Functional Haplotypes in the Promoter of Matrix Metalloproteinase-2 Predict Risk of the Occurrence and Metastasis of Esophageal Cancer

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ABSTRACT

Matrix metalloproteinase-2 (MMP-2) plays important roles in cancer development and aggression. Our previous studies revealed a strong association between the MMP-2 −1306C/T polymorphism and risk of several cancers. A novel −735C/T polymorphism in MMP-2 promoter has been identified but the function is undefined. This study examined our hypothesis that these two polymorphisms might have functional relevance and impact on risk of esophageal squamous cell carcinoma in the context of haplotype. Genotypes and haplotypes were analyzed in 527 cases and 777 controls and odds ratios (ORs) and 95% confidence intervals (CIs) were estimated by logistic regression. The function of the polymorphisms was examined by electrophoretic mobility shift assays, luciferase gene expression assays, and reverse transcriptase-PCR analyses. It was found that the −735C→T transition disrupts an Sp1 site and displays a lower promoter activity. The C−1306C−735C haplotype had 7-fold increased luciferase expression and 3.7-fold increased MMP-2 mRNA levels in esophageal tissues compared with the T−1306T−735T haplotype. A case-control analysis revealed a 1.52-fold (95% CI = 1.17–1.96) or 1.30-fold (95% CI = 1.04–1.63) excess risk of developing esophageal squamous cell carcinoma for the −1306CC or −735CC genotype carriers compared with noncarriers, respectively. A greater association was observed between elevated risk of developing esophageal squamous cell carcinoma and the C−1306C−735C or C−1306T−735T allele containing haplotypes, with the risk being highest for the C−1306C−735C haplotype compared with the T−1306T−735T haplotype (OR = 6.53; 95% CI = 2.78–15.33). The C−1306C−735T haplotype was also associated with increased risk for distant metastasis of esophageal squamous cell carcinoma (OR = 3.34; 95% CI = 1.16–9.63). These findings suggest that the C−1306C−735C haplotype in the MMP-2 promoter contributes to risk of the occurrence and metastasis of esophageal squamous cell carcinoma by increasing expression of MMP-2.

INTRODUCTION

Accumulating evidence has shown that matrix metalloproteinases (MMPs) play a critical role in cancer development and aggression because of their capability to degrade or break down both extracellular matrix and basement membrane, the important physical barriers in preventing against expanding growth and migration of cancer cells (1–3). Moreover, recent studies have demonstrated that MMPs also have many other substrates involving in several steps of cancer development such as apoptosis, cell proliferation, angiogenesis, and immune surveillance (4). MMP-2, a member of MMPs, predominately degrades gelatin and type IV collagen, the major structural component of basement membrane, and thus has been considered as an important factor in cancer invasion and metastasis (5, 6). Nevertheless, this enzyme also has activity toward a spectrum of functional molecules such as growth factor-binding proteins and growth factor receptors. For instance, MMP-2 can cleave insulin-like growth factor-binding proteins and release insulin-like growth factors (7), which are well known to have a strong effect on stimulating cell proliferation and inhibiting apoptosis. MMP-2 can also target fibroblast growth factor receptor 1, yielding active soluble ectodomain of the receptor (8), which has the potential to modulate the mitogenic and angiogenic activities of fibroblast growth factor. These findings suggest that MMP-2 contributes not only to cancer progression but also to cancer development (4).

