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Original Article

A single nucleotide polymorphism in the matrix metalloproteinase 2 promoter is closely associated with high risk of nasopharyngeal carcinoma in Cantonese from southern China

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Abstract

Matrix metalloproteinase 2 (*MMP2*) has been shown to play an important role in several steps of cancer development. The −1306C/T polymorphism of the *MMP2* gene displays a strikingly lower promoter activity than the T allele, and the CC genotype in the *MMP2* promoter has been reported to associate with the development of several cancers. To assess the contribution of the *MMP2* −1306C/T polymorphism to the risk of nasopharyngeal carcinoma (NPC), we conducted a case-control study and analyzed *MMP2* genotypes in 370 patients with NPC and 390 frequency-matched controls using real-time PCR-based TaqMan allele analysis. We found that subjects with the CC genotype had an increased risk (OR = 1.55, 95% CI = 1.05−2.27) of developing NPC compared to those with the CT or TT genotypes. Furthermore, we found that the risk of NPC was markedly increased in subjects who were smokers (OR = 15.04, 95% CI = 6.65−33.99), heavy smokers who smoked ⩾20 pack-years (OR = 18.66, 95% CI = 7.67−45.38), or young (<60 years) at diagnosis (OR = 1.52, 95% CI = 1.01−2.29). Our results provide molecular epidemiological evidence that the *MMP2* −1306C/T promoter polymorphism is associated with NPC risk, and this association is especially noteworthy in heavy smokers.

Key words Matrix metalloproteinase 2 gene, polymorphism, nasopharyngeal carcinoma, smoker, epidemiology

To date, 20 human matrix metalloproteinases (MMPs) have been identified. MMPs degrade a range of extracellular matrix proteins and are implicated in connective tissue destruction and remodeling associated with cancer invasion, metastasis, and cartilage destruction in arthritis[1-3]. Therefore, MMPs were initially believed to be primarily involved in tumor invasion, blood vessel penetration, and metastasis through breakdown of physical barriers[4-6]. Recent work has suggested that, in addition to the historically considered features of promoting invasion and metastasis, MMPs may also be important for multiple steps of cancer development[7,8]. Naturally occurring genetic polymorphisms have been shown to have allele-specific effects on transcription of the *MMP* gene promoters and to be associated with susceptibility to cancers[9-14].

The *MMP2* gene, which maps to 16q13, is 17-kb long and has 13 exons varying in size from 110 to 901 bp and 12 introns ranging in size from 175 to 4350 bp. MMP-2 is secreted as a pro-enzyme whose cleavage leads to the production of a soluble active form. A

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naturally occurring sequence variation in the human *MMP2* gene promoter was reported [15], and this single nucleotide polymorphism (SNP) is a C/T transition at −1306 that disrupts an Sp1-type promoter site (CCACC box) and displays a strikingly lower promoter activity than does the T allele. The CC genotype in the *MMP2* promoter has been reported to associate with the development of gastric cardia adenocarcinoma [10], lung cancer[11], esophageal cancer[12], and breast cancer[16,17].

Nasopharyngeal carcinoma (NPC) is one of the most common head and neck cancers in southern China. Epstein-Barr virus infection, chromosomal alterations, genetic and environmental factors have been reported to be involved in NPC etiology[18-20]. The clinically significant characteristics of NPC include early metastasis to lymph nodes, local invasiveness, and frequent local recurrence after treatment[21]. We recently reported in a molecular epidemiological study that the *MMP2* −1306CC genotype is associated with a several-fold increased risk of lung cancer alone or through interaction with smoking exposure [11]. In this retrospective case-control study, we examined the contribution of the −1306C/T polymorphism in the *MMP2* gene on NPC risk in a large molecular epidemiological study of a southern Chinese population.

