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Matrix metalloproteinase variants associated with risk and clinical outcome of esophageal cancer

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ABSTRACT. We conducted a case-control study to investigate the role of matrix metalloproteinase (*MMP) 2, MMP3,* and *MMP9* single nucleotide polymorphisms on susceptibility to esophageal squamous cell carcinoma (ESCC) in a Chinese population, and their association with environmental factors. A total of 226 patients with ESCC, and 226 age- and gender-matched healthy controls were enrolled in this study. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis was carried out on *MMP2* -1306 C>T (rs243865), *MMP3* -1171 5A>6A (rs3025058), and *MMP9* -1562 C>T (rs3918242) genotypes. Unconditional regression analysis showed that individuals carrying the *MMP2* -1306 TT genotype had a decreased incidence of ESCC compared to those with the CC genotype [odds ratio (OR) = 0.32; 95% confidence interval (CI), 0.10-0.89, P value = 0.02]. Moreover, *MMP9* -1562 CC carriers were associated with an increased ESCC risk compared to those with the TT genotype (OR = 2.71; 95%CI, 1.04-7.87, P value = 0.02). In the Cox proportional hazards model, after adjusting for potential confounding factors, patients carrying the *MMP9* -1562 CC genotype had a significantly increased risk of death from ESCC (hazard ratio = 2.97; 95%CI, 1.25-6.87, P value = 0.005).

In conclusion, this study showed that the *MMP2* -1306 TT and *MMP9* -1562 CC genotypes were associated with increased ESCC, and patients carrying the *MMP9* -1562 CC genotype had a significantly increased risk of death from ESCC.

Key words: Esophageal squamous cell carcinoma; Polymorphism; Matrix metalloproteinases

INTRODUCTION

Esophageal squamous cell carcinoma (ESCC), which occurs at a very high frequency in China, is one of the most common fatal cancers worldwide and the number of deaths is increasing (Thallinger et al., 2012; Shang and Wang, 2013; Lin et al., 2013). Surgical resection is a standard treatment for ESCC patients but its use depends on the stage and development of the tumor. While multi-therapeutic strategies such as chemo- and radiotherapy are employed to tackle ESCC, the overall 5-year survival rate remains low at 10-30% (Stoner et al., 2007; Nakajima and Kato, 2013). In addition, tumor resistance to radio- and chemotherapy has also been reported (Xie et al., 2009).

Therefore, there is an urgent need to find new therapeutic methods to improve the clinical treatment of ESCC patients. It has been demonstrated that some pathogenic factors such as cancer-related genes are associated with the progression and development of ESCC. The matrix metalloproteinases (MMPs) are a family of zinc-dependent proteolytic enzymes capable of degrading the extracellular matrix. MMPs play a key role in the physiological degradation of the extracellular matrix in angiogenesis, tissue repair, and tissue morphogenesis (Freije et al., 2003). They also regulate cell growth and inflammation by cleaving non-matrix proteins such as growth factors, cytokines and chemokines, and their respective receptors. Previous studies show that MMPs play a critical role in the invasion and metastasis of most malignancies (Stetler-Stevenson et al., 1993; Nagase and Woessner, 1999; Stetler-Stevenson and Yu, 2001; Freije et al., 2003). MMP2, MMP3, and MMP9 are three important members of the MMP family, and previous studies reported that polymorphisms of MMP2, MMP3, and MMP9 were associated with the risk and prognosis of several cancers such as bladder, gastric, breast, and oral cancer, as well as osteosarcoma (Pereira et al., 2012; Saeed et al., 2013; Yang et al., 2014; Wieczorek et al., 2014; Yan et al., 2014; Wen et al., 2014). Furthermore, there have been several reports on the association between MMP2, MMP3, and MMP9 polymorphisms, and the development and prognosis of ESCC, but the results are inconsistent (Zhang et al., 2005; Wu et al., 2008; Bradbury et al., 2009; Peng et al., 2010; Li et al., 2013). The discrepancy between these results may be caused by variable genetic distributions in different populations, or dissimilar study designs and sample sizes.

Therefore, we conducted a case-control study to investigate the role of MMP2, MMP3, and MMP9 single nucleotide polymorphisms (SNPs) on susceptibility to ESCC in a Chinese population, and their association with environmental factors.

