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A study on genetic polymorphism in Matrix Metalloproteinases-3 in oral submucous fibrosis patients and in healthy individuals

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Abstract

Background: OSF is a potentially malignant condition affecting the oral cavity and oropharynx. MMP-3 also known as Stromelysin -I is a key member of the MMP family which is responsible for degradation of collagen type II,IV,V,IX and X, proteoglycans, gelatins, fibronectin, laminin and elastin. It plays an important role in activation of pro MMP-1 into the active form of MMP-1 in malignant tissues. MMP-3 expression is low in normal tissues but it is altered during tumour formation, where remodeling of ECM is required.

Purpose of the study: To assess the association of single-nucleotide polymorphisms, Adenosine (Insertion/Deletion) in -1171 5A > 6A in the MMP-3 promoter regions of patients with oral submucous fibrosis and in healthy individuals (controls).

Methods: Thirty cases of OSF were categorized according to Khanna et al classification into four groups and Twenty age and sex matched controls were included in the study. Blood samples were collected in EDTA coated vacutainers and PCR restriction analysis was done. A statistical analysis was done using Chi-square test and Fisher's exact test to assess the frequency and association of the alleles in the case-control group.

Results: The result showed a statistical significance difference between the duration of habit and disease severity with polymorphisms. The result also showed a higher frequency of the 5A allele in the study group as compared to the controls.

Conclusion: A long-term follow up of these patients is mandatory to see the prognosis and their susceptibility to malignancy. The positive outcome of an association of the disease with polymorphisms would result in the development of potential diagnostic and therapeutic possibilities in potentially malignant and malignant lesions.

Keywords: MMP-3, Oral submucous fibrosis, Genetic polymorphism

Background

Potentially malignant disorders of the oral mucosa are pathologies that have an increased potential for malignant transformation at any site in the mouth and oropharynx [1]. Worldwide, estimates of Oral submucous fibrosis (OSF) shows a confinement to Indians and Southeast Asians, with overall prevalence rate in India to be about 0.2 to 0.5% and prevalence by gender varying from 0.2 to 2.3% in males and 1.2–4.57% in females. It is predominantly seen in the second or third decade, and recent data suggest a male predominance; however, both sexes are equally at risk [2].

The condition is thought to be multifactorial in origin with a high incidence in people who chew arecanut, and a significant malignant transformation rate (7–30%)poses global problems for public health. In an epidemiological study on oral cancer and potentially malignant lesions in a rural Indian population, the malignant transformation rate of OSF was 7.6% over a 17-year period observation [3].

OSF is histopathologically characterized by fibrosis of subepithelial connective tissue. Collagens are the major structural component of extracellular matrix (ECM), hence precise regulation of collagen metabolism is

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essential to maintain the normal integrity of connective tissue. OSF is not only due to excessive deposition but also due to decreased collagen degradation. In OSF the equilibrium between Matrix Metalloproteinases (MMPs) and Tissue inhibitor matrix metalloproteinases (TIMP) is disturbed in such a manner that it ultimately results in increased deposition of ECM. Since OSF is considered as a high-risk pre-cancerous condition, knowledge of genetic susceptibility to malignant transformation is of immense importance, especially in developing cancer predictive markers for OSF patients.

Matrix metalloproteinases (MMPs) are a group of structurally related zinc-dependent endopeptidases and have the capability to degrade all components of extracellular Matrix. To date, at least 25 different vertebrate MMPs have been identified, 24 of which are present in humans, including two recently duplicated genes encoding MMP-23 [4]. These MMPs are classified into five main classes (collagenases, gelatinases, stromelysins, membrane-type and others, including the matrilysin) on the basis of their putative substrate specificity and internal homologies.

The evidence that individual characteristics play an important role in physiological and pathological processes has resulted in the targeted study of genetic polymorphisms in the clinical setting. In this context, mediators that degrade ECM, such as matrix metalloproteinases (MMPs), are highlighted in studies involving inflammatory and degenerative diseases, and principally the prognosis and metastasis of cancer [5]. For MMP genes, functional nucleotide polymorphism regulate the movement and metastasis of cancer cells [6].

