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RESEARCH ARTICLE

Role of functional polymorphism of matrix metalloproteinase-2 (-1306 C/T and -168 G/T) and MMP-9 (-1562 C/T) promoter in oral submucous fibrosis and head and neck squamous cell carcinoma in an Indian population

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Abstract
The aim of this study was to explore the association of MMP-2 (-1306 C/T and -168 G/T) and MMP-9 (-1562 C/T) promoter polymorphisms in oral submucous fibrosis (OSMF) and head and neck squamous cell carcinoma (HNSCC) cases. These SNP were genotyped by PCR-RFLP. Total of 1260 individuals were recruited, of which 412 OSMF, 422 HNSCC and 426 were controls. In HNSCC, MMP-2 (-1306 C/T) and MMP-9 (-1562C/T) polymorphism, T allele showed strong association ($p < 0.00$ and $p < 0.01$) as compared to healthy control respectively, but not in case of OSMF and showed significant association with increasing progression of clinico-pathological grading. We concluded that SNPs in the MMP-2 and -9 promoter region may be associated with susceptibility to HNSCC not in OSMF.

Keywords: MMP, SNP, OSMF, HNSCC, genomics

Introduction

Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteases (Mignatti et al. 1993) that are capable of degrading various components of extracellular matrix (ECM) (Nagase et al. 2002). In humans, 24 types of MMP genes are known and these are classified into five different groups based on their substrate specificity and domain structure. These include collagenases (MMP-1, MMP-8 and MMP-13), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3, MMP-10 and MMP-11), matrilysins (MMP-7 and MMP-26) and membrane-type MMPs (MT-MMPs). In the normal steady-state, activity of the most MMPs is very low. Under the normal physiological conditions; degradation of ECM plays an important role for the development, morphogenesis, tissue repair and remodeling, but when disturbance occurs in the ECM, it becomes a cause of many diseases such as arthritis, nephritis, encephalomyelitis, chronic ulcers, fibrosis, apoptosis, tumors invasion and metastasis etc (Egeblad et al. 2002, Wagenaar-Miller et al. 2004, Deryugina et al. 2006).

Overexpression of MMPs genes are transcriptionally induced by oncogenic transformation, cytokines as well as growth factors– including interleukins, interferons, EGF, KGF, NGF, VEGF, PDGF, TNF-α and TGF-β (Nagase et al. 2002, Westermarck et al. 1999). The expression of MMPs is primarily regulated at the level of transcription. Many stimuli increase the expression of c-fos and c-jun proto oncogene products and it activates the activator protein-1 (AP-1) at proximal promoter regions of several

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Abbreviations

ECM, extra cellular matrix
HNSCC, head and neck squamous cell carcinoma
MMP, matrix metalloproteinase
OSMF, oral submucous fibrosis
OR, odds ratio
PCR, polymerase chain reaction
RFLP, restriction fragment length polymorphism
ROC, receiver operating characteristic curve
SNP, single nucleotide polymorphism
95% CI, 95% confidence interval

MMPs such as MMP-1, -3, -7, - 9, -10, -12 and -13 types (Westermarck et al. 1999). The author's group reviewed the molecular functions and polymorphic association of different MMPs such as MMP-1 (-1607 1G/2G), MMP-2 (-1306 C/T), MMP-3 (-1171 5A/6A), MMP-9 (-1562 C/T) and TIMP-2 (-418 G/C or C/C), and also discuss the possible therapeutic aspects of these MMPs in potentially malignant lesions (PML) and malignant lesions (ML) of the head and neck (Chaudhary et al. 2009).

The MMP-2 and MMP-9 gene are located on chromosome 16q13 and 20q11.2-q13.1 respectively. The activity of MMP-2 (gelatinase-A) gene regulated by post transcriptional mechanisms, including the activation of proenzyme and inhibition of enzyme activity (Nagase et al. 2002, Egeblad et al. 2002). Transcriptional regulation of MMP-2 gene is important, because the human MMP-2 promoters contains a number of cis-acting regulatory elements and several transcription factors, including Sp1, Sp3, p53 and AP-2 and participate in the control of its constitutive expression (Bian et al. 1997, Qin et al. 1999). SNP in the promoter (-1306C/T) region of the MMP-2 gene reported by Price et al. The -1306 C/T transition is located in a core recognition sequence of Sp1 (CCACC box), which eliminate the Sp1-binding site and consequently reduced the promoter activity of MMP-2 gene (Price et al. 2001). Many studies suggested that individuals carrying CC genotype may be associated with the higher transcriptional activity (Ye et al. 2000, Miao et al. 2003, Lin et al. 2004).

MMP-9 is an important member of this family. It is also known as gelatinase-B or type IV collagenases, which is a most important member of the basement membrane. MMP-9 has three repetitive fibronectin domains, which allow it to bind to ECM component such as gelatin, collagen and laminin. MMP-9 SNP is located at the position 1562 bp upstream of the transcriptional start site and contains either cytidine (C) or thymidine (T), and according to Zhang et al. DNA-protein interaction assay indicated that T allele-associated promoter activity was higher than the C-allele due to the binding of transcriptional repressor protein (Zang et al. 1999).

A number of studies have demonstrated the polymorphism of MMP-9 (-1562 C/T) in lung cancer (Zhou et al. 2005, Bayramoglu et al. 2009), breast cancer (Lei et al. 2007, Roehe et al. 2007), colorectal carcinoma (Elander et al. 2006, Xu et al. 2007, Woo et al. 2007, Xu et al. 2006, Xing et al. 2007) and oral squamous cell carcinoma (OSCC) (Tu et al. 2007, Vairaktaris et al. 2008). Several studies suggested that individuals carrying T allele were more susceptible such as gastric carcinoma (Matsumura et al. 2005), myocardial ischemia (Medley et al. 2004) & pulmonary disease (Ito et al. 2005). On the other hand in hepatitis C cases, the frequency of C alleles was higher in cirrhosis patients as compared to chronic hepatitis (Okamoto et al. 2005, Lichtinghagen et al. 2003).

