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 electroforetic 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→T haplotype has 7-fold increased luciferase expression and 3.7-fold increased MMP-2 mRNA levels in esophageal tissues compared with the T-1306→T-735 haplotype. A case-control analysis revealed a 1.52-fold (95% CI = 1.17–1.96) and 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 carcinomas and C-1306 or C-735 allele containing haplotypes, with the risk being highest for the C-1306C→C-735C haplotype compared with the T-1306T→T-735 haplotype (OR = 6.53; 95% CI = 2.78–15.33). The C-1306C→C-735C 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→C-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, 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 allele MMP-2 promoter was significantly greater than reporter gene expression driven by the T allele 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 −1306C/T site were determined by PCR-based denaturing high-performance liquid chromatography analysis as described previously (16, 17). MMP-2 genotypes at the −735C/T site were analyzed by PCR-based restriction fragment length polymorphism methods (15). The primers used for amplifying DNA containing the −735C/T site were 5′-ATAAGGTTAACCTCCCCACTT-3′ and 5′-GGTAAATTAGGGCCAGGACCTG-3′, which produce a 300-bp fragment. Digestion of the PCR product with HinFl (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 HinFl 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. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated by unconditional logistic regression as a measure of association with risk of the development and metastasis of esophageal squamous cell carcinoma. All ORs were adjusted for age, sex, and pack-years smoked. Statistical analyses were performed with Statistical Analysis System software (version 6.12; SAS Institute, Cary, NC).

**Electrophoretic Mobility Shift Assay.** Synthetic double-stranded oligonucleotides 5′-GAGAATGGCCACCTCCTCTGGAGGT-3′ and 5′-GGAGATGCCGACCTCTCCTGGAGGT-3′ corresponding to the −735C or −735T sequence from the MMP-2 promoter region were labeled with [γ-32P]ATP (Amersham Pharmacia Biotech, Piscataway, NJ) according to the Gel-Shift Assay Kit protocol (Promega, Madison, WI). For each gel shift reaction, an aliquot of labeled oligonucleotide (>30,000 cpm) was incubated with 10 μg of nuclear extract from HEK293 cells for 20 minutes at room temperature in a 20-μL reaction mixture containing 40 mmol/L HEPES (pH 7.9), 12.5% glycerol, 1.0 mmol/L EDTA, 2 μg poly(dA·dT)·poly(dA·dT) (Life Technologies, Inc., Rockville, MD) as a partner DNA fragment, 100 μg/mL of BSA, and 0.2 μg of sheared salmon sperm. For competition experiments, a 50 to 100-fold molar excess of unlabeled −735C or −735T probe, a Sp1 consensus-binding site (5′-ATTGATCAGGGGCGGCAGC-3′), a Sp1 mutant consensus site (5′-ATTGATCAGTGTGGCCGAC-3′), or a non-specific oligonucleotide (5′-GAGCCGACTACTGATCGAG-3′) was preincubated for 10 minutes at room temperature with the nuclear extracts before the addition of the labeled probe. For supershift experiments, antibody against Sp1 and rabbit IgG (Upstate Biotechnology, Inc., Lake Placid, NY) was used. The antibody or rabbit IgG (2 μg) was incubated with nuclear extracts at 4°C for 30 minutes, followed by an additional incubation for 20 minutes at room temperature with a labeled Sp1 consensus-binding site or sense 35S probe. Samples were run on a nondenaturing 6% polyacrylamide gel in 0.5x Tris-borate EDTA, at 0.5 V/cm for 2 hours. The gels were dried and visualized by autoradiography using Kodak X-Omat film at −80°C.

