

Estimation of Matrix Metalloproteinases-2 Promoter Polymorphism as a Risk Factor for Oral Carcinogenesis in Indian Population

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Abstract

Introduction: Matrix metalloproteinase-2 (MMP-2) can degrade extracellular matrix and basement membrane, and play an important role in the development and progression of multiple carcinomas, including oral squamous cell carcinoma (OSCC). A -1306 C/T polymorphism in the MMP-2 promoter disrupts Sp1-binding site, and results in reduction of transcriptional activity. This study aimed to assess the association of such genotype with the risk of OSCC in patients of Indian ethnicity. We also assessed the relative frequencies of MMP-2 promoter in patients with potentially malignant conditions: oral submucous fibrosis (OSMF) and Oral lichen planus (OLP).

Materials and Methods: Genomic DNA from the blood samples of total 100 patients comprising of OSCC (40 cases), OSMF (40 cases), OLP (20 cases) and 20 controls were amplified by polymerase chain reaction (PCR) and subjected to restriction fragment length polymorphism (RFLP) analysis for genotyping. Chi square test and unconditional logistic regression models were used for statistical analysis.

Result: We found that subjects with the CC genotype was associated with significantly increased risk for developing OSCC compared with those with the variant T allele, suggesting that the C allele could be the risk allele. Also, the frequencies of the CC genotype were significantly higher in patients with OSMF and OLP than that of the Controls.

Conclusion: This is the first paper demonstrating that functional genotype of MMP-2 promoter is a risk factor for oral carcinogenesis in Indian population.

Keywords: Oral carcinogenesis; Oral submucous fibrosis; Polymorphism

Introduction

Oral cancer holds the eighth position in the cancer incidence ranking worldwide and it is the third most common malignancy in south central Asia [1]. India has always been cited as the country with the highest incidence of oral cancer in the world. In India alone over 1,00,000 cases of oral cancer are registered every year. In high risk countries such as Sri Lanka, India, Pakistan and Bangladesh, oral cancer is the most common cancer in men, and may contribute up to 25% of all new cases of cancer [2].

Worldwide, estimates of Oral submucous fibrosis (OSMF) shows a high predilection to Indians and Southeast Asians, with overall prevalence rate in India to be about 0.2% to 0.5%. It has been suggested that ingestion of chillies, areca nut, genetic susceptibility, nutritional deficiencies, altered salivary constituents, autoimmunity and collagen disorders may be involved in the pathogenesis of this condition. The condition is well recognized for its malignant potential rate of 7.6% and is particularly associated with use of areca nut in various forms with significant duration and frequency of chewing habits [3]. Oral lichen planus (OLP) is a chronic inflammatory mucosal disease. The most important complication of OLP is the development of Oral squamous cell carcinoma (OSCC). However, the underlying mechanisms of OLP that initiate the development of OSCC in OLP patients have not been clearly established [4]. Several studies reported an annual malignant transformation risk of 0.04%-1.74% [5].

Although tobacco in any form, alcohol use and nutritional deficiency play a major role in the etiology of Oral cancer, only a fraction of exposed individuals develop the disease, suggesting a genetic susceptibility in the general population. The first degree relatives of head and neck cancer patients have a 2-fold elevated risk of developing cancer, over the general population [6]. Chronic exposure to

carcinogens such as tobacco and alcohol can damage individual genes and larger portions of the genetic material, including chromosomes. Accumulation of such genetic alterations can lead to development of premalignant lesions and subsequent invasive carcinoma [7].

Matrix metalloproteinases (MMPs) are a multigene family of zinc-dependent endopeptidases that share a similar structure and which collectively, have the capacity to degrade most component of the extracellular matrix (ECM) [8]. Matrix metalloproteinases (MMPs) can regulate the tumour microenvironment, and their expression and activation is increased in almost all human cancers compared with normal tissue [9]. On the basis of the functional relevance of MMPs in the pathogenesis of cancers, MMPs may be the excellent biologic candidate susceptible genes for cancers [10]. Among the MMPs, Matrix metalloproteinase-2 (MMP-2, gelatinase A) is a member of the MMP family that primarily hydrolyzes type IV collagen, the major structural component of basement membrane [6]. Over expression of MMP-2 may enable cancer cells to cleave type IV collagen selectively and cross the basement membrane, allowing cancer cell entrance into blood vessels during the early stages of metastasis [11]. Expression of MMP-2 is elevated in carcinomas in association with low differentiation grade and accelerated tumor progression [12].