MATERIAL AND METHODS

Population

A hospital-based case-control study was conducted. A total of 226 ESCC patients

and 226 age- and gender-matched healthy controls from the First Affiliated Hospital of Xi'an Jiaotong University were enrolled into our study, between January 2008 and December 2010. ESCC patients were newly diagnosed and histopathologically confirmed independently by two pathologists. Patients who had secondary or recurrent tumors or a history of other malignant neoplasms, and inadequate organ function were excluded from the case group.

Age- and sex-matched controls (N = 226) were selected from subjects who came to our hospital for a health check-up. Controls who had a history of cancer were excluded. Informed consent was obtained from all cases and control subjects when they agreed to participate in our study. The study protocol was approved by the ethics committee of the First Affiliated Hospital of Xi'an Jiaotong University.

Demographic and related lifestyle factors such as gender, age, dietary habits, alcohol consumption, tobacco smoking, and family history of cancer were collected by a face-to-face questionnaire. These were completed by trained interviewers who were not aware of the study hypothesis.

Smoking status was divided into non-smokers and smokers. The latter were defined as those who smoked more than one cigarette/pipe per day for at least 50% of the year. Alcohol drinking status was divided into non-drinkers, and drinkers who consumed 50 g alcohol (200 mL beer, 100 mL wine, and 50 mL spirits) per week for at least 50% of the year. Family history referred to first- or second-degree relatives such as parents, grandparents, siblings, and offspring.

All patients were followed up until December 30, 2012, with a median follow-up time of 35.6 months (range, 2-60 months). All patients were followed up by telephone every four weeks until death or the end of study. Overall survival (OS) was calculated from the date of enrolling in this study to the date of death or last clinical follow-up.

Genotyping

A 5-mL venous blood sample was collected from each subject following interview. The blood samples were kept at -20°C until needed, with 0.5 mg/mL ethylenediaminetetraacetic acid used as an anticoagulant. Genomic DNA was extracted from whole blood using a TIANamp blood DNA kit (Tiangen Biotech; Beijing, China) according to manufacturer instructions.

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis was carried out on *MMP2* -1306 C>T (rs243865), *MMP3* -1171 5A>6A (rs3025058), and *MMP9* -1562 C>T (rs3918242) genotypes. PCR primers for *MMP2* -1306 C>T (rs243865), *MMP3* -1171 5A>6A (rs3025058), and *MMP9* -1562 C>T (rs3918242) were designed using the Primer 5.0 software according to the manufacturer instructions. Primer pairs for *MMP2* -1306 C>T (rs243865) were 5'-CTGACCCCCAGTCCTATCTGCC-3' and 5'-TGTTGGGAACGC CTGACTTCAG-3'; for *MMP3* -1171 5A>6A (rs3025058) were 5' ACGTTGGATGGTCCTC ATATCAATGTGGCC-3' and 5'-ACGTTGGATGCTATGGTTCTCCATTCCTTTG-3'; and for *MMP9* -1562 C>T (rs3918242) were 5'-GCCTGGCACATAGTAGGCCC-3' and 5'-TTCCTAGCCAGCCGGCATC-3'.

PCR amplification conditions were as follows: an initial denaturation for 5 min at 95°C; followed by 35 step cycles of denaturation at 95°C for 30 s, annealing at 62°C for 30 s, and extension at 72°C for 30 s; followed by a final extension at 72°C for 10 min. PCR products were verified on a 2% agarose gel stained with ethidium bromide and observed under ultraviolet light. The results were confirmed by sequencing the PCR products using an automated sequencing system. For quality control, 10% of the cases and controls were randomly selected to be genotyped again by different investigators and 100% reproducibility was obtained.

Statistical analysis

All analyses were performed with the SPSS Version 16.0 software (SPSS Inc., Chicago, IL, USA). Continuous variables are reported as means ± standard deviation (SD), while categorical variables are shown as frequencies and percentages. Demographic and clinical variables were compared across genotypes using the chi-square test. The association between genetic polymorphisms and the risk of ESCC was estimated by a conditional multiple logistical regression model, and the results are expressed as odds ratios (ORs) and 95% confidence intervals (95%CIs). The OR (95%CI) was adjusted for potential risk factors such as gender, age, smoking and drinking status, and family history of cancer. The association between *MMP7* and *MMP9* mRNA and overall survival of ESCC was estimated using Cox's proportional hazard model; the most frequent genotype was used as the reference group. Statistical significance was defined as a two-sided P value < 0.05.