It has been reported that oral submucous fibrosis (OSMF) and head and neck squamous cell carcinoma (HNSCC) is most prevalent in Asian countries, because of the habit of tobacco, smoking and areca nut chewing (Mehrotra et al. 2008, Ko et al. 2003, Lin et. 2004, Pandya et al. 2009) and viral infections such as high risk and low risk human papilloma virus (HPV) are also responsible for causing these lesions in North Indian population (Chaudhary et al. 2009; Mehrotra et al. 2010, Chaudhary et al. 2010). Chang et al. reported that areca nut ingredient such as arecoline inhibits the gelatinolytic activity of the many matrix metalloproteinases in the mucosal layer (Chang et al. 2010). Thereby, many MMPs genotypes may be associated with the susceptibility to these lesions.

The purpose of this study was to investigate the association of the MMP-2 (-1306 C/T & -168 G/T) and MMP-9 (-1562 C/T) promoter polymorphisms in potentially malignant lesions (PML) such as OSMF and malignant lesions (ML) such as head and neck squamous cell carcinoma (HNSCC) respectively, as well as to find out whether these polymorphisms influence prognosis to risk factor and its susceptibility in histopathological grading criteria.

Materials and methods

Sample collection procedure

A total of 1260 individuals were incorporated in this study, of which 412 with OSMF and 422 HNSCC patients were recruited from the Department of Otorhinolaryngology, Moti Lal Nehru Medical College, Allahabad, India, after obtaining clearance from the institutional ethical committee. Detailed information of each patient was noted in a pretested proforma. Information regarding the patient's name, age, sex and addiction habits was gathered. Emphasis was given to addictions like areca nut chewers (i.e. areca nut chewing with tobacco and slaked lime), never areca nut chewers, smokers (i.e. cigarette and bidi smokers), never-smokers, alcohol drinkers, and never taken alcohol drinks, of the patients. Briefly, individuals who formerly or currently chewed 4 pouches tobacco with areca nut and pan/day or 4 pouches gutkha pan masala, 4 cigarettes or bidi/day and alcohol (≈300 ml) three times/week for at least 2 years were defined as chewers, smokers and drinkers respectively.

Detailed clinical examination of each OSMF patient was done to assess the site, size and type of lesion. Trismus was classified as stage I (> 3cm), stage II (2-3cm) and stage III (<2cm). For confirmation of the clinical diagnosis, histopathological examination was carried out in the biopsy tissues, and the grading for OSMF cases were done according to the classification given by Pindborg and Sirsat (Pindborg et al. 1966). Likewise, for the HNSCC patients after the clinical diagnosis, the hisopathological examination was done. The HNSCC cases were grouped on the basis of tumour site, lymph node position and metastasis (TNM) (Patel et al. 2005). All specimens were examined independently by two different histopathologists in a double blind fashion. If there was any discrepancy, a third opinion was obtained to reach a final diagnosis. As the histpathogical slides were evaluated by histopathologists and the patients were examined by the otorhinolaryngology surgeons, there was no chance of bias. None of the HNSCC patients gave a history of having OSMF prior to developing malignancy.

The 426 healthy control subjects, having no history of any type of pre-malignancy and malignancy or genetic disorder, were recruited from individuals, who came for routine physical checkup in the same hospital and compared for age, sex and addiction habits. After that the blood samples were taken after obtaining the patients informed consent to participate in the study. Blood samples of 5 ml was drawn from each subject into vacutainer tubes containing ethylene-di-amine-tetra-acetic acid (EDTA) and stored at 4°C till they were processed.

Isolation of genomic DNA

Genomic DNA was extracted from the blood samples by using the Qiagen QIAamp DNA Blood Mini Kit (Qiagen Inc. USA). The extracted genomic DNA was quantified and checked for purity spectrophotometrically (Spectro UV-Vis Double Beam PC, UVD Model 2950 LABOMED, Inc. CA, USA) Ethedium bromide (EtBr) stained 0.8% agrose gel electrophoresis was used to confirm the presence of genomic DNA in patient samples and controls.

Genotyping of the MMP-2 (-1306 C/T and -168 G/T) promoter polymorphism

A PCR-based restriction fragment length polymorphism (PCR-RFLP) was performed for MMP-2 genotyping. The PCR primers used for amplifying the MMP-2 (-1306 C/T) polymorphism were: FP 5′ CTG ACC CCC AGT CCT ATC TGC C-3′ and RP 5′-TGT TGG GAA CGC CTG ACT TCA G-3′ (Yu et al. 2002) and for -168 G/T polymorphism the primer sequences were FP: 5′-CTG ACC ATT CCT TCC CGT TC-3′and RP: 5′-CGC CTG AGG AAG TCT GGA T-3′. In MMP-2 (-1306 C/T and -168 G/T) polymorphism, 25µl PCR mixture containing 10X PCR Buffer, 2.5mM $MgCl_2$, 1U of Taq DNA polymerase (Fermentas Inc. Glen Burine MD), 200 nmol of dNTPs and 200nmol of forward and reverse primer and 50 ng of genomic DNA. The PCR condition were 95°C for 5 min then 35 cycles for 30 sec at 95°C, 30 sec at 58.6°C for -1306 C/T polymorphism and 61.5°C for -168 G/T polymorphism, 30 sec for 72°C with a final extension at 72°C for 5 min. After amplification, the PCR products were digested with restriction enzyme *Bse DI* for MMP-2 (-1306C/T and -168 G/T) polymorphism and analysed by 3.0% agarose gel electrophoresis.