MMP-2 is constitutively expressed by a large number of cell types and overexpressed in a wide variety of human cancers, including esophageal squamous cell carcinoma (9–11). Furthermore, the production of this proteinase in tumors is made by not only cancer cells but also normal stromal cells (9–11), suggesting that the overexpression of MMP-2 is probably due to transcriptional changes but not gene amplification or activating mutations. Because the human MMP-2 promoter contains a number of cis-acting regulatory elements, the constitutive and induced expression of this proteinase is likely to be subject to regulation by transcription factors (12, 13). Several single nucleotide polymorphisms (SNPs) in the MMP-2 promoter region have been identified (14). Among them, a C to T transition located at nucleotide −1306 abolishes a Sp1-binding site and consequently diminishes promoter activity. Transient transfection experiments showed that reporter gene expression driven by the C allelic MMP-2 promoter was significantly greater than reporter gene expression driven by the T allelic counterpart both in epithelial cells and in macrophages, indicating the functional significance of this polymorphism (14). Recently, another C to T transition located at nucleotide −735 in the promoter region of MMP-2 has been identified (15), but the functional significance is undefined. Bioinformatic analysis suggests that the −735C/T polymorphism might also disrupt a consensus sequence for Sp1-binding site, implying that this polymorphism might have the potential to alter MMP-2 transcription.

We have previously shown that the functional −1306C/T polymorphism in MMP-2 is associated with susceptibility to cancers of the lung, gastric cardia, and breast (16–18), suggesting that the MMP-2 polymorphism might be a general risk factor for common cancers. On the basis of these findings, we additionally hypothesized that the −735C/T polymorphism in MMP-2 might also have impact on individual susceptibility to cancer. Furthermore, because the −735C/T site is close to the −1306C/T site in the MMP-2 promoter, these two SNPs might be in linkage disequilibrium and act in an interaction manner. To test these hypotheses, we have examined the functional relevance of the −735C/T polymorphism, alone, and in combination with −1306C/T polymorphisms in the context of haplotypes. Moreover, we conducted a case-control study to investigate the relationship between the genotypes and haplotypes of these polymorphisms in MMP-2 promoter and risk of the occurrence and metastasis of esophageal squamous cell carcinoma.

MATERIALS AND METHODS

Subjects for the Case-Control Study. This study recruited 527 patients with esophageal squamous cell carcinoma and 777 healthy controls. All subjects were ethnic Han Chinese. Patients were consecutively recruited from
January 1997 to November 2001 at the Cancer Hospital, Chinese Academy of Medical Sciences (Beijing, China). All patients with histopathologically confirmed esophageal squamous cell carcinoma were enrolled, yielding 92% response rate. A portion of cases (n = 240) was enrolled in our previous molecular epidemiologic studies of esophageal squamous cell carcinoma (19). The pathological stage of esophageal squamous cell carcinoma at the time of diagnosis was classified by senior pathologists of the hospital on the basis of postoperative histopathological examination or biopsy according to the Tumor-Node-Metastasis classification (20).

Healthy controls were cancer-free individuals living in Beijing region, and they were selected from a community cancer-screening program for early detection of cancer conducted during the same period as the cases were collected (17). These controls were randomly selected from a pool of 2800 individuals based on a physical examination, and the response rate was 96%. The selection criteria included no individual history of cancer and frequency matched to esophageal squamous cell carcinoma cases on sex and age (±5 years). At recruitment, informed consent was obtained from each subject, and each participant was then interviewed to collect detailed information on demographic characteristics and lifetime history of tobacco use. This study was approved by the Institutional Review Board of the Chinese Academy of Medical Sciences Cancer Institute.

**MMP-2 Genotyping.** Genomic DNA from controls and most of cases was isolated from blood samples. Approximately 30% DNA samples from cases were isolated from surgically resected normal tissues adjacent to the tumor of esophageal squamous cell carcinoma patients. MMP-2 genotypes at the −1306CT site were determined by PCR-based denaturing high-performance liquid chromatography analysis as described previously (16, 17). MMP-2 genotypes at the −735CT site were analyzed by PCR-based restriction fragment length polymorphism methods (15). The primers used for amplifying DNA containing the −735CT site were 5′-ATAAGGTAAACCTCCCCACATT-3′ and 5′-GGTAAATGGCAGGACACCCT-3′, which produce a 300-bp fragment. Digestion of the PCR product with HinfI (New England Biolabs, Beverly, MA) at 37°C overnight produced one fragment of 300 bp for the CC genotype; three fragments of 300, 254, and 46 bp for the CT genotype; and two fragments of 254 and 46 bp for the TT genotype. The genotypes identified by HinfI digestion were confirmed by DNA sequencing, and sequences were compared with the published MMP-2 promoter sequences (12).