RESULTS

The characteristics of the ESCC cancer patients and controls are shown in Table 1. There was no significant difference between the cases and controls in terms of gender and age. When ESCC patients and healthy controls were compared in terms of demographic characteristics, ESCC patients were more likely to be older, have a smoking and drinking habit, and have a family history of cancer (P < 0.05; Table 1).

Table 1. Demographic and clinical characteristics of esophageal squamous cell carcinoma patients and controls.

Characteristics	Patients N = 226	%	Controls N = 226	%	χ^2-test	P value
Age (years)						
<50	48	21.24	51	22.57		
50-64	130	57.52	131	57.96		
≥50	48	21.24	44	19.47	0.28	0.89
Gender						
Female	138	61.06	144	63.72		
Male	88	38.94	82	36.28	0.34	0.56
Cancer history in first-degree relatives						
No	209	92.48	225	99.56		
Yes	17	7.52	1	0.44	14.81	<0.001
Alcohol drinkers, N (%)						
Never	87	38.5	141	62.39		
Ever	139	61.5	85	37.61	25.81	<0.001
Cigarette smokers, N (%)	120	53.1	78	34.51		
Never	106	46.9	148	65.49		
Tumor TNM stage						
IIA or less	73	32.5				
IIB or more	153	67.5				
Concurrent chemoradiotherapy						
Without taxane	161	71.3				
With taxane	65	28.7				
Operation method						
Tri-incision	188	83.1				
Thoracoabdominal incision	38	16.9				

In our study population, the genotype and allele distributions of *MMP2* -1306 C>T, *MMP3* -1171 5A>6A, and *MMP9* -1562 C>T in the controls were in line with Hardy-Weinberg equilibrium. The genotype and allele distributions of the three SNPs in the ESCC cases and control subjects are shown in Table 2. Unconditional regression analysis showed that individu-

als carrying the *MMP2* -1306 TT genotype had a decreased incidence of ESCC compared to those with the CC genotype (OR = 0.32; 95%CI, 0.10-0.89, P value = 0.02). Moreover, *MMP9* -1562 CC carriers were associated with an increased ESCC risk compared to those with the TT genotype (OR = 2.71; 95%CI, 1.04-7.87, P value = 0.02).

Table 2. Genotype distributions of *MMP2* -1306 C>T, *MMP3* -1171 5A>6A, and *MMP9* -1562 C>T in esophageal squamous cell carcinoma patients and controls.

Gene	Patients N = 226	%	Controls N = 226	%	OR (95%CI)[1]	P value
MMP2 -1306 C>T						
CC	136	60.18	136	60.3	1.0 (Ref.)	-
CT	73	32.30	73	32.4	0.88 (0.58-1.34)	0.54
TT	17	7.52	17	7.3	0.32 (0.10-0.89)	0.02
MMP3 -1171 5A>6A						
5A/5A	163	72.3	168	74.5	1.0 (Ref.)	-
5A/6A	37	16.4	35	15.3	1.09 (0.63-1.88)	0.74
6A/6A	26	11.3	23	10.2	1.17 (0.61-2.23)	0.62
MMP9 -1562 C>T						
TT	164	72.5	173	76.4	1.0 (Ref.)	-
CT	44	19.4	46	20.5	1.01 (0.62-1.65)	0.96
CC	18	8.1	7	3.1	2.71 (1.04-7.87)	0.02

[1]Adjusted for gender, age, alcohol drinking, and cigarette smoking. OR, odds ratio; 95%CI, 95% confidence interval.

We analyzed the effect of *MMP2* -1306 C>T, *MMP3* -1171 5A>6A, and *MMP9* -1562 C>T on the overall survival of ESCC patients (Table 3). During the follow-up period, 74 patients (32.74%) died. In the Cox proportional hazards model, after adjusting for potential confounding factors, patients carrying the *MMP9* -1562 CC genotype had a significantly increased risk of death from ESCC (hazard ratio = 2.97; 95%CI%, 1.25-6.87, P value = 0.005, Figure 1). However, we observed no significant association between the *MMP2* -1306 C>T and *MMP3* -1171 5A>6A polymorphisms, and overall survival of ESCC patients.

Table 3. Effect of *MMP2* -1306 C>T, *MMP3* -1171 5A>6A, and *MMP9* -1562 C>T on the overall survival of esophageal squamous cell carcinoma (ESCC) patients.

