = 13.81; 95% CI, 1.84-103.56; Supplementary Table S3, bottom, analysis G). Our results indicated that the MMP-9 SNP was not as good a prognostic indicator as lymph node metastasis for general gastric cancer but may be an assisting indicator.

Discussion

In the above case-control study, we show that homozygous genotypes of 279R and 574P SNP of MMP-9 gene were not associated with the overall occurrence of gastric cancer but were negatively associated with the occurrence of gastric cancer of no lymph node metastasis in a population in southeast China. Moreover, among the patients, we showed that both SNPs were not associated with any clinicopathologic features of gastric cancer but were significantly associated with lymph node metastasis.

![Fig. 2. Kaplan-Meier survival curves of gastric cancer patients of different MMP-9 genotypes who underwent surgical resection. The curves were drawn after the patients were divided into two groups according to their genotypes of MMP-9 (homozygous 279R-574P and nonhomozygous 279R-574P haplotype).](image-url)
node metastasis. The multivariate analyses by logistic regression modeling and stratification confirmed and reinforced these associations. Our result is similar as in another previous study, where these two MMP-9 SNPs were individually reported to be associated with the metastasis of lung cancer (21), but different from other studies, where 279QQ (not 279RR) was found to be associated with in-transit metastasis in melanoma cancer (22) and associated with higher grade in renal cell carcinoma (23). Despite those differences, the present study adds to the accumulating evidence that germ-line MMP-9 protein variations may affect cancer progression.

In this study, particular attention has been paid to avoid spurious results caused by artificial bias in the study design and by confounding effects. Comparing our results with an association study of MMP-9 SNPs in a population in another city of southeast China, no differences were observed in the allelic frequency in the control (279R, 0.765 versus 0.675; 574P, 0.8 versus 0.703; ref. 21). In addition to having measures to eliminate technical genotyping errors, a multitude of univariate or multivariate analyses has been done to uncover any inconsistencies reflecting the presence of biases. All analyses gave similar results with relatively large OR values for the association of MMP-9 SNPs with lymph node metastasis, confirming our findings.

Elevated MMP-9 expression is associated with the overall occurrence of gastric cancer because overexpression of MMP-9 has been reported to be present in a large percentage of gastric cancers, ranging from 40% to 60% (7–9, 27, 28). However, in our study, the SNPs did not show any association with the development (occurrence) of overall gastric cancer. Interestingly, we observed negative association with the occurrence of gastric cancer with no lymph node metastasis. These data lead us to suspect that the homozygous 279R and 574P variants of MMP-9 might have an inhibiting role in the initiation of a subgroup of gastric cancer that has a weaker propensity for lymph node metastasis, and this could be explained by the involvement of MMP-9 in activating the TGFβ1 pathway (3). TGFβ1 is a protein known to have dual functions, suppression of tumor initiation (29) and promotion of tumor progression, and is capable of inducing MMP-9 expression (30). A large body of data has indicated that a higher expression level of MMP-9 is closely related to progression of gastric cancer, such as metastasis and invasion (6, 8, 31). Aberrant chromosomal amplification most likely provides a genomic basis and a main pathway for the gastric tumor cells to achieve this capability (15, 32, 33). Chromosome 20q, where MMP-9 is located, is amplified in as many as 66% of gastric cancer (16). The SNP located at the promoter of MMP-9 may also be a genetic factor involved in progression of gastric cancer through its effects on the transcription of MMP-9 mRNA (11). Although at present overexpression is largely accepted to be the main factor affecting the progression of gastric cancer, the finding in this study provides the first evidence that qualitative variants of MMP-9 may be important. Interestingly, in this study, we have shown that the increased risk of lymph node metastasis associated with the homozygous 279R-574P haplotype is mainly in the diffuse type of gastric cancer. Several reports from other laboratories have suggested that the MMP-9 overexpression mainly occurs in the intestinal type (7, 34) and that the association of overexpression with lymph node metastasis is restricted to the intestinal type (20). Combined with the findings from this study, a hypothesis can be constituted that the quantitative and qualitative aspects exert their effects complementarily. Whereas in the intestinal type the overexpression of MMP-9 mainly contributes to the cancer progression, in the diffuse type the genotype of MMP-9 becomes an important factor involved in the lymph node metastasis. To test this hypothesis and examine in a more specific way how these two aspects of regulation interact with each other in controlling the cancer progression is an important objective of future studies. For this purpose, the expression level of MMP-9 is currently being examined and more cases are being recruited.

In this study, the Kaplan-Meier survival analysis did not give significant results due to small sample size and high amount of censored data, which reduced the power of statistical analysis. The analysis of 1-year survival showed that homozygous 279R-574P haplotype was associated with mortality ($P_{\text{adjusted}} = 0.034$; OR$_{\text{adjusted}} = 5.8$; 95% CI, 1.15-29.40), suggesting that the short-term postoperative survival is influenced by the MMP-9 variants. In this study, we confirmed correlation of lymph node metastasis with the serosal invasion of the tumor ($P = 0.006$; Supplementary Table S2). However, surprisingly, although the double homozygous 279R-574P genotype is associated with increased risk of lymph node metastasis, it was not associated with the serosal invasion with an OR of $<1$, which hints at a potential negative association ($P = 0.34$; OR, 0.65; Table 2). Therefore, it seems that nodal metastasis and invasion could differ in certain aspects during cancer progression, in which MMP-9 variants have different functions.