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MMP-2 is tightly regulated at the transcriptional and post-transcriptional levels. Functional single nucleotide polymorphism (SNP) in the promoter region of MMP-2 has been reported, that may influence gene transcription and expression level in potentially malignant and malignant lesions. MMP-2 SNP is located at -1306 upstream of the transcriptional site and contains either a cytosine (C) or thymidine (T) [13]. The C/T transition located at nucleotide -1306 abolishes the Sp1-binding site and consequently diminishes promoter activity [6].

Studies on the MMP-2 -1306 promoter polymorphism have been carried out in cancers of lungs, breast, esophagus and colorectum and have demonstrated its inconsistent association in subjects with different ethnicity [14-17]. We investigated the association of -1306 promoter polymorphism in MMP-2 with susceptibility to develop OSCC in Indian population. We also investigated the distribution of MMP-2 promoter genotypes in patients with potentially malignant oral diseases namely OSMF and OLP.

Materials and Methods

Selection of cases and controls

Total one hundred newly diagnosed, previously untreated patients with OSCC (n=40), OSMF (n=40) and OLP (n=20) from the Department of Oral and Maxillofacial Pathology, Government Dental College and Hospital at Nagpur, India were recruited between November 2010-May 2011. Those with second primary Head and Neck Squamous Cell Carcinoma, primaries of the nasopharynx or sinonasal tract or primaries outside the upper aerodigestive tract and cervical metastasis of unknown origin were excluded. The diagnosis was confirmed by histopathological examination. Twenty control subjects were selected from people who came to the hospital for routine physical checkups or had non-neoplastic operations in the same hospital, and frequency-matched to the cases by age (± 5 years), sex, and tobacco/alcohol use status. The frequency matching was used to evaluate the main effect of the polymorphisms. Those with previous diagnosis of any cancer type, autoimmune disorders, and blood diseases were excluded from the control group. All subjects were ethnically homogenous Indians and from the same region of India. All enrolled subjects were consented and were investigated by author with designed standard protocol that involved history, clinical and histopathological examination. The study was approved by Research and Ethics Committees of Maharashtra University of Health Sciences (MUHS), Nashik (ECM/8344-53).

MMP-2 genotyping

Five ml of venous blood samples were drawn into EDTA containing tubes and processed. A leukocyte cell pellet obtained from the buffy coat was used for genomic DNA extraction with a standard phenol-chloroform method. The 193 bp sequence of MMP-2 promoter containing -1306C/T was amplified by polymerase chain reaction (PCR) using sense primer: 5' CTG AGA CCT GAA GAG CTA AAG AGC T 3' and antisense primer 5' CTT CCT AGG CTG GTC CTT ACT GA 3' [18]. The PCR reaction mixture (50 μ l) contained 50 ng genomic DNA, 2 μ M each primer, 2.5 mM dNTP (Applied Biosystems), 5.0 unit proenzyme DNA polymerase (Sigma, India) with 1X PCR buffer. The reaction was carried out under the following conditions: an initial melting step of 2 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 64°C and 45 s at 72°C and a final elongation of 10 min at 72°C. Restriction fragment length polymorphism (RFLP) was performed on amplified PCR buffer by subsequent digestion with BstUI restriction

enzyme (New England Biolabs, USA) and separated on a 2.5% agarose gel stained with ethidium bromide to determine genotype.

Statistical analysis

The distribution of genotypes in all groups was tested deviation of Hardy-Weinberg Equilibrium. The Chi square test was applied to examine differences in genotypic and allelic distribution between patients and controls. Moreover, the Odd's ratio and 95% confidence interval (95% CI) were calculated by unconditional logistic regression. A p value <0.05 was considered statistically significant.

Results

Amongst 40 cases of OSCC, the allele frequencies for the MMP-2 -1306 C and -1306 T were 81.25% and 18.75% in OSCC patients, compared with 57.5% and 42.5% in controls (p=0.0042) (Table 1). The frequencies of the CC genotype was significantly higher in OSCC patients than in controls (72.5% versus 20%; p=0.0001).