MMP-3 also known as stromelysins I is a key member of this MMP family which is responsible for degradation of collagen type II,IV,V,IX and X, proteoglycans, gelatins, fibronectin, laminin and elastin. It plays an important role in activation of pro MMP-1 into the active form of MMP-1 in malignant tissues. MMP-3 expression is low in normal tissues but it is altered during tumour formation, where remodeling of ECM is required [7].

Common bi-allelic single nucleotide polymorphisms are found in promoter region of several MMPs. The promoter regions control transcription of the gene function. The promoter region of MMP3 is characterized by a 5A/6A promoter polymorphism at position -1171 in which one allele has six adenosine (6A) and the second has five adenosine (5A). A single adenosine insertion/deletion polymorphism (5A/6A) at position -1171 of the MMP-3 promoter region causes different transcription of MMP-3 expression. Such genetic polymorphisms are vital because they can be used as biomarkers that indicate for prognosis of potentially malignant and malignant lesions and thus may be involved in early intervention and diagnosis in patients at high risk [7].

The presence of the 5A allele has been associated with susceptibility for coronary heart disease, abdominal aneurysm and inflammatory disorders such as areca-related oral submucous fibrosis, celiac disease as well as ovarian and breast carcinomas [8–11]. By contrast, the 6A allele has been associated with carotid intima-media thickening and the progression of coronary artery disease, restenosis after balloon angioplasty, angiographic coronary atherosclerotic lesion growth and stenosis suggesting that a lower expression may result in matrix accumulation, faster arterial wall thickening and plaque progression [6, 12].

Thus, we conducted a cross- sectional case control study with the purpose of evaluating the association of promoter(-1171 5A- > 6A) polymorphism in the MMP-3 gene in patients suffering from Oral submucous fibrosis. The main aim and objective of this study was to correlate the association of -1171 5A > 6A polymorphism in the MMP-3 gene in OSF patients and healthy individuals (controls) and to to assess the association of single-nucleotide polymorphisms, Adenosine (Insertion/Deletion) polymorphism in -1171 5A > 6A in the MMP-3 promoter regions of patients with oral submucous fibrosis.

Methods

The study was conducted to assess the genetic polymorphism of MMP-3 gene in oral submucous fibrosis(OSF). This study included patients with OSF and healthy individuals as controls. They were selected at random from among the patients visiting the Department of Oral Medicine and Radiology, SDM College of Dental Sciences, Sattur, Dharwad, India. Prior to the collection of clinical samples ethical clearance was obtained from the institution review board (**IRB No.2013/P/OM/16**). This case-control study group included 50 participants of which 30 patients suffered from OSF and 20 controls were apparently healthy individuals devoid of any habits related to tobacco chewing or smoking or alcohol consumption. The study was conducted during the period of June 2014 to September 2015.

Inclusion criteria for the study group comprised of clinically and histopathologically proven OSF and for controls age and sex matched apparently consenting healthy cases.

Exclusion criteria included cases with prior history of cancer, pregnant women, previously treated or under the course of treatment of OSMF, hematological and autoimmune disorders.

Along with the samples patient's information pertaining to socio-demographic factor such as age, sex, religion, habits, education, occupation, income, marital status, diet, family history were collected as per

predesigned data collection sheet. A consent form in vernacular was prepared to obtain the informed consent from the subjects.

Peripheral Blood Mononuclear Cell (PBMC) samples were collected from consenting subjects after obtaining their informed consent. EDTA coated vacutainers were used for the collection of 3–4 ml PBMC. The collection tubes were coded and transported to the laboratory in ice-cold condition (4 °C).

DNA isolation

DNA was isolated using standard isolation kit (DNeasy Blood and Tissue Kit, Cat. No. 69506, QIAGEN, USA). After optimization, genomic DNA was isolated. The extracted DNA was quantified and purity was assayed using UV Spectrophotometer at 260 and 280 nm.

Genotyping of the MMP-3 promoter polymorphism

The primers used for the amplification of the region of the MMP-3 gene that contained the -1171 5A- > 6Apolymorphism Table 1.

Polymerase chain reaction

The 25 μl PCR reaction mixture was prepared that had 0.2 μM forward and reverse primer each; 200 μM of dNTPs (NEB #NO447L); 1X Taq Buffer, 1unit Taq enzyme (NEB #MO273L); about 1000 ng of DNA made upto volume with nuclease free water.