**Construction of Reporter Plasmids.** A PCR-based approach was used to generate a construct encompassing −1691 to +10 of human MMP-2 promoter as described previously (14). The primers used for amplification of this DNA fragment were 5′-GTCAAGACTTTAAACTGACTCTGGAAAGCTCA-3′ and 5′-GTCAAGTACCCTCTGGATGGCTGACCGGAAACGAG-3′, which contain 5′- HindIII and 5′-KpnI cloning sites (italized sequences), respectively. To ensure high fidelity amplification, Pfu Turbo polymerase (Promega) was used in the PCR. The PCR product was digested with HindIII and KpnI and ligated into an appropriately digested pGL3-Basic vector (Promega). The resulting construct was designated as pT−T because sequence analysis demonstrated that it contains a T at both −1306 and −735 polymorphic sites. This pT−T product was subsequently used as a template to generate other three constructs containing a C at −1306 and/or −735 positions by using the site-specific mutagenesis, respectively, which were denominated as pC−T−pC−T−pC−C. All constructs used in this study were restriction mapped and sequenced to confirm their authenticity.

**Transient Transfections and Luciferase Assays.** HEK293 cells were grown in RPMI 1640 supplemented with 10% (v/v) heat-inactivated FCS, 2 mmol/L l-glutamine, 100 units/ml penicillin, and 100 units/ml streptomycin at 37°C and 5% CO2 in a humidified incubator. For transient transfection experiments, 5 × 103 cells were plated in 10-mm 24-multiwell plates and grown to 60 to 70% confluence. Transfection was carried out using LipofectAMINE Reagent (Life Technologies, Inc., Rockville, MD) according to the manufacturer’s protocol. Cells were cotransfected with 0.5 μg of reporter plasmid and 0.1 μg of pRL-SV40 (Luciferase Assay System; Promega); the latter was used to standardize transfection efficiency. Luciferase activity was determined according to the manufacturer’s protocol using a luciferase assay system (Promega). Briefly, cells were scraped into lysis reagent, transferred to microfuge tubes and centrifuged for 30 seconds at 12,000 × g. Luciferase activity was measured using a manual luminometer (Turner Designs; TD 20/20) by mixing 100 μL of luciferase assay reagent with 20 μL of 1:10 dilution of the lysate, and the value for each sample was recorded three times at 10-second intervals. For each plasmid construct, three independent transfection experiments were performed, and each was done in triplicate. Results were expressed as a ratio of luciferase activity to pRL-SV40. Differences were determined by t test, and P < 0.01 was considered significant.

**Reverse Transcriptase-PCR Analysis.** Fifty-two normal esophageal tissue samples 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′-TTCAGGACCCGTT-TCATTTGGGCGACAGTG-3′ and 5′-TTCAAAACTTCAAGTCCGTTAACCTCGGAG-3′, which produce a 493-bp fragment, and for β-actin were 5′-CTGGTTCGCTTCTGCGGCAACT-3′ and 5′-CTGGTTCGCTTCTGCGGCAACT-3′, which generate a 601-bp fragment. PCR was carried out 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.
RESULTS

Disruption of Sp1-Binding Site in the MMP-2 Promoter by the −735T SNP. By using the AliBaba 2 software,1 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, 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 expression

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1 Internet address: http://www.gene-regulation.com/pub/programs.html#alibaba2.
driven by the −735C allelic MMP-2 promoter was ~3-fold greater than that driven by the −735T allelic counterpart (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 −735CT and −1306CT SNPs on transcriptional activity were further assessed. Interestingly, the expression levels of reporter gene driven by the pT−T, pT−C, pC−T, and pC−C 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 individual esophageal tissues (Fig. 4). MMP-2 mRNA levels were significantly lower in individuals with the −1306CT or TT genotype than that 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−735T 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

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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></td>
<td>No. (%)</td>
<td>No. (%)</td>
<td></td>
</tr>
<tr>
<td><strong>MMP-2 −1306CT</strong></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><strong>MMP-2 −735CT</strong></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>
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</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>
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</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.
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).

**Haplotype 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 ($\chi^2 = 26.63, P < 0.0001, df = 3$). Compared with the T−1306−C−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 dissection 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 ($P < 0.001$).

**Haplotype 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 frequencies 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>T−1306−C−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−T−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−1306−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.
† P <0.0001, test for trend.

**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/T 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 genes 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/T and −1306CT/T 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
many cancer types, additional studies on other types of common cancers would be warranted in different ethnic populations.

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

Chunyuan Yu, Yifeng Zhou, Xiaoping Miao, et al.

Cancer Res 2004;64:7622-7628.