Materials and Methods

Patients and samples

A total of 370 patients with NPC and 390 healthy controls, all ethnic Cantonese in southern China, were enrolled in this case-control study. All the patients with histopathologically confirmed NPC were untreated incident cases and were consecutively recruited between February 2000 and September 2003 at the Sun Yat-sen University Cancer Center. Disease staging was performed in accordance with the 1992 Chinese TNM staging classification[22]. Population controls were cancer-free individuals living in the Guangdong (Canton) region who were selected from a community cancer screening program for early cancer detection. The selection criteria included no individual history of cancer, and frequency matching to NPC cases was by age (± 5 years), sex, smoking status, and ethnicity (Cantonese). However, because of the limited number of controls, we ultimately matched controls to NPC only by age and ethnicity (Cantonese). Overall, 385 eligible cases and 424 controls agreed to further risk factor interviews administered by a trained nurse interviewer, with the final study consisting of 370 cases (96% of eligible) and 390 controls (92% of eligible). Some subjects were excluded due to the failure of collecting blood samples from them. At recruitment, informed consent was obtained from each subject, and each participant was interviewed to solicit detailed information on demographic characteristics and lifetime history of tobacco use. Information was collected on the number of cigarettes smoked per day, the age at which the subjects started smoking, and the age at which ex-smokers stopped smoking. Smokers were considered to be current smokers if they smoked up to one year before the date of diagnosis for cases or up to the date of the interview for controls. The study included four ex-smokers, and all ex-smokers were in the light-smokers group. Because the number was so small, we recruited them into the current smokers group. This study was approved by the Hospital Review Board of the Sun Yat-sen University Cancer Center.

MMP2 genotyping

Genomic DNA was isolated from the peripheral blood of controls and NPC patients. Real-time polymerase chain reaction (PCR)−based TaqMan allele analysis was used to determine *MMP2* genotypes. Primers amplifying a 63-bp fragment of the *MMP2* promoter containing the −1306C/T site were *MMP2*-2F, 5'-AACATTCCCCATA-TTCCCCAC-3'; *MMP2*-2R, 5'-TTCTGAGCTGAGACCT-GAAGAGC-3'; *MMP2* −1306C genotype probe, 5'-VIC-AGCACTCCACCTCTT-MGB-3'; and *MMP2* −1306T genotype probe, 5'-FAM-CAGCACTCTACCTCTTT-MGB-3'. TaqMan PCR was in a 25-μL reaction mixture containing 20 ng DNA, 1× TaqMan Master Mix (ABI, USA), 5 μL (0.9 μmol/L) of each primer, and 0.2 μmol/L FAM- and VIC-labeled probes. Reaction conditions were as follows: 10 min at 95℃, followed by 40 cycles of 15 s at 92℃ and 1 min at 60℃. The *MMP2* −1306C/T allele analysis was performed by using SDS software automatically on an Applied Biosystems 7900 System, and all patients and controls were genotyped twice.

Statistical analyses

Pearson's chi-square test was used to examine differences in demographic variables, smoking status, and *MMP2* −1306C/T polymorphism genotype distribution between patients and controls. Association between the *MMP2* polymorphism and the risk of NPC was calculated by unconditional logistic regression models and estimated by odds ratios (ORs) and 95% confidence intervals (CIs). Light or heavy smokers were categorized by the 50th percentile pack-year value among controls, i.e., <20 or ⩾20 pack-years (cigarettes per day ÷ 20 × years smoked)[23]. ORs were adjusted for age, sex, and pack-years smoked. If combined risk was greater than the two independent risk factors, we considered the additive joint effects [24]. The Bonferroni correction was used for multiple comparisons. All

analyses were carried out with Statistical Analysis System software (version 6.12; SAS Institute, Cary, NC). A value of $P < 0.05$ was considered significant.

Results

Population characteristics

The distributions of age, sex, and smoking status of study subjects are shown in Table 1. No significant differences were observed between patients and controls in age distribution. The mean age was 46.3 years for the patient group and 43.0 years for the control group, and median age was 45 years for the patient group and 41 years for the control group. However, more smokers (including four ex-smokers) were present in the patient group than in the control group (68.7% vs. 28.5%, $P < 0.05$). The patient group included more males than did the control group ($P < 0.001$). Moreover, the distribution in light (< 20 pack-years) and heavy smokers (⩾20 pack-years) between the patient and the control groups was significantly different ($P < 0.05$).

MMP2 −1306C/T polymorphism and risk of NPC

The allelic frequencies were 0.89 for *MMP2* −1306C and 0.11 for −1306T among the 390 controls, and 0.92 and 0.08 for the 370 NPC patients, respectively (Table 2). The frequencies of three *MMP2* genotypes in controls were 78.5% for CC, 20.5% for CT, and 1.0% for TT, which collectively fit the Hardy-Weinberg equilibrium law ($P = 0.77$). The frequencies of the three genotypes among all patients were 84.1% for CC, 15.4% for CT, and 0.5% for TT, which were significantly different from those among controls ($P < 0.03$). Since the TT homozygote was rare in our study in both patient and controls, this genotype was combined with the CT genotype as the reference group for subsequent risk estimation using logistic regression analysis. Multivariate analysis showed that subjects carrying the CC genotype were at slightly increased risk for NPC (OR = 1.55, 95% CI = 1.05–2.27) compared to subjects carrying at least one variant T allele, suggesting that the C allele could be the risk allele.