The PCR was performed in three steps: first, 5 min at 94 °C; then, 35 cycles of 30 s at 94 °C, 30 s at 57 °C, and 90 s at 72 °C; lastly, 1 h at 72 °C. The amplicons were denatured for 5 min at 100 °C, and mixed with formamide-containing stop buffer, and then subjected to electrophoresis on 4% polyacrylamide gel.

After the PCR amplification, the amplicons were run through 4% agarose gel electrophoresis and the DNA bands were observed in gel documentation (Figs. 1 and 2).

Restriction enzyme digestion

All PCR products (10 μl) were digested with 10units of *BstUI* (NEB # R0518)/ *Tth111I* (NEB # R0185) restriction endonuclease enzyme. Restriction enzyme digestion was carried out by incubating at 37 °C for 15 min. The restriction enzyme digestion was subjected to agarose gel electrophoresis.4% agarose was prepared in 1X TAE buffer (300 ml). To this 30 μl of ethidium bromide was added.5 μl of restriction enzyme digest product was mixed with 3 μl of loading dye bromophenol blue. The electrophoresis was conducted at a constant voltage of 100 V for 60 min. The observed bands were analysed under the gel documentation chamber (Fig. 3).

Statistical analysis

The association of the variants of case-control group and the MMP-3 genotypes were analysed with Chi-Square test. Differences between the values were considered significant when $P < 0.05$.

The frequency of distribution and associations between the MMP-3 genotypes and the risk of disease genesis were estimated by odds ratio (OR), and 95%confidence interval (CI) were calculated by the Fisher's exact test.

Results

This research was taken up with the intention to study the association and correlation of single nucleotide polymorphism in OSF, healthy individuals and also to assess the association of this polymorphism, Adenosine (Insertion/Deletion) in **-1171 5A > 6A** in the MMP-3 promoter regions OSF patients. The results show the association of three genotypes **5A/5A, 5A/6A, 6A/6A and the variables.**

The quantity of the products consumed showed a significant difference with the 5A/5A genotypes. The study group that showed the presence of the 5A/5A genotype, consumed less than 5 packets of tobacco per day.

On computing the chi-square test of association for duration, frequency, stages of OSF and genotypes, the association of duration of the habit and disease severity was significant with the genotypes.

On computing the fisher's exact test we found a statistical significant association of the 5A allele and 6A allele with the study and control group. The frequency of the 5A allele in OSF cases was 84.6%and the frequency of the 6A allele was 56.32%. Thus, it was noted that the frequency of 5A genotype is higher in OSF when compared with controls.

Discussion

Fibrosis is a dynamic change between fibrogenesis and fibrolysis with a net output toward fibrogenesis. OSF is a disease which can be induced by areca chewing producing disruption in stromal tissue and cytokine regulation.

Table 1 Primers

Primer Name	Sequence (5′–3′)	GC Content (%)	Tm (°C)	Product Size
MMP F	GGTTCTCCATTCCTTTGATGGGGGGAAAGA	50	77.7	129
MMP R	CTTCCTGGAATTCACATCACTGCCACCACT	50	76.6	

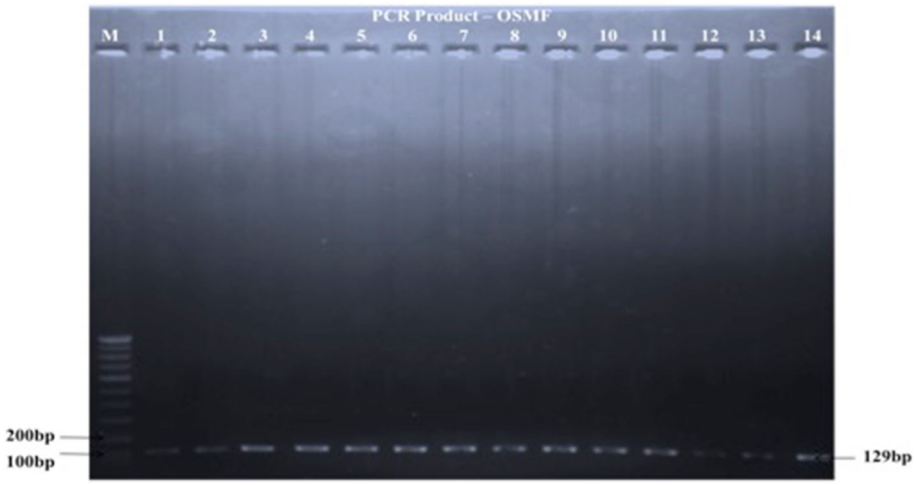

Fig. 1 1% agarose gel image of PCR products of OSMF samples. Line M – DNA ladder of 100 bp. Line 1–14 – OSMF sample 1 to 14