All of the genotyping was performed with blinded to case/control status, and blinded quality control samples were inserted to validate genotypes. Concordance for blinded samples was 100%.

**Statistical Analysis.** χ² test was used to compare the distribution of MMP-2 genotypes and haplotypes between cases and controls and between metastatic and nonmetastatic cases. Lighter or heavier smokers were categorized by the approximate 50th percentile pack-year value among controls, i.e., <26 or ≥26 cigarettes per day (CPD). Hardy–Weinberg equilibrium was tested by a goodness-of-fit χ² test. Haplotype frequencies and linkage disequilibrium coefficient were estimated using PHASE (21) and EH (EH-plus) software (22), respectively. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated by unconditional logistic regression analysis using Stata 8.2 software (StataCorp, College Station, TX). The χ² test and P < 0.05 were considered significant.

**Reverse Transcriptase-PCR Analysis.** Fifty-two normal esophageal tissues adjacent to the tumors were obtained from surgically removed specimens of individual patients. The normal tissues sampled at least 5 cm away from the margin of the tumor were immediately placed in liquid nitrogen and then stored at −80°C before analysis. Total RNA was isolated from tissues using the Trizol Reagent (Life Technologies, Inc.). An aliquot of total RNA (2 μg) from each specimen was reverse transcribed into single-strand cDNA using oligo (dT)₁₅ primer and Superscript II (Life Technologies, Inc.). Each single-strand cDNA was diluted for subsequent PCR amplification of MMP-2 and β-actin, with the latter being used as an internal quantitative control. The primers used for amplification of MMP-2 were 5′-TTCAAAGCCGGT-TCACTTGGCGCACTTTGC-3′ and 5′-TTCAAAACTTTGCTGCTTTGCTTAC-3′, which generate a 493-bp fragment, and for β-actin were 5′-CTGGCTGAACCTTGCTGGCCGACG-3′ and 5′-CTAGAAGCTTGGGTTAGAGG-3′, which generate a 601-bp fragment. PCR was performed under the following conditions: an initial denaturing step of 5 minutes at 95°C, followed by 30 cycles of 40 seconds at 94°C, 40 seconds at 60°C, and 40 seconds at 72°C, and a final elongation step of 7 minutes at 72°C. PCR products were separated and visualized in 1.5% agarose gel containing ethidium bromide and quantified by using a UVP GDS-8000 image analysis system (UVP, Inc., Upland, CA). The relative density of the MMP-2 band was calculated based on the density of the β-actin band in each sample.
DISRUPTION OF SP1-BINDING SITE IN THE −735T SNP. By using the AliBaba 2 software, we found that the −735C/T polymorphism may alter a consensus sequence for SP1-binding site (CCCTCC→CTCTCC). Electrophoretic mobility shift assays were thus designed to examine whether the Sp1 consensus sequence is abolished by the presence of a T at the −735 site. As shown in Fig. 1, a clear DNA-protein complex was detected with the −735C probe (Fig. 1, Lane 2) but not the −735T probe (Fig. 1, Lane 9) in the assays. To determine the sequence specificity of this DNA-protein complex, competition experiments were performed. The band was competed by 50- and 100-fold excess of unlabeled −735C probe (Fig. 1, Lanes 3 and 4) but not by the same concentrations of unlabeled −735T probe (Fig. 1, Lanes 5 and 6). The specificity of this band was additionally confirmed by the addition of 100-fold excess of nonspecific competitor (Fig. 1, Lane 7). These assays clearly demonstrated the ability of the −735C allele, but not the −735T allele, to bind specifically the nuclear protein. We next performed electrophoretic mobility shift assays using above described −735C probe and an Sp1 consensus probe, and the results are shown in Fig. 2A. The labeled Sp1 consensus probe formed a specific DNA-protein complex (Fig. 2A, Lane 2), which was identical to that observed with the −735C probe (Lane 9) and was eliminated by excess unlabeled probe (Fig. 2A, Lanes 3, 10, and 14, respectively). Lanes 6 and 13 in Fig. 2A demonstrate the specificity of the DNA-protein complex by competition experiments with 100-fold excess of unlabeled mutated Sp1 consensus probe. Furthermore, the ability of the −735C but not the −735T allele to compete specifically for Sp1 binding was confirmed by additional competition experiments using 100-fold excess of unlabeled −735T probe (Fig. 2A, Lanes 4 and 12), 50- or 100-fold excess of unlabeled −735C probe (Fig. 2A, Lanes 5 and 7), and 100-fold excess of unlabeled Sp1 consensus probe (Lane 11). Super-shift assays were then performed to confirm Sp1 binding using the Sp1 consensus probe (Fig. 2B, Lanes 1–4) or −735C probe (Fig. 2B, Lanes 5–8) in either the absence (Fig. 2B, Lanes 1 and 5) or presence of antibody against Sp1 or rabbit IgG (Fig. 2B, Lanes 2, 3, 6, and 7, respectively). The DNA-protein complex was successfully supershifted with the anti-Sp1 antibody (Fig. 2B, Lanes 2 and 6) but not the rabbit IgG (Fig. 2B, Lanes 3 and 7). Taken together, these results clearly demonstrate that nucleotide −735C/T in the MMP-2 promoter is within a Sp1-binding sequence and the C to T substitution disrupts the binding site.