By using logistic regression analysis, we evaluated the association between the MMP-2 -1306C/T polymorphism and risk of head and neck cancer. Because the MMP-2 -1306TT homozygotes were extremely rare, this genotype was combined with the MMP-2 -1306CT genotype for estimation of oral cancer risk. We found that subjects with the MMP-2 CC genotype was associated with significantly increased risk [adjusted OR, 10.54; 95% confidence interval (95% CI), 2.88-38.57] for developing HNSCC compared with those with the variant T allele, suggesting that the C allele could be the risk allele (Table 2).

Characteristic	OSCC	OSMF	OLP	CONTROL
Number	40	40	20	20
Mean age (years) \pm SD ^a	47.8 \pm 9.8	27.7 \pm 7.9	46.7 \pm 11.2	43.7 \pm 7.4
Sex				
Male	25(62.5%)	32(80%)	8(40%)	14(70%)
Female	15(37.5%)	8(20%)	12(60%)	6(30%)
Site of primary tumour				
Buccovestibular complex	18(45%)			
Alveolus	14(35%)			
Tongue	4(10%)			
Floor of mouth	2(5%)			
Gingiva	2(5%)			
Tumour stage(T ^b)				
T1	9(22.5%)			
T2	18(45%)			
T3	13(32.5%)			
T4				
Lymph nodes (N ^c)				
N0	26(65%)			
N1	12(30%)			
N2	2(5%)			
N3				
Clinical stage				
I	32(80%)			
II	6(15%)			
III	2(5%)			
IV				
Histopathological grade				
Well differentiated SCC ^d	33			
Moderately differentiated SCC	5			
Poorly differentiated SCC	2			

Data presented as n (%) unless otherwise stated

^aSD: Standard Deviation

^bT: primary tumour

^cN: regional lymph node

^dSCC: Squamous Cell Carcinoma

Table 1: Clinical characteristics of cases and controls.

Amongst 40 cases of OSMF, the allele frequencies for the MMP-2 -1306 C and -1306 T were 76.25% and 23.75%, compared with 57.5% and 42.5% in controls ($p=0.0189$). The frequencies of the CC genotype was significantly higher in patients with OSMF than that of the controls (62.5% versus 20%; $p=0.0014$) [adjusted OR, 6.66; 95% confidence interval (95% CI), 1.87-23.71] (Table 3). Amongst 20 cases of OLP, the allele frequencies for the MMP-2 -1306 C and -1306 T were 70% and 30% in OLP patients, compared with 57.5% and 42.5% in controls ($p=0.2013$). The frequencies of the CC genotype was higher in patients with OLP than that of the controls (55% versus 20%; $p=0.0128$) [adjusted OR, 4.88; 95% confidence interval (95% CI), 1.19-19.94] (Table 4).

Discussion

Completion of the human genome project has revealed more than ten million single nucleotide polymorphisms; however, the significance of most of them in health and disease states is still elusive [19]. Genetic polymorphisms have emerged in recent years as important determinants of disease susceptibility and severity. Research considering genetic alterations jointly with environmental exposures could be relevant for a better understanding of HNC in the betel quid chewing area. Genetic polymorphism may play a significant role in person-to-person variability in cancer susceptibility, raising the intriguing possibility that some individuals could be predisposed to HNC development [20]. During the last few years, a number of polymorphisms influencing the expression of genes encoding for factors implicated in tumor invasion and metastasis have been correlated with increased risk of developing oral malignancies [7].

Subjects	Genotype			Allele	
	CC	CT	TT	C	T
OSCC patients	29(72.5%)	7(17.5%)	4(10%)	65(81.2%)	15(18.75%)
Control	4(20%)	15(75%)	1(5%)	23(57.5%)	17(42.5%)
<i>p value</i> ^b	0.0001			0.0042	
Adjusted OR ^a (95% CI)	10.54 (2.88-38.57)				

Data presented as n (%) unless otherwise stated
^aOdd ratio and 95% CI were calculated by logistic regression
^bChi square test
 OSCC: Oral Squamous Cell Carcinoma
 OR: Odd's Ratio
 CI: Confidence Interval

Table 2: Genotypic and allele frequencies at -1306 loci of MMP-2 in OSCC patients and control subjects.