The risk of NPC relative to the *MMP2* polymorphism was further examined by stratification according to smoking status and pack-years smoked (Table 3). NPC

Table 1. Characteristics of 370 nasopharyngeal carcinoma (NPC) patients and 390 control subjects

Variable	No. of patients (%)	No. of controls (%)	*P*
Gender			<0.001
Male	282 (76.2)	189 (48.5)	
Female	88 (23.8)	201 (51.5)	
Age (years)			0.53
<60	315 (85.1)	343 (87.9)	
⩾60	55 (14.9)	47 (12.1)	
Mean age [a]	46.3 (12.1)	43.0 (16.0)	
Smoking status			<0.001
Non-smokers	116 (31.3)	279 (71.5)	
Smokers [b]	254 (68.7)	111 (28.5)	0.002
<20 pack-years	87 (23.5)	58 (14.9)	
⩾20 pack-years	167 (45.2)	53 (13.6)	
Mean pack-years smoked	24.3 (14.3)	23.7 (14.1)	
Median pack-years smoked	20	20	

[a]The values in parentheses are standard deviation. [b]Smokers included 4 ex-smokers.

Table 2. *MMP2* genotypes in NPC patients and controls and its association with risk of NPC

Genotype	No. of patients	No. of controls	Adjusted OR (95% CI) [a]	*P*
CT + TT	59	84	1.00	
CC	311	306	1.55 (1.05–2.27)	0.027
T allele frequency	0.08	0.11		

[a]Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated by logistic regression, with the *MMP2* variant genotypes (CT + TT) as the reference group, and adjusted for age, sex, and smoking status.

risk was markedly increased in subjects who were light smokers (including four ex-smokers) (OR = 15.04, 95% CI = 6.65–33.99) and heavy smokers (OR = 18.66, 95% CI = 7.67–45.38). The ORs of single risk factors were 3.40 (95% CI = 2.63–4.40) for smokers, 2.78 (95% CI = 1.96–3.93) for light smokers, and 3.88 (95% CI = 2.87–5.24) for heavy smokers compared to non-smokers. Since the ORs for both smoking and the –1306CC genotype were greater than the sum minus 1 of the OR for smoking and the OR for the corresponding genotype, these data suggested more than additive joint effects between the genotype and smoking [24]. Thus, we observed more than additive joint effects between the *MMP2* –1306CC genotype and smoking status.

MMP2 –1306C/T polymorphism and clinical characteristics of NPC

The correlation between *MMP2* genotype and NPC risk stratified by sex and age is given in Table 4. NPC risk associated with the CC genotype was significantly higher in subjects who were young (<60 years) at diagnosis (OR = 1.52, 95% CI = 1.01–2.29). The distribution of the CC genotype showed no significant difference between males and females (Table 4), T stage, N stage, M stage, or TNM stage (Table 5).

Discussion

The relationship between the –1306C/T polymorphism in the *MMP2* gene promoter and risk of human malignancies has been documented. Populations carrying the *MMP2* –1306CC genotype have increased risk of gastric cancer [10], lung cancer [11], esophageal carcinoma [12], colorectal cancer [25], and oral cancer [26]. In this investigation, our results revealed a significant difference in the distribution of the *MMP2* allelic variant CC in NPC patients and cancer-free controls. Subjects carrying the CC genotype had a 1.55-fold increased risk for NPC. In addition, we observed more than additive joint effects between this genetic polymorphism and cigarette smoking on increased risk of NPC, with an OR of 7.49 among light smokers and 18.66 among heavy smokers with the CC genotype. This study provides

Table 3. Risk of NPC related to *MMP2* genotypes and smoking status

MMP2 genotype	Smoking status	No. of patients	No. of controls	Adjusted OR (95% CI)[a]	*P*
CT + TT	Non-smokers	15	58	1.00	
CC	Non-smokers	101	221	2.04 (1.06– 3.96)	0.03
CT + TT	Smokers	46	26	5.79 (2.25–14.91)	<0.001
CC	Smokers [b]	208	85	15.04 (6.65–33.99)	<0.001[c]
CT + TT	Pack-years <20	11	11	2.81 (0.88– 8.99)	0.06
CC	Pack-years <20	76	48	7.49 (3.16–17.78)	<0.001[c]
CT + TT	Pack-years >20	35	15	8.04 (2.77–23.32)	<0.001
CC	Pack-years >20	132	37	18.66 (7.67–45.38)	<0.001[c]

[a] ORs and 95% CIs were calculated by logistic regression, with the CT or TT genotype as the reference group, and adjusted for age and sex.
[b] Smokers included 4 ex-smokers.
[c] *P* value remained significant after Bonferroni correction.

