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Original article

Prognostic Significance of *MMP2* and *MMP9* Functional Promoter Single Nucleotide Polymorphisms in Head and Neck Squamous Cell Carcinoma

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Abstract

Objective(s)
Matrix metalloproteinases comprise a family of enzyme that is able to degrade components of extra cellular matrix. There are single nucleotide polymorphisms in the promoter regions of several genes with ability to influence cancer susceptibility. The aim of this study was to analyses association between *MMP2* and *MMP9* promoter polymorphisms and head and neck squamous cell carcinoma occurrence and progression.
Materials and Methods
A case- control study was performed including 80 head and neck squamous cell carcinoma patients and healthy controls for *MMP2* and 86 head and neck squamous cell carcinoma patients and 72 healthy controls for *MMP9*. Blood samples were genotyped for *MMP2* and *MMP9* using polymerization chain reaction–restriction fragment length polymorphism method (PCR-RFLP). Statistical analysis was performed using SPSS 12.0 software.
Results
Our results showed that distribution of *MMP2* genotype between controls and patients was significantly different (χ^2= 10.3, *P*= 0.005). Comparison between CC genotype in HNSCC patients and controls showed that C allele modified the risk of HNSCC progression (OR= 2.6, 95% CI, 1.0046–6.729). The *MMP9* genotype distribution among HNSCC patients was significantly different (χ^2= 14.56, *P*= 0.0007). The frequency of TT genotype in HNSCC patients was different from healthy controls and was more common genotype in HNSCC cases (OR= 2.18, 95% CI, 0.7052–6.7854).
Conclusion
Our results suggested an association of the *MMP2* and *MMP9* SNP with the development of HNSCC. Also, our results showed that *MMP, MMP9* genotypes and smoking were related to HNSCC progression.

Keywords: Extra cellular matrix, Head and neck squamous cell carcinoma, Matrix metalloproteinase

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Introduction

The matrix metalloproteinases (MMPs) constitute a family of proteases that can selectively degrade a wide spectrum of both extracellular matrix and non matrix proteins (1). At least twenty six MMPs have been identified, which are classified according to their substrate specificity and structural similarities as collagenases, stromelysins, gelatinases, and membrane-type MMPs (2, 3). With regard to the cancers, MMPs can regulate the tumor microenvironment, and their expression and activation are increased in almost all human cancers compared with normal tissue. Originally, MMPs were considered to be important almost exclusively in invasion and metastasis; however, some studies document that MMPs are involved in several steps of cancer development, including cancer-cell growth, differentiation, apoptosis, migration, invasion, and in the regulation of tumor angiogenesis and immune surveillance (1, 4). On the basis of the above functional relevance of MMPs in the pathogenesis of cancers, we hypothesize that MMPs may be the excellent biologic candidate susceptibe genes for cancers. Expressions of most MMPs are normaly low in tissues and induced when remodeling of the ECM is required (5). Structural and functional analyses of the promoter regions from a number of MMP genes have provided a better understanding of the mechanisms that regulate their expression. These studies show that the expression of these genes is affected by single nucleotide polymorphisms (SNPs) in their promoters (6). One of these SNPs identified in *MMP1* promoter creates an ETS binding site for transcription factors and stimulates several cancers (7). Additional SNPs influencing cancer susceptibility have also been reported in the promoter of other MMPs such as *MMP2* and *MMP9* (8, 9).

MMP2 classified as gelatinase A. This gene is localized on 16q13. The gene is 17 kb long with 13 exons varying in size from 110 to 901 bp and 12 introns ranging from 175 to 4350 bp. Alignment of introns showed that introns 1-4 and 8-12 of the *MMP2* gene coincide with intron location in the *MMP1* and *MMP3* genes, indicating a close structural relationship of these metalloproteinase genes (10). Yet, 6 polymorphisms were identified in the promoter of *MMP2* gene, 1 in the 5' un-translated region, 6 in the coding region, 1 in the intronic sequence and 10 in the 3' un-translated region (11). However, this study is about -735C/T promoter polymorphism. Bioinformatics analysis suggests that the -735 C/T polymorphism might disrupt a consensus sequence for Sp1- binding site, implying that this polymorphism might have the potential to alter *MMP2* transcription (12).

The promoter region of *MMP9* also called gelatinase B contains a C/T single nucleotide polymorphism at position -1562. The T allele of *MMP9* C-1562T polymorphism is associated with higher level of gene expression. This may be because the T allele has a lower binding affinity for a repressor of transcription (13).