Genotyping of the MMP-9 (-1562 C/T) promoter polymorphism

A PCR based restriction fragment length polymorphism was performed for MMP-9 genotyping. The PCR primers used for amplifying and FP 5′-GCC TGG CAC ATA GTA GGC CC-3′ and RP 5′-CTT CCT AGC CAG CCG GCA TC-3′ (Morgan et al. 2003) (Table 1). PCR was performed in a 25 µl volume containing 50 ng of genomic DNA template, 2.5 µl of 10X PCR buffer, 2.5 mmol of $MgCl_2$, 1 U of Taq DNA polymerase (Fermentas Inc. Glen Burnie MD), 200µmol of dNTPs and 200 nmol of forward and reverse primer. The PCR cycling conditions were 5 min at 95°C followed by 34 cycles of 30 s at 95°C, 30 s at 59°C and 30 s at 72°C and with a final step at 72°C for 10 min to allow for the complete extension of all PCR fragments. For a negative control, instead of DNA sample, distilled water was used in a PCR reaction mixture. A 10 µl aliquot of PCR product was digested at 37°C overnight, in a 20 µl reaction containing 5 U of *Pae I*/*Sph I* and 1X reaction buffer. After digestion, the products were separated on a 3.5% agarose gel stained with EtBr.

Statistical analysis

The Chi-square test (χ^2 test) was used to find out the difference in genotype distribution of MMP-1 promoters between the OSMF, HNSCC and control subjects. A p value of < 0.05 was considered as statistically significant. Hardy–Weinberg equilibrium was tested in the control subjects to rule out sampling bias. For each parameter, the OSMF histopathological grade I, II, III,IV

Table 1. Single nucleotide polymorphic (SNP) sites, forward and reverse primer (FP and RP) sequences, annealing temperature (Ta), detection method, restriction enzyme (RE), PCR product size (bp) and references.

Gene	SNPs	Primer sequences (forward and reverse primers)	Annealing temp (°C)	Detection method	Restriction enzyme
MMP-2	-168 G/T	5′-CTG ACC ATT CCT TCC CGT TC-3′, 5′-CGC CTG AGG AAG TCT GGA T-3′	52.5°C	PCR-RFLP	*Bse GI*
	-1306 C/T	5′-CTTCCTAGGCTGGTCCTTACTGA-3′, 5′-CTGAGACCTGAAGAGCTAAAGAGCT-3′	61.5°C	PCR-RFLP	*Bse DI*
MMP-9	-1562 C/T	5′-GCCTGGCACATAGTAGGCCC-3′, 5′-CTTCCTAGCCAGCCGGCATC-3′	69.2°C	PCR-RFLP	*Pae I/SphI*

and HNSCC cases risk was analyzed by odds ratios (OR) and 95% Confidence Intervals (95% CI). The statistical analysis was performed using the SPSS 15.0 software package (SPSS Japan Inc. Tokyo, Japan).

Results

A total 834 cases (average age 48.0 ± 17.6 years) were included in this study, of which 412 patients suffered from OSMF (252 males and 160 females) and 422 patients from HNSCC (292 males and 130 females). Four hundred and twenty six cases (average age 47.01 ± 15.2 years, 297 males, 129 females) were healthy controls. MMP-2 and MMP-9 promoter polymorphism in patients with OSMF, HNSCC and healthy controls were analyzed with respect to gender, age and habits like areca nut chewing, smoking and alcohol intake etc. Histopathological grades of OSMF and HNSCC are shown in Table 2.

Genotypic distributions of MMP-2 (-168 G/T &-1306 C/T) and MMP-9 (-1562C/T) promoter polymorphism in OSMF and HNSCC cases:

In the case of MMP-2 (-168 G/T &-1306 C/T) and MMP-9 (-1562 C/T) genotypic distribution in OSMF, HNSCC and controls were analyzed through polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP). The frequency of 'T' allele did not show significant MMP-2 (-168 G/T) polymorphic association in case of OSMF (T allele frequency = 0.19, OR = 1.19, 95% CI = 0.94–1.53 and $p = 0.17$), and as well as in cases of HNSCC (T allele frequency = 0.16, OR = 1.12, 95% CI = 0.86–1.45 and $p = 0.42$), while in the case of MMP-2 (-1306 C/T) genotypic distribution the frequency of T allele did not show significant polymorphic association in case of OSMF (T allele frequency = 0.14, OR = 0.796, 95% CI = 0.61–1.03 and $p = 0.10$). In case of MMP-2 (-1306 C/T) polymorphism, T allele showed a strong association (T allele frequency = 0.25, OR = 1.86, 95% CI = 1.15–1.85 and $p < 0.00$) in HNSCC cases as compared to healthy control. In case of MMP-9 (-1562C/T) polymorphism, T allele showed a significant association (T allele frequency = 0.18, OR = 1.17, 95% CI = 1.06–1.79 and $p < 0.01$) in HNSCC, while in case of OSMF, T allele did not show significant association ($p = 0.14$) as compared to healthy control (Table 3).

Table 2. Demographic distribution and clinical parameters among the OSMF and HNSCC cases.