Effects of MMP-2 −735C/T and −1306C/T SNPs on Transcriptional Activity. To directly determine the allele-specific effects of Sp1 binding on native promoter activity, four luciferase reporter gene constructs were generated by PCR, spanning −1691 to +10 of the MMP-2 promoter region, with either a T or C at the −735 and −1306 polymorphic sites (Fig. 3A), and they were used to transfect transiently HEK293 cells. As shown in Fig. 3B, reporter gene expression

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1 Internet address: http://www.gene-regulation.com/pub/programs.html#alibaba2.
Fig. 3. Transient reporter gene expression assays with constructs containing full-length MMP-2 promoter. A, schematic of reporter gene constructs having a full-length MMP-2 promoter with the only difference between the four constructs being a T or C at the −1306 and −735 polymorphic sites. B, luciferase expression of the four constructs in HEK293 cells cotransfected with pRL.SV40 to standardize transfection efficiency. Luciferase levels of pGL-3 Basic and pRL-SV40 were determined in triplicate and standardized for transfection efficiency. Fold increase was measured by defining the activity of the empty pGL-3 Basic vector as 1. Data shown are the means fold increase ± SD from three independent transfection experiments, each done in triplicate. **, P < 0.0001 compared with the pT–T construct.

Fig. 4. Representative gel picture showing reverse transcriptase-PCR analysis of MMP-2 mRNA in esophageal tissues from individuals with different MMP-2 promoter genotypes. The expected length of PCR products was 601 bp for β-actin and 493 bp for MMP-2. Lane M, DNA size marker; Lanes 1–11, individual samples. Individual’s genotype designation: Lanes 1–7, 9, and 10, −735CC/−1306CC; Lanes 4 and 11, −735TT/−1306CT; and Lanes 5 and 8, −735CT/−1306TT.

Table 1  Genotype and allele frequencies of MMP-2 among cases and controls and their contributions to the risk of esophageal squamous cell carcinoma

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Cases (n = 527)</th>
<th>Controls (n = 777)</th>
<th>OR* (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-2 −1306CT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>6 (1.1)</td>
<td>18 (2.3)</td>
<td>1.00 (reference)</td>
</tr>
<tr>
<td>CT</td>
<td>112 (21.3)</td>
<td>220 (28.3)</td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>409 (77.6)</td>
<td>539 (69.4)</td>
<td>1.52 (1.17–1.96)</td>
</tr>
<tr>
<td>C allele frequency</td>
<td>0.882</td>
<td>0.836</td>
<td></td>
</tr>
<tr>
<td>MMP-2 −735CT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>25 (4.7)</td>
<td>39 (5.0)</td>
<td>1.00 (reference)</td>
</tr>
<tr>
<td>CT</td>
<td>179 (34.0)</td>
<td>313 (40.3)</td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>323 (61.3)</td>
<td>425 (54.7)</td>
<td>1.30 (1.04–1.63)</td>
</tr>
<tr>
<td>C allele frequency</td>
<td>0.783</td>
<td>0.749</td>
<td></td>
</tr>
</tbody>
</table>