Table 4. Association between *MMP2* polymorphisms and NPC risk stratified by sex and age

Genotype	Male				Female			
	No. of patients	No. of controls	Adjusted OR (95% CI)[a]	*P*	No. of patients	No. of controls	Adjusted OR (95% CI)[a]	*P*
CT + TT	48	42	1.00		11	42	1.00	
CC	233	147	1.36 (0.86–2.17)	0.15	78	159	1.41 (0.88–2.25)	0.08
Genotype	Age <60 years				Age ⩾60 years			
	No. of patients	No. of controls	Adjusted OR (95% CI)[b]	*P*	No. of patients	No. of controls	Adjusted OR (95% CI)[b]	*P*
CT + TT	58	80	1.00		5	8	1.00	
CC	257	263	1.52 (1.01–2.29)	0.05	50	39	1.83 (0.50–6.68)	0.36

[a]ORs and 95% CIs were calculated by logistic regression, with the *MMP2* variant genotypes (CT + TT) as the reference group, and adjusted for age and smoking status; [b]ORs and 95% CIs were calculated by logistic regression, with the *MMP2* variant genotypes (CT + TT) as the reference group, and adjusted for sex and smoking status.

Table 5. Risk of NPC related to *MMP2* genotypes and TNM stage

Group	Number[a]	Percentage (%)	Adjusted OR (95% CI)[b]	*P*
All cases	311/370	84.1	1.55 (1.05–2.27)	0.027
T stage				
T1	26/28	92.9	1.00	
T2 + T3 + T4	285/342	83.3	0.61 (0.18–2.13)	0.45
N stage				
N0	69/81	85.2	1.00	
N1 + N2 + N3	242/289	83.7	0.97 (0.48–1.97)	0.91
M stage				
M0	307/365	84.1	1.00	
M1	4/5	80.0	0.93 (0.22–1.82)	0.79
TNM				
I + II	78/89	87.6	1.00	
III + IV	233/281	82.9	0.72 (0.35–1.49)	0.35

[a] Number of patients with the CC genotype/total number of patients for each stratum.
[b] ORs and 95% CIs were calculated by logistic regression, with the CT or TT genotype as the reference group, and adjusted for age, sex, and smoking status.

substantial evidence that the *MMP2* −1306CC genotype, together with cigarette smoking, enhances NPC susceptibility and may be important for NPC development. Another study has reported that *MMP2* −1306CC is related to NPC risk in 239 patients in a Cantonese population (OR = 2.19, 95% CI = 1.21–3.96) [27]. Our results confirmed the susceptibility of *MMP2* −1306CC carriers to NPC in a larger population of 370 patients and further showed this susceptibility was more significant in subjects who were smokers, especially heavy smokers. In addition, we found that the risk of NPC was significantly increased in young (<60 years) subjects with the −1306CC genotype (OR = 1.52, 95% CI = 1.01–2.29). We also analyzed the relationship between −1306CC genotype and TNM stage but found no association between them.

The *MMP2* −1306C/T polymorphism impacts cellular function *in vivo* because the C→T transition at −1306 purportedly disrupts an Sp1-type promoter site (CCACC box), resulting in a strikingly lower promoter activity than does the T allele of the *MMP2* gene [15]. The Sp1 site, with other promoter elements such as the AP-2 site, has been shown to be necessary for regulating constitutive expression of *MMP2* [28]. Transient transfection experiments *in vitro* demonstrated that the Sp1 promoter site in the *MMP2* −1306C allele enhances the *MMP2* transcription [15], indicating that MMP-2 protein expression would be higher in individuals with the CC genotype than in those with the TT or CT genotype. Since MMP-2 and other MMPs may contribute in multiple ways to all stages of carcinogenesis[8], the increased level of this enzyme over a lifetime may increase host susceptibility to cancer development. Experimental cancer models showed that mice lacking the *MMP3*, *MMP1*, or *MMP9* genes developed fewer carcinogen-induced cancers than did wild-type mice [2,29], and transplanting *MMP9*-expressing bone marrow cells prevented development of squamous cell carcinomas in mice lacking the *MMP9* [30]. *MMP2*-deficient mice were more susceptible to colonization by cancer cells injected in lung veins than wild-type mice [31], and overexpression of MMPs in transgenic mice resulted in elevated cancer susceptibility [15,32]. Similarly, our epidemiological study showed that the *MMP2* −1306CC genotype may result in high expression of MMP-2 over a lifetime and increase NPC susceptibility.