Clinico-pathogical parameter	OSMF cases (412)	HNSCC cases (422)
Gender		
Male	252 (61.2%)	292 (69.2%)
Female	160 (38.8%)	130 (30.8%)
Age (Years ±SD) (Range)	42 ± 12.6	56 ± 15.6
Location		
Lip (lower & upper)	77 (18.7%)	52 (12.3%)
Tongue	108 (26.3%)	47 (11.1%)
Hard pallet	43 (10.4%)	30 (07.1%)
Soft palate	82 (19.9%)	52 (12.3%)
Buccal mucosa	102 (24.7%)	47 (11.2%)
Larynx	–	70 (16.6%)
Pharynx	–	54 (12.8%)
Hypo pharynx	–	23 (05.5%)
Tonsil	–	47 (11.1%)
OSMF Grade		–
I	126 (30.6%)	–
II	117 (28.4%)	–
III	92 (22.3%)	–
IV	77 (18.7%)	
T category		
T1–2	–	273 (64.7%)
T3–4	–	149 (35.3%)
N category		
N0	–	268 (63.5%)
N1–3	–	154 (36.5%)

Association between MMP-2 (-168 G/T &-1306 C/T) and MMP-9 (-1562 C/T) promoter polymorphisms and various clinico-pathological parameters

In the case of OSMF grading (Grade I, II, III & IV) polymorphism of MMP-2 (-168 G/T) promoter polymorphism did not show significant association promoter polymorphism, while in the case of MMP-2 (-1306 C/T) and MMP-9 (-1562 C/T) promoter polymorphism T allele showed a significant association ($p < 0.02$ and $p < 0.04$ respectively) with the increasing progression of OSMF (Table 4). In the case of HNSCC grading, polymorphism of MMP-2 (-168 G/T)) promoter polymorphism did not show significant association promoter polymorphism, while in the case of MMP-2 (-1306 C/T) and MMP-9 (-1562 C/T) promoter polymorphism T allele showed a significant association ($p < 0.003$ and $p < 0.00$ respectively) with the increasing progression of HNSCC, mentioned in Table 5.

Genotypic distribution of MMP-2 (–168 G/T & -1306 C/T) and MMP-9 (-1562 C/T) promoter polymorphisms and age group in OSMF, HNSCC and control groups

The distribution of MMP-2 (-168 G/T & -1306 C/T) promoter polymorphisms did not show significant association in case of age (<45 & ≥45) group, while in MMP-9 (-1562 C/T) promoter polymorphism T and C allele, both showed significant association ($p < 0.00$ and $p < 0.01$respectively) in case of age (<45 & ≥45) group distribution between the OSMF and healthy controls (Table 6). In case of HNSCC, the distribution of MMP-2 (-168 G/T) promoter polymorphisms did not show significant association in case of age (<45 & ≥45) group distribution between the OSCC and healthy control, while in case of MMP-2 (-1306 C/T) and MMP-9 (-1562 C/T) promoter polymorphisms T allele showed significant association ($p < 0.01$ and $p < 0.03$ respectively) between the HNSCC and healthy control (Table 7).

Genotypic distribution of MMP-2 (-168 G/T -1306 C/T) and MMP-9 (-1562 C/T) gene in OSMF and HNSCC with relation to various addiction habits

The association between MMP-2 (*-168 G/T* -1306 C/T) and MMP-9 (-1562 C/T) genotypes in the OSMF, HNSCC and

control groups and various addiction habits are shown in Tables 8, 89, and 10. In the OSMF and HNSCC group, the genotypic association of MMP-2 (-168 G/T) genotype do not show any association in relation to various addiction habits. In the case of MMP-2 (-1306 C/T) polymorphism, the C/T, T/T genotype showed a significant 2.4 fold ($p < 0.01$) risk in case of areca nut chewers, 2.1 fold ($p < 0.01$) risk in case of chewers & smokers and 3.2 fold ($p < 0.04$) risk in combination of chewer, drinker & smokers for the development of OSMF. While in case of HNSCC, the C/T, T/T genotype showed significantly 3.2 fold ($p < 0.00$) risk in case of areca nut chewers, 3.9 fold ($p < 0.001$) risk in

Table 3. Genotypic distribution of MMP-2 (-168 G/T and -1306 C/T) and MMP-9 (-1562 C/T) polymorphism in control, OSMF and HNSCC cases.

MMP-2 [-168 G/T]	G/G	G/T	T/T	T allele frequency	*p* value	OR	95% CI
Control (422)	290 (68.7%)	121 (28.7%)	11 (2.6%)	0.17	–	1	Referent
OSMF (412)	275 (66.7%)	112 (27.1%)	25 (6.1%)	0.19	0.17	1.19	0.94–1.53
HNSCC (426)	222 (52.1%)	168 (39.4%)	36 (8.5%)	0.16	0.42	1.12	0.86–1.45
MMP-2 [-1306 C/T]	**C/C**	**C/T**	**T/T**	**T allele frequency**	***p* value**	**OR**	**95% CI**
Control (422)	298 (70.6%)	104 (24.7%)	20 (4.7%)	0.14	–	1	Referent
OSMF (412)	314 (76.2%)	80 (19.4%)	18 (4.4%)	0.17	0.10	0.796	0.61–1.03
HNSCC (426)	299 (70.2%)	87 (20.4%)	40 (9.3%)	0.25	<0.00	1.86	1.15–1.85
MMP-9 [-1562 C/T]	**C/C**	**C/T**	**T/T**	**T allele frequency**	***p* value**	**OR**	**95% CI**
Control (422)	315 (74.6%)	96 (22.7%)	11 (2.6%)	0.14	–	1	Referent
OSMF (412)	307 (74.5%)	78 (18.9%)	27 (6.5%)	0.16	0.14	1.17	0.89–1.53
HNSCC (426)	311 (73.0%)	80 (18.7%)	35 (8.2%)	0.18	**<0.01**	1.39	1.06–1.79

Table 4. Genotypic distribution of MMP-2 (-168 G/T and -1306 C/T) and MMP-9 (-1562 C/T) polymorphism in OSMF cases according to the clinico-pathological grading. [Pearson's Chi squared test]