SNPs	ESCC	%	Death N = 74	%	5-year survival rate (%)	HR (95%CI)	P value
MMP2 -1306 C>T							
CC	136	60.3	42	56.4	69.12	1.0 (Ref.)	-
CT	73	32.4	25	34.5	64.38	1.15 (0.63-2.10)	0.62
TT	17	7.3	7	9.1	58.82	1.33 (0.44-3.67)	0.55
MMP3 -1171 5A>6A							
5A/5A	163	72.3	52	70.3	68.10	1.0 (Ref.)	-
5A/6A	37	16.4	13	16.9	64.86	1.10 (0.50-2.32)	0.79
6A/6A	26	11.3	9	12.8	65.38	1.09 (0.42-2.58)	0.85
MMP9 -1562 C>T							
TT	164	72.5	43	58.7	73.78	1.0 (Ref.)	-
CT	44	19.4	17	22.6	61.36	1.47 (0.72-2.94)	0.24
CC	18	8.1	14	18.7	22.22	2.97 (1.25-6.87)	0.005

HR, hazard ratio; 95%CI, 95% confidence interval.

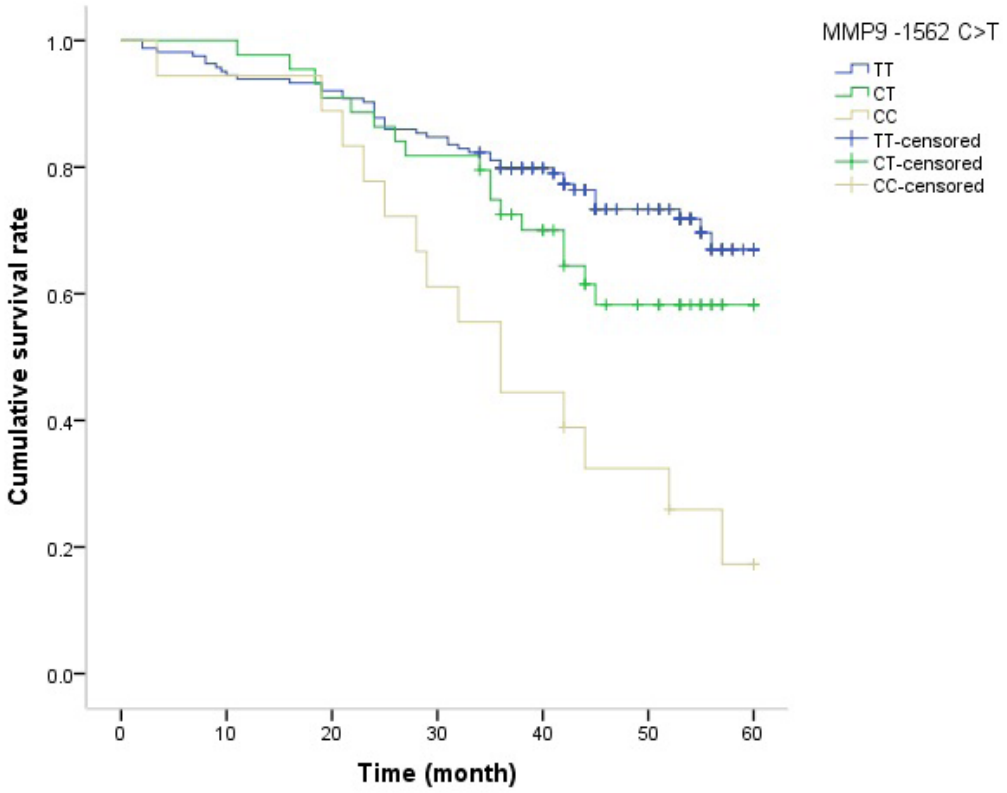

Figure 1. Kaplan-Meier analysis of overall survival for *MMP9* -1562 C>T.

DISCUSSION

Identification of the genes involved in the genetic predisposition or progression of cancer has an important role in clinical practice and basic medicine research. In this hospital-based case-control study of ESCC, we investigated the association of *MMP2* -1306 C>T, *MMP3* -1171 5A>6A, and *MMP9* -1562 C>T polymorphisms with the development and prognosis of ESCC in a Chinese population. Our multivariate logistic analysis showed that the *MMP2* -1306 TT and *MMP9* -1562 CC genotypes were associated with increased ESCC, and patients carrying the *MMP9* -1562 CC genotype had a significantly increased risk of death from ESCC.