Previous studies were about the relationship between polymorphism mentioned above in *MMP2* and *MMP9* and various cancers such as lung (13), breast (14), Nasopharyngeal Carcinoma (3) and HNSCC (15), but there aren't any report about these polymorphisms in Iran. Therefore, because of mortality and morbidity of HNSCC in the world and many malignancies associated with it (16, 17), we conducted a case - control study in Fars province population in Iran to assess the effects of these polymorphisms on the development and clinical staging of this tumor.

Materials and Methods

Subjects

MMP2 gene promoter sequence was obtained from 80 patients with HNSCC and healthy controls. *MMP9* gene promoter sequence was obtained from 86 patients with HNSCC and 72 controls from south of Iran. Cases were collected from Fars Cancer Research Institute. Controls were healthy volunteers and age matched. Information on sex, age, smoking habit and family history was obtained from cancer patients and healthy controls. For smoking habit, former and present smoking status, the number of cigarettes smoked per day and the time of starting and quitting were determined. Individuals who formerly or currently smoked 5 cigarettes/day for at least 2 years were defined as smokers.

The list of clinical and histological characteristics of HNSCC is reported in Tables 1 and 2.

DNA extraction

Three milliliters of venous blood from each

subject was drown into vacutainer tubes containing EDTA and stored at 4 °C. Genomic DNA was extracted within 1 week after sampling using DNA Isolation kit for Mammalian Blood (Roche diagnostics, GmbH, Mannheim, Germany).

MMP2 promoter SNP genotyping

The *MMP2* polymorphism was detected by PCR using forward:
5′-ATAGGGTAACCTCCCCACATT-3′
and reverse:
5′-GGTAAAATGAGGCTGAGACCTG-3′
primers. PCR was performed in a 25 µl volume containing 100 ng of DNA template, 2.5 µl 10X PCR buffer, 0.4 µl $MgCl_2$ (100 mM), 1U of *Taq* DNA polymerase (BIORON), 0.5 µl dNTPs (10mM) and 0.2 µl of forward and reverse primer (1 mM). The PCR cycling conditions were in first cycle 5 min at 95 °C, 45 sec at 58.5 °C, 45 sec at 72 °C, followed for 35 cycle 45 sec at 95 °C, 45 sec at 58.5 °C, 45 sec at 72 °C and one cycle with 45 sec at 95 °C, 45 sec at 58.5 °C and final extension was 7 min at 72 °C.

An 8 µl PCR product was digested overnight at 37 °C in a 10 µl reaction containing 5 U of *Hinf*I (G/ANTC, BIORON) and 1X reaction buffer. The CC genotype was detected on a 2% agarose gel electrophoresis stained with ethidium bromide by the band with size 300 bp, TT genotype with 245 and 46bp bands and CT genotype with 300, 245 and 46 bp bands.

MMP9 promoter SNP genotyping

The *MMP9* genotype was determined by PCR-RFLP assay. The PCR primers used to amplify the *MMP9* polymorphism were 5′-GCCTGGCACATAGTAGGCCC–3′ (forward primer) and 5′-CTTCCTAGCCAGCCGGCATC -3′ (reverse primer). PCR performed in conditions like others. The PCR cycling conditions were 5 min at 94 °C followed by 35 cycles of 30 sec at 94 °C, 30 sec at 61 °C and 30 sec at 72 °C, with a final step at 72 °C for 5 min to allow for complete extension of all PCR fragments. An 8 µl PCR product was digested overnight at 37 °C in a 10 µl reaction containing 5 U of *Sph*1 (GCATG/C, BIORON) and 1X reaction buffer. The products were separated on a 3% agarose gel stained with ethidium bromide. As a result, the CC genotype was represented by DNA bands 0f 435 bp and TT genotype by a DNA band of 247 bp and 188 bp, the heterozygote displayed by a DNA band of 435, 247 and 188 bp (Figure 1).

Statistical analysis

Statistical analysis was performed using SPSS12.0 software package. Hardy-Weinberg analysis was performed to compare the observed and expected genotype frequencies using χ^2 test. Comparison of the *MMP2* and *MMP9* genotypes distribution in the

Table 1. Selected characteristics of *MMP2* in HNSCC patients and healthy controls.