Because these two SNPs are in relatively tight linkage disequilibrium, it is difficult to discern the functional differences caused by these two SNPs in this study. However, larger ORs suggest that MMP-9 R279Q is more closely associated with lymph node metastasis than P574R. This result seems to be similar with the result of the association study of MMP-9 SNPs in melanoma, in which R279Q but not P574R had a significant association with metastasis (22). Combined analysis of genotypes of both SNPs suggested that the double homozygous 279R-574P haplotype was more closely associated with lymph node metastasis and 1-year postoperative mortality of the patients, suggesting that both SNPs are functional and may act synergistically. However, due to the limited scale of this study, these observations need further validation.

There are currently 24 members in the MMP family, all sharing a catalytic domain coordinated by zinc, catalyzing the degradation of protein components of extracellular matrix (4, 35) as well as activating latent growth factors, cell surface receptors, and adhesion molecules (36). In MMP-9, R279Q polymorphic amino acid is located in the second fibronectin type II domain (FN2; three tandem FN2 internal repeats totally) of the catalytic domain. The FN2 of MMP-9 was found to have an affinity for denatured collagen, suggesting that this domain has an important role in substrate binding (4, 37). P574R is located in the hemopexin-like domain, which is necessary for the collagenase activity of MMP-9 in cleaving triple-helical collagen and is involved in the binding of MMP-9 with its specific inhibitor (TIMP-1; ref. 2). The saline change in the chemical property between two amino acids (from the positively charged arginine to uncharged glutamine in R279Q, and uncharged proline to the positively charged arginine in P574R) suggests that both SNPs might be able to induce changes in the biological functions of MMP-9.
To further predict the potential differences in biological activities among these MMP-9 variants, the conservation status of these polymorphisms was examined by multiple alignment among orthologues and paralogues. R279Q does not seem to be a variation incompatible with the essential functions of MMP-9, but 574R may be an odd substitution based on limited number of orthologues available for the alignment. R279Q is not located in the putative gelatin (may also collagen)-binding pocket of the FN2 module. The putative critical residues for ligand binding are believed to include 294F, 311Y, 313W, and 320Y, 326F, and 328F. R279Q is not located at the position of critical residues for the maintenance of the module fold and solvent-exposed hydrophobic surface (38, 39). PolyPhen is a recently developed web-interfaced program to predict the phenotypic effects of nonsynonymous SNPs (40). The prediction is based on a comprehensive algorithm combining straightforward empirical rules that are applied to the sequence, phylogenetic, and structural information characterizing the substitution. The analyses on these two SNPs with PolyPhen produced similar results, with R279Q being benign and P574R being damaging by a modest chance. The homology modeling using the Swiss-Model program on R279Q (not P574R for lack of a close template), however, came up with interesting clues. We notice that 279R is immediately adjacent (within 3Å) to 328F, within a 5-Å distance to 311Y and 328F, and within a 7-Å distance to 311Y, 326F, and 328F. More interestingly, the side chains of 279R (but not 279Q) and 280D can form a strong (double position) salt bridge (Supplementary Fig. S1), which potentially have indirect effects on the shape and stability of the ligand-binding pocket and the FN2 module. Better understanding of functional effects of these two MMP-9 SNPs requires more detailed and complete functionally related structural characterization in the physiologic setting (complex with native collagen IV and TIMP-1) and more sophisticated modeling.

Lymph node metastasis of cancer itself is a multistep event, including escape of tumor cells, invasion into lymphatic vessels, and proliferation. During the development of gastric cancer, there are two sources of MMP-9: one is the tumor cells and the other is the adjacent normal cells. Most gastric cancer has abnormalities in the genome. The genotype we have identified for each case only represents the genotype of MMP-9 generated from the normal cells. In patients of heterozygous genotype of MMP-9, if there is allelic preferential loss or amplification of the SNP locus, the genotype of MMP-9 proteins produced by cancer cells may be homozygous. In other words, MMP-9 variants from two resources can be different. How MMP-9 from the two sources functions in the progression of gastric cancer remains to be elucidated. It will be interesting to investigate if there is preferential expression of MMP-9 variants in cancer cells of patients with heterozygous genotype of MMP-9 and the relative expression levels of MMP-9 between the cancer cells and normal cells in gastric cancer patients of different MMP-9 genotypes. These results will provide insights into the molecular mechanisms whereby MMP-9 and MMP-9 variants affect cancer progression and may distinguish between tumor-produced or normal tissue–produced MMP-9 pathways.

In summary, through this association study for MMP-9 SNPs in gastric cancer, we determined that MMP-9 R279Q and P574R are significantly associated with lymph node metastasis, especially the diffuse-type of gastric cancer. This study also has uncovered other association rules among MMP-9 SNPs and several clinicopathologic features. The present study provides clues from an epidemiologic and statistical point of view to better understand the complex mechanisms involved in the development and progression of gastric cancer. Direct illustration of biological significance of these findings about MMP-9 variants awaits further studies at the level of molecular biology and biochemistry.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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