Our study found that the *MMP2* -1306 C>T polymorphism is associated with the development of ESCC. It is well known that *MMP2* -1306 C>T contains a C>T transition at the -1306 position upstream of the transcriptional site, and this polymorphism can abolish the SpI-binding site and downregulate transcriptional activity. Previous studies reported that the *MMP2* -1306 T allele was associated with lower expression of the *MMP2* gene compared with the C allele (Price et al., 2001). A previous meta-analysis indicated that the *MMP2* -1306 CT and TT genotypes were associated with a decreased risk of ESCC compared with the CC genotype (Zhang et al., 2013). Furthermore, several studies reported that overexpression of

MMP2 was associated with the development and aggressiveness of several cancers such as gastric, bladder, lung, and head and neck cancers (Zhang et al., 2013; Hu et al., 2013; Yan et al., 2014; Yang et al., 2014). Three studies reported the association between the *MMP2* -1306 C>T polymorphism and risk of ESCC (Lin et al., 2004; O-Charoenrat and Khantapura, 2006; Zhou et al., 2007). Lin et al. (2004) conducted a study in Taiwan, and showed that subjects carrying the CT or TT genotypes had a decreased risk of developing ESCC compared with those having the CC genotype. O-Charoenrat and Khantapura (2006) reported that *MMP2* -1306 CT and TT genotypes were associated with a decreased risk of ESCC, and were also correlated with adverse clinicopathological variables. Our findings confirmed those of previous studies (Lin et al., 2004; Zhou et al., 2007).

The *MMP9* -1562 C>T polymorphism is located 1562 bp upstream of the transcriptional start site and contains either C or T, and this allele variation could influence the transcriptional activity of the *MMP9* gene. A previous study conducted transient transfections and DNA-protein interaction assays, and found that the T allele of *MMP9* -1562 C>T had higher promoter activity compared with the C allele due to the binding of a transcriptional repressor (Zhang et al., 1999). Several previous studies found that the *MMP9* -1562 C>T polymorphism was associated with an increased risk of lung and bladder cancer and ESCC (Vairaktaris et al., 2008; Lei et al., 2009; Wieczorek et al., 2013). Lei et al. (2009) reported a meta-analysis of eight case-control studies, and discovered that the *MMP9* -1562 C>T polymorphism was associated with lung cancer risk. Wieczorek et al. (2013) reported that the *MMP9*-1562 T allele was linked with increased bladder cancer risk. One study reported that the *MMP9*-1562 C>T polymorphism was associated with increased risk of ESCC, and conversely, another report demonstrated no association (Zhou et al., 2007). The discrepancy between these results may be explained by differences in ethnicities, sources of control subjects, sample size, and also chance. Further studies are needed to confirm the association between the *MMP9* -1562 C>T polymorphism and ESCC risk.

In our study, we found that the *MMP9* -1562 C>T polymorphism is correlated with ESCC prognosis. Previous studies also reported that the -634 G/C polymorphism was associated with the clinical outcome of colorectal cancer and non-small cell lung cancer (Rollin et al., 2007; Langers et al., 2012). One previous meta-analysis found that overexpression of *MMP-9* is associated with a poor prognosis for ESCC (Zeng et al., 2013). Further studies are required to confirm this association.

There are several limitations in our study. First, cases and controls were selected from one hospital. Therefore, there is a risk of selection bias because the cases and controls were not a random sample of the ESCC population, and may not accurately represent all patients with ESCC. Second, due to the rarity of ESCC, only a small number of cases were selected. The relatively small sample size could limit the statistical power to find differences between the groups. Third, other genetic factors may affect the development and prognosis of ESCC, and *MMP* genes may interact with them. Therefore, further studies with large samples are needed to confirm the association between *MMP* polymorphisms and the development and prognosis of ESCC.

In summary, this study showed that the *MMP2* -1306 TT and *MMP9* -1562 CC genotypes were associated with an increased risk of ESCC, and patients carrying the *MMP9* -1562 CC genotype had a significantly increased risk of death from ESCC. This suggests that *MMP* polymorphisms are predictive markers of risk and clinical outcome for ESCC in the Chinese population.

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