Groups	Control		HNSCC		
MMP2	n= 80	%	n= 80	%	*P* value
Sex					
Male	32	40	72	90	0.421
Female	48	60	8	10	
Smoking					
Miss	30	37.5	8	10	0.000
Available	80		80		
Smoker	22	27.5	52	65	
Non smoker	28	35	20	25	
Staging					
I			8	10	0.915
II			6	7.5	
III			32	40	
IV			14	17.5	
Miss			20	25	
Tumor size					
≥ 2 cm			28	35	0.835
<2 cm			8	10	
Miss			44	55	

HNSCC, head and neck squamous cell carcinoma, *P* value for χ^2 test.

Table 2. Selected characteristics of *MMP9* in HNSCC patients and healthy controls.

Groups	Control		HNSCC		
MMP9	n= 86	%	n= 72	%	*P* value
Sex					
Male	30	34.88	54	75	0.841
Female	56	65.11	18	25	
Smoking					
Miss	36	41.8	6	8.3	0.007
Available	86		72		
Smoker	28	32.5	42	58.3	
Non smoker	22	25.5	24	33.3	
Staging					
I			12	16.6	
II			10	13.8	0.980
III			32	44.4	
IV			14	19.4	
Miss			4	5.5	
Tumor size					
≥ 2 cm			40	55.5	0.848
<2 cm			14	19.4	
Miss			18	25	

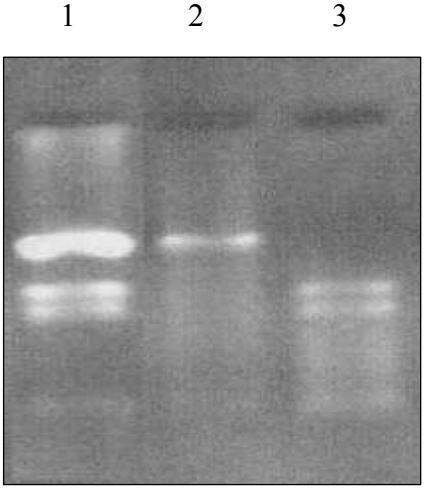

Figure 1. Agarose gel electrophoresis of the *MMP*-9 promoter PCR products and digested PCR fragments. Lane 1. C/T heterozygote's, lane 2. CC homozygote's and lane 3. homozygote for TT genotype.

study groups was using χ^2 test. Comparison of the MMP 2 and *MMP9* genotypes distribution in the study groups was performed by means of two – sided contingency table using the χ^2 test. The odds ratio (OR) and 95% confidence interval (CI) were calculated using an unconditional regression model and adjusted by age and sex accordingly. A probability level of 5% was considered significant.

Results

MMP2 promoter SNP with susceptibility to HNSCC

At the time of HNSCC diagnosis, the mean age of patients was 59.00±11.4 years (range 13-85) and controls were age matched to the cases (57.00±10.2 years). Overall 90% of patients were men compared with 40% in controls (χ^2 = 1.72, *P*= 0.42). The *MMP2* genotyping was performed in 80 patients and controls. Genotype distribution didn't deviate from Hardy Weinberg equilibrium (χ^2= 5.6, *P*= 0.06). *MMP2* genotype and allele frequencies were not significantly different between patients and controls (χ^2_g= 10.3, P_g= 0.005 and χ^2_a= 5.9, P_a= 0.01, Table 3). The frequency of C allele in HNSCC patients was more common among controls (32.50 Vs 21.25). Comparison between CC genotype and CC + CT genotypes in HNSCC patients and controls showed that C allele modified the risk of HNSCC progression (OR= 2.6, 95% CI, 1.0046–6.729, OR= 1.85, 95% CI, 1.0524–3.277, Table 3). The smoker individuals were common in patients rather than controls (65 Vs 27.5, χ^2= 54, *P*= 0.0). Respectively, most of the smokers in HNSCC patients were with CC or CT genotypes (OR =4.9, 95% CI, 0.4877–49.2287, OR= 3.7, 95% CI, 0.7795–17.8801). All cases with available information were with malignant tumor. Most of the HNSCC patients with CC genotype were with tumor size greater than 2 centimeters (OR_{CC}= 1.75, 95% CI, 0.0844 – 36.2889) and most of them were with

Table 3. The genotype and allelotype frequencies of *MMP2* in HNSCC patients and healthy controls.

MMP2	Control		HNSCC		
	n= 80	%	n= 80	%	*P* value
MMP2 genotype					
CC	6	7.5	12	15	0.005
CT	22	27.5	28	35	
TT	52	65	40	50	
MMP2 allelotype					
C	17.0	21.25	26.0	32.5	
T	63.0	78.75	54.0	67.5	0.01

OR= 2.6, 95% CI, 1.0046 – 6.729.