Subjects	Genotype			Allele	
	CC	CT	TT	C	T
OSMF patients	25(62.5%)	11(27.5%)	4(10%)	61(76.2%)	19(23.7%)
Control	4(20%)	15(75%)	1(5%)	23(57.5%)	17(42.5%)
<i>p value</i> ^b	0.0014			0.0189	
Adjusted OR ^a (95% CI)	6.66 (1.87-23.71)				

Data presented as n (%) unless otherwise stated
^aOdd ratio and 95% CI were calculated by logistic regression
^bChi square test
 OSMF- Oral SubMucous Fibrosis
 OR: Odd's Ratio
 CI: Confidence Interval

Table 3: Genotypic and allele frequencies at -1306 loci of MMP-2 in OSMF patients and control subjects.

Subjects	Genotype			Allele	
	CC	CT	TT	C	T
OSMF patients	11(55%)	6(30%)	3(15%)	28(70%)	12(30%)
Control	4(20%)	15(75%)	1(5%)	23(57.5%)	17(42.5%)
<i>p value</i> ^b	0.0128			0.2013	
Adjusted OR ^a (95% CI)	4.88 (1.19-19.94)				

Data presented as n (%) unless otherwise stated.
^aOdd ratio and 95% CI were calculated by logistic regression.
^bChi square test
 OLP: Oral Lichen Planus
 OR: Odd's Ratio
 CI: Confidence Interval

Table 4: Genotypic and allele frequencies at -1306 loci of MMP-2 in OLP patients and control subjects.

MMP-2 is 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 base pair (bp) and 12 introns ranging from 175 to 4350 bp [10]. The global role of functional genotype of MMP-2 for the risk of various neoplasms needs to be studied [21]. In the present study, we examined the relationship between the functional polymorphisms in the promoters of MMP-2 and oral cancer susceptibility in Indian population using PCR-RFLP.

The MMP-2 plays an important role in multiple stage carcinogenesis. The -1306 C/T transition in the promoter region of MMP-2 disrupts the Sp1 binding site and lead to a remarkable lower promoter activity [21]. The Sp1 is a ubiquitously expressed transcription factor that binds to GC/GT-rich elements and is critical for regulating MMP-2 in a constitutive or inducible manner. The "CC" allele binds substantially more Sp1 transcription factor and has significantly higher transcriptional activities than the "CT" or "TT" allele [6]. The presence of Sp1 consensus sequence at MMP-2 promoter may enhance transcription, which produces higher levels of MMP-2 in subjects carrying the "CC" genotype than those carrying the variants. Thus, it is reasonable to assume that subjects carrying germ line "CC" genotype would have increased expression of this enzyme for long period and they may be more susceptible to cancer [21].

Our data suggest that subjects carrying CC genotype were at a higher risk of developing OSCC in Indian population. This was in accordance with Lin SC et al. [21] and O-Charoenrat P and Khantapura [6] who demonstrated that subjects with the MMP-2 CC genotype was associated with significantly increased risk for developing OSCC compared with those with the variant genotype, in Taiwanese and Thai population respectively. This could be interpreted by the fact that in these subjects there would be an increased MMP-2 promoter transcriptional activity leading to increased production of the enzyme.

Lin SC et al. [21] investigated the relative frequencies of MMP-2 promoter genotypes in OSMF patients and found a lack of association. We also investigated the frequency of various genotypes of MMP-2 promoter in OSMF as well as OLP patients and found an increased frequency of CC genotype in Indian population. Various environmental factors, diet, tobacco and alcohol intake, combined with genetic heterogeneity in subjects of Indian ethnicity may influence the MMP-2 promoter polymorphism's contribution in oral carcinogenesis. We suggest that there may be a higher risk of malignant transformation in these subjects due to higher transcriptional activity. This may be confirmed in future studies with a larger sample size.

In conclusion, the present study provides evidences for the first time that -1306C/T polymorphism in MMP-2 promoter is a risk factor for oral carcinogenesis in Indian population, with the CC genotype being associated with the increase of risk. Also, it is the first study to demonstrate an association of increased frequency of CC genotype in OSMF and OLP patients. To more precisely establish the contribution of the MMP-2 promoter polymorphism to oral cancer incidence, further examination of the prevalence of these variants in populations of other ethnic origin is required.

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