		OSMF grade (422)				
SNP Genotypes	OSMF Cases *N* = 412	OSMF Grade I *N* = 126 (30.6%)	OSMF Grade II *N* = 117 (28.4%)	OSMF Grade III *N* = 92 (22.3%)	OSMF Grade IV *N* = 77 (18.7%)	*p* value
MMP-2 [-168 G/T]						
G/G	275 (66.7%)	87 (69.04%)	82 (70.1%)	60 (65.2%)	46 (59.7%)	0.45
G/T	112 (27.2%)	35 (27.8%)	28 (23.9%)	24 (26.08%)	25 (32.4%)	0.57
T/T	25 (6.1%)	04 (3.17%)	07 (6.0%)	08 (8.69%)	06 (7.79%)	0.43
MMP-2 [-1306 C/T]						
C/C	314 (76.2%)	113 (89.7%)	81 (69.2%)	76 (82.6%)	44 (57.1%)	0.37
C/T	80 (19.4%)	8 (30.8%)	34 (29.1%)	14 (15.2%)	24 (31.2%)	0.07
T/T	18 (4.4%)	5 (3.9%)	2 (1.7%)	2 (2.2%)	9 (11.7%)	**<0.02**
MMP-9 [-1562 C/T]						
C/C	307 (74.5%)	86 (68.2%)	88 (75.2%)	67 (72.8%)	66 (85.7%)	0.86
C/T	78 (18.9%)	38 (30.2%)	20 (17.1%)	16 (17.4%)	4 (5.2%)	0.22
T/T	27 (6.5%)	02 (1.6%)	09 (7.7%)	09 (9.78%)	07 (9.09%)	**<0.04**

Table 5. Genotypic distribution of MMP-2 (-168 G/T and -1306 C/T) and MMP-9 (-1562 C/T) polymorphism in HNSCC cases according to the clinico-pathological grading. [Pearson's Chi squared test]

		T category		N category		
SNP Genotypes	HNSCC Cases *N* = 426	T 1-2, *N* = 277 (64.7%)	T 3-4, *N* = 149 (35.3%)	N 0, *N* = 268 (63.5%)	N 1-3, *N* = 158 (36.5%)	*p* value
MMP-2 [-168 G/T]						
G/G	222 (52.1%)	154 (69.4%)	68 (30.6%)	157 (70.7%)	65 (29.3%)	0.84
G/T	168 (39.4%)	107 (63.7%)	61 (36.3%)	90 (53.6%)	78 (46.4%)	0.07
T/T	36 (8.4%)	16 (5.8%)	20 (13.4%)	21(7.8%)	15 (9.5%)	0.24
MMP-2 [-1306 C/T]						
C/C	299 (70.2%)	203 (67.9%)	96 (32.1%)	215 (71.9%)	84 (28.1%)	0.33
C/T	87 (20.4%)	48 (17.3%)	39 (76.1%)	41 (15.2%)	46 (29.1%)	0.28
T/T	40 (9.3%)	26 (65.0%)	14 (35.0%)	12 (30.0%)	28 (70.0%)	**<0.003**
MMP-9 [-1562 C/T]						
C/C	311 (73%)	224 (80.8%)	87 (58.3%)	205 (76.4%)	106 (67%)	0.09
C/T	80 (18.7%)	38 (13.7%)	42 (28.1%)	36 (13.4%)	44 (27.8%)	0.75
T/T	35 (8.2%)	15 (5.4%)	20 (13.4%)	27 (10%)	08 (5.1%)	**<0.00**

case of chewers & smokers, and 3.3 fold ($p < 0.03$) risk in combination of chewer, drinker & smokers for the development of HNSCC (Tables 8 and 9).

In the case of MMP-9 (-1306 C/T) polymorphism, the C/T, T/T genotype showed a significant 2.5 fold ($p < 0.04$) risk in case of areca nut chewers, 3.2 fold ($p < 0.04$) risk in case of chewers & smokers, and 3.8 fold ($p < 0.03$) risk in combination of chewer, drinker & smokers for the development of OSMF. While in the case of HNSCC, the C/T, T/T genotype showed a significant 3.3 fold ($p < 0.00$) risk

Table 6. Association between MMP-2 (-168 G/T and -1306 C/T) and MMP-9 (-1562 C/T) polymorphism and OSMF cases in various age groups.

SNP Genotype	Control (422)		OSMF (412)		
Age (year old)	**<45 yrs *N* = 212**	**≥45 yrs *N* = 210**	**<45 yrs *N* = 268**	**≥45 yrs *N* = 144**	*p value
MMP-2 $_{[-168\ G/T]}$					
G/G	120 (56.6%)	170 (80.9%)	180 (67.2%)	95 (65.9%)	0.19
G/T	86 (40.6%)	35 (16.6%)	76 (28.4%)	36 (25.0%)	0.59
T/T	06 (2.8%)	05 (2.4%)	12 (4.5%)	13 (9.1%)	0.71
MMP-2 $_{[-1306\ C/T]}$					
C/C	153 (72.2%)	145 (69.1%)	212 (79.1%)	102 (70.8%)	0.50
C/T	51 (24.1%)	53 (25.2%)	45 (16.8%)	35 (24.3%)	0.33
T/T	08 (3.7%)	12 (5.7%)	11 (4.1%)	07 (4.8%)	0.19
MMP-9 $_{[-1562\ C/T]}$					
C/C	144 (67.9%)	171 (81.4%)	230 (85.8%)	77 (53.5%)	**<0.00**
C/T	64 (30.2%)	32 (15.2%)	26 (9.7%)	52 (36.1%)	**<0.01**
T/T	4 (1.9%)	7 (3.3%)	12 (4.5%)	15 (10.4%)	0.64

*X^2=Chi-square analysis, OSMF=Oral Sub mucous fibrosis.

Table 7. Association between MMP-2 (-168 G/T and -1306 C/T) and MMP-9 (-1562 C/T) polymorphism and HNSCC cases in various age groups.