*ORs and 95% CIs were calculated by unconditional logistic regression with the MMP-2–variant genotypes (CT or TT) as the reference group and adjusting for sex, age, smoking status, and other genotype where it was appropriate.

HAPLOTYPES OF MMP-2 PREDICT ESOPHAGEAL CANCER

Driven by the −735C allele, MMP-2 promoter was ~3-fold greater than that driven by the −735T allele; SNP counterparts (3.54 ± 0.71 versus 1.24 ± 0.38, P < 0.0001), indicating the biological significance of this SNP on promoter activity via recruitment of Sp1 transcriptional factor. The combined effects of the −735C/T and −1306C/T SNPs on transcriptional activity were further assessed. Interestingly, the expression levels of reporter gene driven by the pT–T, pT–C, pC–C, and pC–T alleles increased gradually, with the values being 1.24 ± 0.38, 3.54 ± 0.71, 5.28 ± 0.84, and 8.79 ± 1.01, respectively (Fig. 3B). These results indicate a strong synergic effect between −735T and −1306T SNPs in the context of haplotype on reducing transcriptional activity of MMP-2. The effect of these two SNPs on MMP-2 transcriptional activity was additionally examined by reverse transcriptase-PCR analysis of MMP-2 mRNA in esophageal tissues (Fig. 4). MMP-2 mRNA levels were significantly lower in individuals with the −1306CT or TT genotype than in those with the −1306CC genotype [0.08 ± 0.06 (n = 19) versus 0.20 ± 0.28 (n = 33); P = 0.078]. We also compared MMP-2 mRNA levels in function of haplotypes, and individuals with the MMP-2 C−1306C−735C haplotype had significantly higher mRNA levels in their esophageal tissues compared with those with the haplotype consisting of at least one T allele at the −1306 or −735 site [0.26 ± 0.32 (n = 23) versus 0.07 ± 0.06 (n = 29); P = 0.003].

Genotypes and Risk of Esophageal Squamous Cell Carcinoma. Having the functional consequences of these two SNPs, we next conducted a case-control study to examine the association of them with risk of esophageal squamous cell carcinoma. There were no significant differences between cases and controls in sex distribution (75 versus 72% males). The mean age (±SD, years) for cases and controls were 58.3 ± 9.7 and 57.6 ± 7.6, respectively (P = 0.187). More smokers were presented in cases compared with controls (60.3 versus 47.4%, P < 0.0001). Of the 486 patients who had detailed pathological data, 41 (8.4%) patients had stage I esophageal squamous cell carcinoma, 190 (39.1%) had stage II esophageal squamous cell carcinoma, and 239 (49.2%) had stage III esophageal squamous cell carcinoma, whereas only 16 (3.3%) patients had stage IV disease with distant metastasis. The genotyping results are shown in Table 1. The allele frequencies for −1306C and −735C were 0.836 and 0.749 in controls, compared with 0.882 and 0.783 in cases, respectively. The observed genotype frequencies of both −1306C/T and −735C/T sites in controls conformed to the Hardy-Weinberg equilibrium (P = 0.367 and 0.836, respectively). The frequencies for the −1306CC, CT, and TT genotypes in cases differed significantly from those in controls (χ² = 11.45, P = 0.003, df = 2), with the CC homozygotes being higher in cases than in controls (77.6 versus 69.4%, P = 0.001). The difference in genotype frequencies at the −735C/T site between cases and controls was borderline significant (χ² = 5.75, P = 0.056, df = 2). Because of functional significance of the heterozygous

[0.8 ± 0.06 (n = 19) versus 0.20 ± 0.28 (n = 33); P = 0.078]. We also compared MMP-2 mRNA levels in function of haplotypes, and individuals with the MMP-2 C−1306C−735C haplotype had significantly higher mRNA levels in their esophageal tissues compared with those with the haplotype consisting of at least one T allele at the −1306 or −735 site [0.26 ± 0.32 (n = 23) versus 0.07 ± 0.06 (n = 29); P = 0.003].