Similar to OSF, liver cirrhosis, radiation induced fibrosis, and systemic sclerosis also show imbalance in fibrotic hemostasis. Under different areca exposure history, polymorphisms of genes associated with collagen homeostasis were reported to be correlated with the risks of OSF [9].

India is the fourth largest consumer of tobacco/betel quid in the world and the third largest producer of tobacco/betel quid after China and Brazil. The synergistic effect on the carcinogenic potency of tobacco/betel quid in oral cancer by alcohol consumption is well-documented in the literature. There are about 250 million tobacco/betel quid users in India who account for about 19% of the betel quid users /betel quid users [13].

MMPs play an important role in fibrosis of the oral cavity. Various types of MMPs are known to be expressed and activated in patients with OSF and in HNSCC. OSF is a potentially malignant disorder, which can be induced by areca nut chewing. Due to excess chewing of areca nut; arecoline may increase the production of collagen fibers in the sub epidermal layer of buccal cavity and causes fibrosis. Chiu et al reported that areca chewing as well as polymorphism of genes associated with collagen homeostasis was correlated with risk of OSF [5]. Shin et al suggested that polymorphism of transforming growth factor (TGF-a) related to an innate immune response was also found to associated with the risk of OSMF [14].

MMP-3 has a wide substrate specificity for various ECM components and the expression of MMP-3 is regulated at the transcription level where the promoter region of the gene corresponds to growth factors, cytokines and some environmental factors associated with ECM [7]. Thus in our study the objective was to determine the association and correlation of single nucleotide polymorphism in OSF, healthy individuals and also to assess the association of this polymorphism, Adenosine (Insertion/Deletion) in -1171 5A > 6A in the MMP -3 promoter regions OSF patients.

Patients of OSF were diagnosed on the basis of clinical signs including trismus, presence of fibrotic bands in the oral cavity and were histopathologically confirmed. Emphasis was given to addictions like areca nut, tobacco and alcohol. MMP -3 promoter genotypes in patients

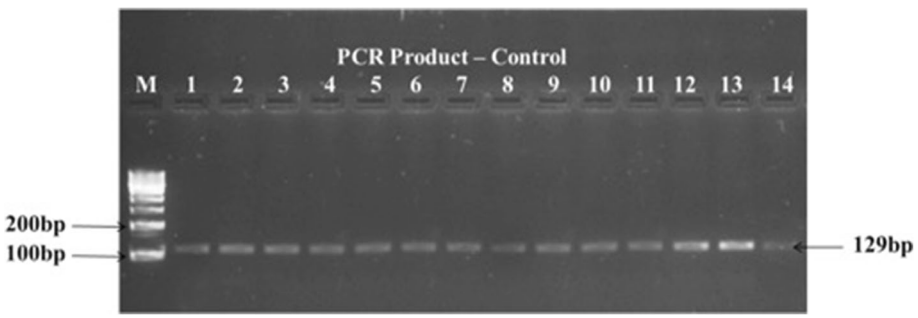

Fig. 2 1% agarose gel image of PCR products of Control samples. Line M – DNA ladder of 100 bp. Line 1–14 – Control sample 1 to 14