SNP Genotype	Control [422]		HNSCC [426]		
Age (year old)	**<45 yrs *N* = 196**	**≥45 yrs *N* = 226**	**<45 yrs *N* = 185**	**≥45 yrs *N* = 241**	*p value
MMP-2 $_{[-168\ G/T]}$					
G/G	130 (66.3%)	160 (70.8%)	97 (52.4%)	125 (51.8%)	0.79
G/T	62 (31.6%)	59 (26.1%)	91 (49.2%)	77 (31.9%)	0.62
T/T	4 (2.1%)	7 (3.1%)	19 (10.3%)	17 (7.1%)	0.34
MMP-2 $_{[-1306\ C/T]}$					
C/C	141 (71.9%)	157 (69.5%)	128 (69.2%)	171 (70.9%)	0.25
C/T	41 (20.9%)	63 (27.8%)	42 (22.7%)	45 (18.6%)	0.22
T/T	14 (7.1%)	6 (2.6%)	15 (8.1%)	25 (10.4%)	**<0.01**
MMP-9 $_{[-1562\ C/T]}$					
C/C	140 (71.4%)	175 (77.4%)	118 (63.8%)	193 (80.1%)	0.09
C/T	52 (26.5%)	44 (19.5%)	42 (22.7%)	38 (15.7%)	0.82
T/T	4 (2.4%)	7 (3.1%)	25 (13.5%)	10 (4.1%)	**<0.03**

*X^2=Chi-square analysis, HNSCC=Head and neck squamous cell carcinoma.

Table 8. Addiction habit distribution in relation to MMP-2 [-168 G/T] alleles in OSMF, HNSCC cases and healthy controls.

MMP-2 Genotype [-168 G/T]	Control (n=426)	OSMF (n=412)	Trend test (p value)	OR	HNSCC (n=412) (p value)	OR	Trend test (p value)
Non areca nut chewer but smokers and drinkers G/G, G/T, T/T	20/39 (51.2%) 19/39 (48.8%)	21/45 (46.6%) 24/45 (53.3%)	0.67	0.65	25/37 (67.5%) 12/37 (27.1%)	0.89	0.053
Only areca nut chewer, G/G, G/T, T/T	49/121 (40.4%) 72/121 (59.5%)	64/108 (59.2%) 44/108 (40.7%)	0.26	1.10	91/119 (76.4%) 28/119 (22.6%)	0.82	0.98
Non smoker but chewer and drinker, G/G, G/T, T/T	13/36 (36.1%) 23/36 (63.8%)	12/24 (50%) 12/24 (50%)	0.28	0.34	23/39 (58.9%) 16/39 (38.4%)	0.23	0.11
Only smoker, G/G, G/T, T/T	47/102 (46.1%) 53/102 (51.9%)	53/78 (76.8%) 25/78 (32.0%)	0.58	0.61	72/95 (75.7%) 23/95 (23.2%)	0.98	0.07
Non Drinker but chewer and smoker, G/G, G/T, T/T	16/31 (51.6%) 15/31 (48.3%)	33/54 (61.1%) 21/54 (38.8%)	0.08	1.10	22/33 (66.6%) 11/33 (30.3%)	0.71	0.07
Only Alcohol Drinker, G/G, G/T, T/T	9/40 (22.5%) 31/40 (77.5%)	17/49 (34.6%) 32/49 (65.2%)	0.20	0.56	36/59 (61.1%) 23/59 (33.8%)	0.71	0.97
Chewer, drinker and smoker, G/G, G/T, T/T	17/57 (29.8%) 44/57 (72.1%)	13/54 (24.1%) 41/54 (75.9%)	0.64	0.23	25/40 (62.5%) 15/40 (22.5%)	0.43	0.06

in the case of areca nut chewers, 3.1 fold ($p < 0.01$) risk in case of chewers & smokers, and 4.1 fold ($p < 0.002$) risk in combination of chewer, drinker & smokers for the development of HNSCC (Table 10).

Discussion

OSMF is a chronic disorder and a potentially malignant condition of the oral cavity (Rajalalitha et al. 2005). It is most prevalent in Asian countries, because of the fashionable habit of areca nut chewing (Ko et al. 2003, Lin et al. 2004). OSMF is prominent in India, occurring exclusively in areca nut chewers with tobacco and gutkha pan masala (Pandya et al. 2009). It is characterized by deposition of collagen in the oral submucosa. The betel nut has an alkaloid called arecoline and plays a major role in the pathogenesis of OSMF by causing an abnormal increase in the collagen production (Canniff et al. 1981).The decreased degradation of collagen due to increased cross-linking of the fibers and reduced collagenase activity are found in OSMF (Shieh et al. 1992). This evidence implies that OSMF may be considered a collagen-metabolic disorder resulting due to the use of areca nuts (Rajalalitha et al. 2005).

MMPs are characterized by disturbances in the homeostatic balance occurring between synthesis and degradation of ECM including collagen. Chang et al. reported that arecoline, inhibits the gelatinolytic activity of MMP-2. Thereby, MMP-2 genotype may be associated with the susceptibility of OSMF (Chang et al. 2002). In this study, in the case of MMP-2 (-1306 C/T) polymorphism, the C/T, T/T genotype showed significantly 2.4 fold ($p < 0.01$) risk in case of areca nut chewers, 2.1 fold ($p < 0.01$) risk in case of chewers & smokers, and 3.2 fold ($p < 0.04$) risk in combination of chewer, drinker & smokers for the development of OSMF. While in the case of HNSCC, the C/T, T/T genotype showed significantly 3.2 fold ($p < 0.00$) risk in case of areca nut chewers, 3.9 fold ($p < 0.001$) risk in case of chewers & smokers, and 3.3 fold ($p < 0.03$) risk in combination of chewer, drinker &smokers for the development of HNSCC. Therefore, this study concluded that addiction habits such as areca nut chewers and tobacco smokers may be associated with MMP-2 -1306 C/T polymorphism in OSMF and HNSCC cases. Similar results were reported by Chang et al. (36) in the case of OSMF, and Lin et al in the case of oral squamous cell carcinoma (OSCC) (Lin et al. 2004). In case of lung carcinoma Yu et al. reported that the frequency of the CC genotype was

Table 9. Addiction habit distribution in relation to MMP-2 (-1306 C/T) alleles in OSMF and HNSCC cases and healthy controls.