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HAPLOTYPES OF MMP-2 PREDICT ESOPHAGEAL CANCER

Genotypes (−1306CT and −735CT), they were combined with the respective homozygous −1306TT or −735TT genotypes for risk estimation (Table 1). Multivariate logistic regression analysis showed that the −1306CC carriers had a 1.5-fold excess risk for developing esophageal squamous cell carcinoma compared with noncarriers (adjusted OR = 1.52; 95% CI = 1.17–1.96). Similarly, the −735CC carriers also had increased risk for esophageal squamous cell carcinoma compared with noncarriers (adjusted OR = 1.30; 95% CI = 1.04–1.63). In the stratification analysis, age, sex, and smoking had no effect on the risk of esophageal squamous cell carcinoma related to the MMP-2 genotypes (data not shown).

**Haplotypes and Risk of Esophageal Squamous Cell Carcinoma.** The interactions of multiple SNPs within a haplotype may have impact on biological phenotype (23). We thus analyzed the effect on risk of esophageal squamous cell carcinoma by these two SNPs in the MMP-2 promoter in the context of haplotypes. The haplotype frequencies were computed from unphased genotypes using PHASE software and the results are presented in Table 2. We observed a significant difference in haplotype frequencies between cases and controls ($\chi^2 = 26.63, P < 0.00001, df = 3$). Compared with the T−1306−T−735 haplotype, each of the other haplotype containing at least one −1306C or −735C allele was associated with increased risk of esophageal squamous cell carcinoma. The adjusted ORs of esophageal squamous cell carcinoma for the T−1306−C−735, C−1306−T−735, and C−1306−C−735 haplotypes were OR = 5.19 (95% CI = 2.14–12.59), OR = 6.04 (95% CI = 2.52–14.48), and OR = 6.53 (95% CI = 2.78–15.33), respectively (trend test, $P < 0.001$). A linkage disequilibrium of these two SNPs was observed. The $\chi^2$ test of statistical significance for a two-locus disequilibrium gave a test statistic value of 18.2 ($D' = 0.68$) for the cases, 11.0 ($D' = 0.33$) for the controls, and 22.6 ($D' = 0.40$) for all subjects. The linkage disequilibrium was statistically significant ($P < 0.001$).

**Haplotypes and Disease Status of Esophageal Squamous Cell Carcinoma.** The association between the MMP-2 −1306CT and −735CT polymorphisms and esophageal squamous cell carcinoma disease stage at the time of diagnosis was additionally evaluated. We did not observe any significant association between the disease stage and −735CT polymorphism alone or in combination with −1306CT polymorphism (data not shown). However, the advanced esophageal squamous cell carcinoma stage appeared to be associated with the haplotype, including these two polymorphisms. The frequency of the C−1306−C−735 haplotype in patients with stage IV esophageal squamous cell carcinoma was 87.5% (28 of 32), which was significantly higher than that in patients with stage I–III tumor (67.1%, 631 of 940; $\chi^2 = 4.99, P = 0.026$). Multivariate logistic regression analysis showed that patients carrying the MMP-2 promoter C−1306−C−735 haplotype had >3-fold increased risk for developing distant metastasis of esophageal squamous cell carcinoma, compared with other haplotypes consisting of at least one −1306T or −735T allele (adjusted OR = 3.34; 95% CI = 1.16–9.63).