Cigarette smoking is a major cause of a variety of malignancies including cancers of the larynx, oral cavity and pharynx, esophagus, bladder, and lung. Numerous studies have consistently shown that cigarette smoking may be an important environmental etiological factor in NPC development in China[33,34]. In this study, we found a significantly higher risk for NPC related to the *MMP2* −1306CC genotype among smokers (including four ex-smokers) (OR = 15.04) and heavy smokers (OR = 18.66). Previous studies showed that cigarette smoking-induced NF-κB activation and NF-κB–regulated gene expression in human non–small cell lung carcinoma cells was suppressed by curcumin through inhibition of NF-κB kinase [35]. NF-κB activation blocks apoptosis, promotes proliferation, and mediates tumorigenesis. Cigarette smoking also induces 5-lipoxygenase (5-LOX) expression, which is important for activation of MMP-2 and vascular endothelial growth factor (VEGF), key proteins that induce angiogenic processes and promote inflammation-associated adenoma formation in mice [5,36]. We found that the association between the *MMP2* −1306CC genotype and

NPC risk appeared to be more pronounced in young subjects (<60 years old). These results are consistent with the result of a previous study on gastric adenocarcinoma [10], which also supports the hypothesis that genetic susceptibility is often associated with an early age of disease onset. In summary, our investigation suggested that the *MMP2* −1306CC genotype and the cigarette smoking environmental factor may cooperate in increasing NPC risk, especially in a young Cantonese population.

One explanation of these findings is that, in addition to higher constitutive expression from gaining an Sp1 promoter site, the smoking inducibility of the C allele of *MMP2* may also be higher than the T allele, which lacks an Sp1 site. Given these conditions, smokers, especially heavy smokers, who carry the CC genotype are expected to be at the highest risk for developing NPC. Another explanation for a higher risk of NPC among smokers and heavy smokers with the CC genotype is that these subjects had larger numbers of transformed cells caused by tobacco carcinogens in the target tissue, consequently increasing the possibility that one of these cells will form a malignancy under the condition of higher *MMP2* expression.

Although the design of the hospital-based case-control study has potential drawbacks such as selection bias, the results in this study are unlikely to be attributable to selection bias because of the large sample size that included >90% of all eligible cases, solid and reproducible genotyping procedures, and significantly increased ORs with very small *P* values. Genotype frequencies among the control population fit the Hardy-Weinberg law, further supporting the randomness of our control selection.

Local overexpression of MMP-2 is correlated with invasion and metastasis of certain cancers, including gastro-esophageal cancers. In this study, we found no significant association between *MMP2* genotype and sex or TNM stage, suggesting that the CC genotype may not be a relevant genetic factor in inducing local overexpression of MMP-2. This study included only five cases with remote metastasis, and the smaller number of metastatic cases may have diminished the association between the *MMP2* −1306CC genotype and TNM stage of NPC. Several studies reported that functional polymorphisms in other MMP genes including *MMP1* (2G allele) and *MMP3* (5A allele) are linked to susceptibility to certain human cancers [7,13]. Other reports and our results suggest that the *MMP2* −1306C/T polymorphism might be a general, but not specific, risk factor for common cancers, further supporting the likelihood that MMPs profoundly influence early tumor initiation and development. However, the data on clinical outcomes should be considered preliminary because of our limited sample size. Additional examinations of larger patient series with more detailed clinicopathologic features and clinical outcome, especially survival rate, may be required.

Conclusions

In summary, our data provide molecular epidemiological evidence that the *MMP2* −1306C/T promoter polymorphism is associated with NPC risk. In particular, our studies show that the CC genotype is associated with increased risk of NPC in the Cantonese population from southern China. This association is especially noteworthy in young individuals and heavy smokers. These findings further support the hypothesis that *MMP2* is important in carcinogenesis and may ultimately help in identifying high-risk populations for NPC.

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