MMP-2 Genotype -1306 C/T	Controls ($n=426$)	OSMF ($n=412$)	Trend test (p value)	OR	HNSCC ($n=422$) (p value)	OR	Trend test (p value)
Non areca nut chewer but smokers and drinkers, C/C, C/T, T/T	23/41(56.1%) 18/41 (43.9%)	33/58 (56.8%) 25/58 (37.9%)	0.98	0.56	34/47 (72.3%) 13/47 (27.6%)	0.34	0.918
Only areca nut chewer, C/C C/T, T/T	45/118 (38.1%) 73/118 (58.4%)	56/104 (53.8%) 48/104 (38.4%)	**<0.01**	2.4	81/109 (74.3%) 28/109 (25.9%)	3.2	**<0.00**
Non smoker but chewer and drinker, C/C C/T, T/T	23/38 (60.5%) 15/38 (36.8%)	10/31(32.2%) 21/31 (61.2%)	0.98	0.36	33/50 (66%) 27/50 (54.0%)	0.76	0.98
Only smoker, C/C C/T, T/T	55/97 (56.7%) 42/97 (41.2%)	40/65 (61.5%) 25/65 (33.8%)	0.54	0.65	60/84 (71.4%) 24/84 (28.5%)	0.76	0.836
Non drinker but chewer and smoker, C/C C/T, T/T	20/34(58.8%) 16/34 (41.2%)	33/53 (62.2%) 20/53 (37.7%)	**<0.01**	2.1	31/43 (72.1%) 12/43 (27.9%)	3.9	**<0.001**
Only alcohol Drinker, C/C C/T, T/T	31/43 (72.1%) 12/43 (23.2%)	33/55 (60%) 22/55 (40%)	0.21	0.45	26/40 (65%) 14/40 (35%)	0.32	0.457
Chewer, drinker and smoker, C/C, C/T, T/T	44/55 (73.3%) 16/55 (21.6%)	22/46 (47.8%) 24/46 (47.8%)	**<0.04**	3.2	36/49 (71.4%) 13/49 (22.4%)	3.3	**<0.03**

Table 10. Association of promoter polymorphism of MMP-9 (-1562 C/T) gene in OSMF and HNSCC with addiction habits.

MMP-9 (-1562 C/T)	Controls ($n=426$)	OSMF ($n=412$)	Trend test (p value)	OR	OSCC ($n=422$)	OR	Trend test (p value)
Non areca nut chewer but smokers and drinkers, C/C, C/T,T/T	38/51 (76.5%) 13/51 (23.4%)	55/68 (80.8%) 13/68 (19.1%)	0.57	0.67	35/47 (74.4%) 12/47 (25.5%)	0.07	0.65
Only areca nut chewer, C/C, C/T, T/T	86/108 (81.4%) 22/108 (18.5%)	74/94 (78.7%) 20/94 (21.2%)	**<0.04**	2.5	90/109 (82.5%) 19/109 (17.4%)	**<0.00**	**3.3**
Non smoker but chewer and drinker, C/C, C/T,T/T	37/48 (70.1%) 11/48 (21.8%)	16/21 (28.5%) 5/21 (23.8%)	0.93	0.76	33/49 (67.3%) 16/49 (32.6%)	0.16	0.45
Only smoker, C/C, C/T, T/T	65/87 (74.7%) 22/87 (25.2%)	64/80 (80%) 16/80 (20%)	0.41	0.53	62/85 (72.9%) 23/85 (27.1%)	0.08	0.44
Non drinker but chewer and smoker, C/C, C/T, T/T	29/44 (68.1%) 15/44 (31.8%)	30/43 (69.7%) 13/43 (30.2%)	**<0.04**	3.2	17/28 (60.7%) 11/28 (39.2%)	**<0.01**	**3.1**
Only alcohol Drinker C/C, C/T, T/T	16/30 (53.3%) 14/30 (46.6%)	38/70 (54.2%) 32/70 (45.7%)	0.93	0.56	47/69 (68.1%) 22/69 (31.8%)	0.96	0.74
Chewer, drinker and smoker, C/C, C/T, T/T	43/58 (74.1%) 15/58 (25.8%)	24/36 (66.6%) 12/36 (33.3%)	**<0.03**	3.8	21/35 (57.1%) 14/35 (42.9%)	**<0.002**	**4.1**

significantly higher in lung carcinoma, when compared with controls (Yu et al. 2002). Earlier Chaudhary et al. have reported that addiction habits such as areca nut chewing and alcohol abuse may enhance the expression of the 2G allele of MMP-1 genes in OSMF and HNSCC cases (Chaudhary et al. 2010).

The MMP-2 plays an important role in multiple stage carcinogenesis. Over expression of MMP-2 has been reported in oral squamous cell carcinoma (OSCC) (Franchi et al. 2002). Many researchers reported that MMP-2 plays critical roles in invasion and metastasis of these malignancies (Kusukawa et al. 1993, Kawamata et al. 1997, Kawamata et al. 1998, Kurahara et al. 1999). Expression of MMP-2 predicts poor prognosis in OSCC including tongue carcinoma (Yorioka et al. 2002, Yoshizaki et al. 2001). A number of studies have shown that MMP-2 is overexpressed in various cancer tissues and its involvement in tumor initiation, invasion, angiogenesis and metastasis (Yu et al. 2002, Price et al. 2001). In the case of MMP-2 (-168 G/T) polymorphism is not associated with a risk for development of OSMF ($p = 0.17$), as well as HNSCC ($p = 0.42$). While in the case of MMP-2 (-1306 C/T) polymorphism, it was found that TT allele may be associated as a risk factor for the development of HNSCC ($p < 0.00$), but not with OSMF. Therefore, it was concluded that MMP-2 (-1306 C/T) promoter may play as a risk factor for head and neck carcinogenesis.