Table 4. The genotype and allelotype frequencies of *MMP9* in HNSCC patients and healthy controls.

MMP9	Control		HNSCC		
	n= 86	%	n= 72	%	*P* value
MMP9 genotype					
CC	30	35	29	40	
CT	52	60	34	47.5	0.0007
TT	4	5	9	12.5	
MMP9 allelotype					
C	56.0	65	46.0	63.75	
T	30.0	35	26.0	36.25	0.7

OR= 2.18, 95% CI, 0.7052 – 6.7854.

metastasis. The frequency of invasive tumors with CC genotype or at least with one C allele was common in HNSCC patients (OR_{CC}= 2.4, 95% CI, 0.2911– 19.7852 and OR_{CT}= 2.16, 95% CI, 0.2989–15.7059) also, most of them were with high stage in their tumors (OR_{CC}= 2.88, 95% CI, 0.3247–25.7027 and OR_{CT+CC}= 2.66, 95% CI, 0.3467–20.5092). The mean age of onset of HNSCC initiation was lower in patients with CC genotype (52.85 Vs 58.45).

MMP9 promoter SNP with susceptibility to HNSCC

The mean age of HNSCC patients was 56.2±12.33 (range 19 to 76) at diagnostic time and controls was 57±10.2. The gender distribution between cases and controls was different, 75% men in HNSCC patients compared with 34.88% in controls. The *MMP9* gene promoter was genotyped in 72 HNSCC patients and 86 controls. The genotype distribution among HNSCC patients and healthy control didn't deviate from that expected by Hardy – Weinberg equilibrium (χ^2= 0.94, *P*= 0.61). The frequency of C and T alleles was 63.75 and 36.25% in HNSCC patients and 65 and 35% in healthy controls (χ^2= 0.07, *P*= 0.7). The frequency of different genotype in cases was 40% CC in patients and 35% in controls, 47.5% CT in patients and 60% in controls and 12.5% CC in patients and 5% in controls (χ^2= 14.56, *P*= 0.0007, Table 4). The frequency of TT genotype in HNSCC patients was different from healthy controls and was more common genotype in HNSCC cases (OR= 2.18, 95% CI, 0.7052–6.7854, Table 4). Almost, all of the tumors in HNSCC patients with at least one T allele were malignant. Information on smoking status from 36 healthy controls and 6 HNSCC patients was unavailable. The proportion of smokers among HNSCC patients was significantly different from healthy controls (χ^2= 7.18, *P*= 0.007). Most of the smoker individuals among HNSCC patients were with at least one T allele. Thus, in smokers TT and TC genotypes increased the risk of developing HNSCC, compared with the CC genotype (OR= 1.1, 95% CI, 0.2661-4.5469). The frequency of TT and TC genotypes was more common in HNSCC patients with III or IV stages (OR_{TT}= 2.33, 95% CI, 0.3558 and OR_{CT+TT} = 1.86, 95% CI, 0.4368-7.9781) and most of them were with tumor size greater than 2 centimeters (OR= 1.51, 95% CI, 0.1225-18.3638). According to the results of this study it is demonstrated that TT genotype

could have an important effect on the age of onset of HNSCC initiation (51.75 Vs 53.75).

Discussion

The ability of cancer cells to invade other tissues and spread to distinct organs is an often–fatal characteristic of malignant tumors. Proteolytic enzymes play a fundamental role in cancer progression providing an access for tumor cells to the vascular and lymphatic systems, which support tumor growth and constitute an escape for further dissemination (18). However, among all the proteolytic enzymes potentially associated with tumor invasion, the members of the MMP family have reached an outstanding importance due to their ability to cleave virtually any component of the ECM and basement membranes, thereby allowing cancer cells to penetrate and infiltrate the subjacent stromal matrix (6).

Many studies show association of MMP polymorphism with initiation of cancer and especially with invasion risk and metastatic activity of different types of cancer such as breast, lung, endometriosis and colorectal (2, 5, 19). Our previous studies showed the relationship of *MMP1* and *MMP3* polymorphism with invasion risk of colorectal and breast cancer in Iranian population (20-22). We also analyzed relationships between *MMP1* and *MMP3* promoters and SNPs and HNSCC cancers in Iran (not published). But the relationship between the *MMP2* and *MMP9* promoter polymorphisms and risk of the development of HNSCC has not been documented in Iran. This is the first study on the relationship between *MMP2* and *MMP9* and HNSCC patients in the Iranian population.