**Table 2** Risk estimates for extended MMP-2 haplotypes in esophageal squamous cell carcinoma cases and controls

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Cases (n = 527)</th>
<th>Controls (n = 777)</th>
<th>aOR (95% CI)†</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>T−1306−T−735</td>
<td>6 (0.6)</td>
<td>52 (3.3)</td>
<td>1.00 (reference)</td>
<td></td>
</tr>
<tr>
<td>C−1306−T−735</td>
<td>118 (11.2)</td>
<td>204 (13.1)</td>
<td>5.19 (2.14–12.59)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>C−1306−C−735</td>
<td>223 (21.2)</td>
<td>339 (21.8)</td>
<td>6.04 (2.52–14.48)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>C−735</td>
<td>707 (67.1)</td>
<td>959 (61.7)</td>
<td>6.53 (2.78–15.33)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

† Adjusted for sex, age, and smoking status.

**Discussion**

Esophageal squamous cell carcinoma is one of the most common cancers in the world. The prognosis of this cancer is poor with the overall 5-year survival rate being ~20% (24). Tobacco smoke, alcohol, nutritional deficiency, and exposure to certain chemical carcinogens are thought to be risk factors for esophageal squamous cell carcinoma. However, not all exposed individuals develop the disease, suggesting that genetic factors may play important roles in esophageal carcinogenesis (25). We sought to identify genetic factors that confer individual susceptibility to the cancer. We analyzed 527 esophageal squamous cell carcinoma patients and 777 controls for the −1306CT and −735CT polymorphisms in the MMP-2 promoter and observed that these two polymorphisms were associated with increased risk for developing the cancer. The −1306CC and −735CC genotypes appear to have strong interaction within a haplotype to influence the cancer risk; carriers of the C−1306−C−735 haplotype had >6-fold increased risk for developing esophageal squamous cell carcinoma compared with noncarriers. These extended results are consistent with our previous findings showing that the −1306CT polymorphism may be genetic risk factor for cancers of the lung, gastric cardia, and breast (16–18). Moreover, we also observed a significant association between the MMP-2 haplotype and distant metastasis of esophageal squamous cell carcinoma. Patients with the C−1306−C−735 haplotype were at >3-fold increased risk for having distant metastasis of the cancer at the time of diagnosis.

Our functional analysis suggested that the association of MMP-2 haplotypes with increased risk of the occurrence and metastasis of esophageal squamous cell carcinoma might be attributed to gain-of-function of this gene resulting from the promoter SNPs. We found that the −735CT SNP locates in a core recognition sequence of Sp1 in the MMP-2 promoter region. Through electrophoretic mobility shift assays, the DNA-Sp1 complex was detected as binding to the −735C allele but not the −735T allele. Competition assays combined with supershift analysis additionally confirmed that the protein binding to this region is Sp1. The potential cis-acting regulatory elements in the MMP-2 promoter region have been extensively investigated by Qin et al. (13). However, they did not find an Sp1 site around nucleotide −735 in their study. Although the reason for this difference between their study and ours is not known, it is most likely that the MMP-2 promoter they used as starting material in their study was the −735TT genotype in which the Sp1 site was not present. For the −1306CT polymorphism, a similar result showing disruption of an Sp1 promoter site by the C→T transition has been shown previously by Price et al. (14). Because Sp1 is a ubiquitously expressed transcriptional factor that regulates a variety of gene in a constitutive or inducible manner (13, 26–28), it is clear that sequence variations that destroy the Sp1-binding sites such as the MMP-2 −735CT and −1306CT polymorphisms may alter the level and specificity of gene transcription. Our luciferase assays and MMP-2 mRNA analysis in esophageal tissues indeed demonstrated a significant difference in transcriptional activity between the −735C and −735T alleles. More importantly, we observed a remarkably elevated transcriptional activity when the −1306C and −735C alleles, C−1306−C−735 haplotype are concomitantly presented in the MMP-2 promoter, indicating an interaction between these two SNPs within a haplotype. Taken together, these data strongly suggest that the presence of Sp1-binding sequences in the −1306C and −735C alleles enhance MMP-2 transcription, which in turn produces higher levels of MMP-2 protein in C−1306−C−735 carriers than in noncarriers. Because MMP-2 plays important roles in all stages of cancer initiation and development (4), it would be expected that individuals who carry the C−1306−C−735 haplotype and...
therefore have elevated expression of this enzyme over lifetime may be at highest susceptibility to carcinogenesis.