MMP-9 has three repetitive type II fibronectin domains, which allow it to bind to ECM components, such as gelatin, collagen, and laminin (Visse et al. 2003). A functional cytosine (C) to thymidine (T) single nucleotide polymorphism at position -1562 in the MMP-9 promoter was reported. Transient transfection and DNA-protein interaction assays indicated that T allele-associated promoter activity (due to the preferential binding of a putative transcriptional repressor protein) was higher than the C allele-associated promoter activity (Zang et al. 1999). Many researchers have shown that this functional polymorphism was correlated to increased susceptibility to certain diseases. MMP-9 degrades type IV collagen, a major component of the basement membrane. The T allele in the MMP-9 promoter was associated with the invasive phenotype or susceptibility to gastric cancers (Matsumura et al. 2005). Conversely, breast cancer patients carrying the T allele had better prognosis compared to homozygous C/C carriers (Grieu et al. 2004) and Franchi et al. reported MMP-9 overexpression in head and neck cancers (Franchi et al. 2002). In our study, MMP-9 (-1562C/T) promoter polymorphism for T allele showed a significant association ($p < 0.01$) in HNSCC cases as compared to healthy controls. Therefore, it was concluded that MMP-9 (-1562C/T) promoter may also play as a risk factor for the development of head and neck squamous cell carcinoma (HNSCC) in Indian population. Similarly, Vairaktaris et al. concluded that MMP-9 polymorphism has an association with increased risk in oral cancer and observed that T allele carriers having an increased risk factor for the development this cancer (Vairaktaris et al. 2008). Recently author groups reported that SNPs in the MMP-1 promoter region may be associated with susceptibility to OSMF, as well as HNSCC cases in an Indian population (Chaudhary et al. 2010).

Tu et al. concluded that no strong correlation of the functional MMP-9 (-1562 C/T polymorphism was found in case of OSCC and OSMF in male areca chewers (Tu et al. 2007). While in our study MMP-9 (-1306 C/T) polymorphism, the C/T genotype showed a significant 2.5 fold ($p < 0.04$) risk in case of areca nut chewers, 3.2 fold ($p < 0.04$) risk in case of chewers & smokers and 3.8 fold ($p < 0.03$) risk in combination of chewer, drinker &smokers for the development of OSMF. While in the case of HNSCC, the C/T genotype showed significantly 3.3 fold ($p < 0.00$) risk in case of areca nut chewers, 3.1 fold ($p < 0.01$) risk in case of chewers & smokers, and 4.1 fold ($p < 0.002$) risk in combination of chewer, drinker &smokers for the development of HNSCC. In this study we concluded that MMP-9 (-1306 C/T) polymorphism may be associated with the various addiction habits in Indian population.

In this case control study, it was observed that genotypic distribution of MMP-9 (-1562 C/T) promoter polymorphism and age group less or more that 45 years of age, results showed significant association in genotypic distribution C/C, as well as C/T genotype ($p < 0.00$ & <0.01) distribution between the age group (<45 & ≥ 45) of potentially malignant lesions (OSMF) and healthy control, while in the HNSCC cases, only T allele showed a significant association between age groups (<45 & ≥ 45). Interestingly, in the older carcinoma subset, the T allelic frequency was significantly higher in case of both OSMF and HNSCC in Indian population. Similarly, Tu et al. reported that in older OSCC subset, the T allelic frequency was significantly higher in patients with buccal mucosa (BM) OSCC, than in patients with non-buccal mucosa OSCC (Tu et al. 2007). Recently we reported that receiver operating characteristics (ROC) analysis between the MMP-3 genotype (5A/6A) and age was to be significant in patients both over and less than 45 years of age in case of OSMF and HNSCC and also concluded that the expression of MMP-3 genotype may be associated with the 5A alleles in these lesions in Indian population (Chaudhary et al. 2010).

In the case of OSMF grading (Grade I, II, III & IV) with polymorphism of MMP-2 (-168 G/T), T allele did not show a significant association, while in the case of MMP-2 (-1306 C/T) and MMP-9 (-1562 C/T) promoter polymorphism T allele showed a significant association ($p < 0.02$ and $p < 0.04$ respectively) with the increasing progression of OSMF grading. In case of HNSCC grading, polymorphism of MMP-2 (-168 G/T)) T allele did not show significant association, while in the case of MMP-2 (-1306 C/T) and MMP-9 (-1562 C/T) promoter polymorphism T allele showed a significant association ($p < 0.003$ and $p < 0.00$ respectively) with the increasing progression of HNSCC grading. The non-keratinizing squamous epithelium on the buccal mucosa surface is the most susceptible target

for the damaging effects of areca nut. The disproportionate prevalence of OSCC among areca chewers might be explained by the fact that areca can induce MMP-9 expression and activity in saliva (Lin et al. 2006) and the induction might involve the enzyme encoded by the T allele (Lin et al. 2002). On the other hand, Vairaktaris et al. reported that T allele carriers have an increased risk for developing oral cancer only in initial stages, but not in advanced ones, and it may be due to the role of MMP-9 in the inhibition of angiogenesis by generating angiostatin from plasminogen (Vairaktaris et al. 2008).

Conclusions

In this study, results concluded that SNPs in MMP-2 (-1306 C/T) and MMP-9 (-1562 C/T) promoter region may be associated with susceptibility to HNSCC, and addiction habits such as areca nut chewing and tobacco smoking may enhance the polymorphic association of C/T allele of the MMP-2 and MMP-9 gene polymorphisms in an Indian population. This polymorphism could be a prognostic maker in head and neck cancer.

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Declaration of interest

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