It has been shown that human *MMP2* promoter contains a number of cis – acting regulatory elements, and the constitutive and induced expression of this proteinase is subjected to regulation by transcription factors (3). Several polymorphisms in the *MMP2* promoter region have been identified. Among them, a C to T transition at -1306 disrupts a Sp1 type promoter motif (CCACC box) and consequently display a strikingly lower promoter activity with the T allele (11). Recently another C to T transition at -735 also destroys a Sp1 binding element, with the T allele being associated with significantly diminished promoter activity (9). In this study, we found that functional polymorphism in the promoter of *MMP2* gene, C-735 T, was significantly associated with susceptibility to HNSCC, because the frequency of C allele was more common in patients (OR= 1.85, 95% CI, 1.0524 – 3.277). To our knowledge, this is the first report of the genetic association between *MMP2* susceptibility to HNSCC, confirming this polymorphism may play a role in the pathogenesis of this malignancy. Over expression of *MMP2* has been extensively described in several forms of human cancer associated with increased cell proliferation, apoptosis, angiogenesis and immune surveillance (23). Recent study showed that *MMP1* and *MMP2* genes were up regulated in head and neck cancers (24). Local over expression of MMPs, including *MMP2*, have been shown to be extensively related to advanced tumor stage, increased invasion and metastasis and poor prognosis of certain cancer (25). In the present study all cases were with malignant tumors and most of them were with high tumor stages. Comparison between metastasis and invasiveness factors and various *MMP2* genotypes showed that C alleles were common in positive groups (OR= 2.16, 95% CI, 0.2911–19.7852). Furthermore, we found a significantly high risk for HNSCC related to the *MMP2* polymorphism and among heavy smokers. The frequency of C allele was high in smoker HNSCC patients (OR= 3.7, 95% CI, 0.7795–17.8801). These results are consistent with previous findings from studies for several cancers including gastric cardiac adenocarcinoma (8) and lung cancer (26) and further support the hypothesis that the *MMP2* polymorphisms seem to interact with tobacco smoking. There are many hypothesis about this result, one hypothesis is that a higher risk of HNSCC among heavy smokers with the CC genotype (OR= 4.9, 95% CI, 0.4877–49.2287) may attribute to the risk of occurrence of larger numbers of transformed cells caused by smoking in the target tissue, which in turn increases the possibility that one of these cells

will become malignant under the condition of higher expression of *MMP2*. Alternatively because MMPs expression can be induced by smoking (27), another suggested explanation is that in addition to higher constitutive expression because of gain of a Sp1 type promoter motif, the inducibility by smoking of the *MMP2* C allele may also be higher than that of the T allele, which loses an Sp1 binding element. Thus it would be expected that heavy smokers, who carried the -735 CC genotype were more susceptible to develop HNSCC.

The T allele of the *MMP9* C-1562T polymorphism was associated with higher levels of gene expression in a reporter gene assay and this may be because the T allele has a lower binding affinity for a repressor of transcription (13). Puokolainen *et al*, in study about the expression of various proteins in HNSCC with immune staining method, noticed that in positive cases, the immunoreactive protein was prominent in cancer cells (15). Over expression of *MMP9* immunoreactive protein was detected in 82% of cases. The study didn't show any correlation between the *MMP9* positivity and the stage of disease, tumor size and lymph node metastasis. In our results, we found that there wasn't any correlation between HNSCC metastasis and *MMP9* genotyping, but almost all of the cases with III and IV stages and tumor size greater than 2 centimeters were with T allele. This is also supported by Hong *et al*, showing an association between *MMP9* and metastasis in oral squamous cell carcinoma, but in other studies of HNSCC, the *MMP9* mRNA or protein has failed to correlate with invasion and metastasis (28, 29). Also, in our study we found that most of the smoker patients were with TT genotype or at least one T allele, suggesting that *MMP9* over expression in TT or TC genotype may affects on HNSCC development.

The role of gelatinases in progression of neoplasia is far from clear. *MMP2* is more often identified as a prognostic factor than *MMP9*. In fact, the effect of *MMP9* on survival is in some way contradictory.

Scorilas *et al* have reported that *MMP9* over expression in breast cancer was associated with a favorable prognosis in node – negative patients (30). Our study suggests that *MMP9* can not be important, at least for the progression of HNSCC in Iran.

Conclusion

The discovery of new prognostic markers could promote entirely new treatment possibility, especially in adjuvant treatment. For instance, synthetic MMP inhibitor has been introduced as an option in cancer therapy. Moreover, our study promotes additional clinical investigation on MMPs.

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