The association between high level of constitutive expression of MMP-2 and susceptibility to tumor formation has been tested in several studies with genetically modified animals. It was found that when induced by carcinoangiogenic stimulus, mice that lack the Mmp2 or Mmp9 gene developed fewer tumors than mice having these genes (29). Cancer cells injected via vein were found to be less capable of colonizing the lungs of Mmp2-knockout mice than the lungs of wild-type mice (30). Conversely, transgenic mice that overexpress membrane type MMP-1, a known activator of pro-MMP-2, were at increased susceptibility to mammary tumor formation and metastasis (31). These data strongly support our observation that the genetic polymorphisms resulting in high expression of MMP-2 over a lifetime may increase cancer susceptibility. In addition, functional polymorphisms in some other MMP genes have also been reported to be associated with risk of certain cancers. For instance, a single guanine insertion polymorphism in the MMP-1 promoter region (1G to 2G), which creates an Ets binding site and enhance transcriptional activity, has been associated with increased risk for lung (32), colorectal (33, 34), endometrial (35), and ovarian cancer (36). A single adenine insertion polymorphism in the MMP-3 promoter (6A allele), which has half the transcriptional activity of the 5A allele, has been linked to reduced susceptibility to breast cancer (37).

Local overexpression of MMP-2 has been shown to be related to lymph node metastasis and poor prognosis of certain cancers, including esophageal squamous cell carcinoma (5, 6, 10, 11, 38). Several studies has suggested that genetic polymorphism in the promoter of MMP-1 (1G/2G) or MMP-3 (5A/6A), which alters the transcription activity of the genes, may influence invasiveness or metastasis of some types of cancer (33, 37, 39). In the present study, we did not find any significant association between pathological stage of esophageal squamous cell carcinoma and MMP-2 polymorphisms at either −1306C/T or −735C/T site. These findings are consistent with our previous observation in gastric cancer study showing that −1306C/T polymorphism was not associated with lymph node metastasis (17). However, in the present study, we did observe a statistically significant association between distant metastasis of esophageal squamous cell carcinoma and the two-site C(−1306)C/−735T haplotype of the −1306C/T and −735C/T polymorphisms. Again, these findings are in agreement with functional analysis of the polymorphisms and may reflect the fact that haplotype may be more precise and powerful than genotype as genetic marker for risk estimate. Additional examinations of larger patient series with more detailed clinic outcome especially the survival rate are warranted. In addition, it would be interesting to investigate haplotypes tagging more SNPs in MMP-2 or other related genes as genetic risk factors for the occurrence and progression of esophageal squamous cell carcinoma and other cancers.

Our case-control study has some drawbacks. Because patients were recruited from only one hospital and controls were from a cancer-screening program, the study subjects may not be representative of the general population. However, because we used incident cases and recruited a large number of subjects, our results are unlikely to be attributable to selection bias. Nevertheless, it would be important to confirm these findings in multicenter case-control studies or in population-based prospective studies.

In summary, our study identified a novel Sp1 regulatory element in the promoter of MMP-2 and suggests that the haplotype for the −1306C/T and −735C/T polymorphisms in the MMP-2 promoter is a genetic susceptibility factor for the occurrence and metastasis of esophageal squamous carcinoma in a Chinese population. These molecular epidemiologic findings are consistent with the results obtained from functional analysis. Because MMP-2 is overexpressed in many cancer types, additional studies on other types of common cancers would be warranted in different ethnic populations.

REFERENCES


Functional Haplotypes in the Promoter of Matrix Metalloproteinase-2 Predict Risk of the Occurrence and Metastasis of Esophageal Cancer

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