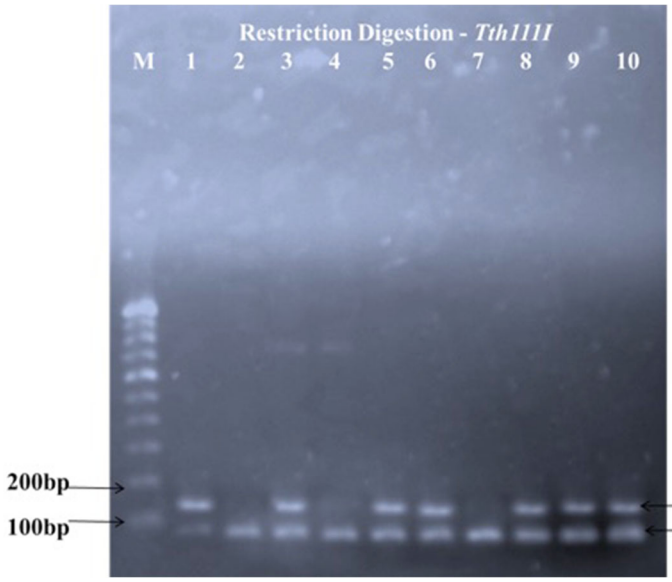

Fig. 3 4% agarose gel image of Restriction digestion PCR products with *Tth111I* enzyme. Line M – DNA ladder of 100 bp. Line 2,4 and 7 – 5A/5A genotype showing only 97 bp fragment in OSMF sample Number 11,17 and 26 respectively. Line 1,3,5,6,8,9 and 10 – 5A/6A genotype showing 129 and 97 bp fragments in OSMF sample Number 6,14,19,22 and 29 respectively and control sample number 10 and 16 respectively

with OSF and controls were analysed with respect to gender, age, habits like betel quid chewing with or without tobacco and alcohol intake.

OSF patients in the study group were 30 and 20 in the control group. Total number of males included were 46 and 4 females. The finding of male predominance of OSF cases was similar to other studies elsewhere in India where the male: female ratios of OSF cases were 3:1, 2.3:1,10:1 5:1 respectively [15–17]. In our study we assessed the association of the genotypes with gender predilection. This study showed the presence of the 5A/5A, 5A/6A genotype only in the male population. Considering, a lesser number of female patients in the study group as well as in the control group, we found no significant difference between polymorphisms and gender Fig. 4 and Table 2.

Chang et al found that arecoline acted not only as an inhibitor of gelatinolytic activity of MMP-2, but also a stimulator for TIMP- 1 activity in OSMF. Their study concluded that MMPs transcription activity might be associated with genesis of OSF in younger areca nut chewers [18]. In our study the distribution of the individuals was based on the mean age which was 33.71 years. It was noted that the age group between 19 and 34 years were in majority in the study group, which was similar to studies by Rupak et al, Katharia et al and Maher et al. [19–21]. More than 64% of cases were below 35 years of age in accordance with a study by Sinor et al [22]. People less than 35 years were 5.3 times more likely to develop OSMF as compared to the older population [23–25]. Interestingly this finding also coincides with the WHO criteria IARC monographs on oral cancer [26]. However, in our study there was no statistical significance between the age groups and polymorphisms (Fig. 5).

OSF has a malignant transformation rate of 7–30% and the pathogenesis is thought to be multifactorial. One of the main pathogenesis is the chewing of areca-nut. The carcinogenic effects of tobacco acting in synergy with areca-nut is well known, but the second report on betel quid by the International Agency for Research on Cancer (IARC) identified areca-nut as a "group one carcinogen [2].

In a study conducted by Seedat et.al it was interesting to note that some patients developed OSF after a short areca-exposing time. However the study concluded that chewing betel nut will lead to pathological changes in the mucosa, but, neither the frequency nor the duration of the habit, are accurate predictors of the extent of these changes or when they are likely to occur [27].

In a previously conducted study by Choudhary et.al with the MMP-3 gene polymorphism in OSF and HNSCC there was a significant difference in the MMP-3 genotype distribution between the areca nut with tobacco chewers and the non - chewers both in OSMF and HNSCC [28].

In our study 73.4% of the patients in the study group consumed betel quid with tobacco and 13.3% were solely betel quid chewers. The remaining 13.3% consumed tobacco with betel quid along with alcohol. Thus, we analysed the association of the type of habits in the population with the genotypes. However in our study there was no significant

Group	Gender	Frequency	Percentage (%)
Study group (OSF)	Male	28	93.3
	Female	2	6.7
	Total	30	100
Control group	Male	18	90
	Female	2	10
	Total	20	100

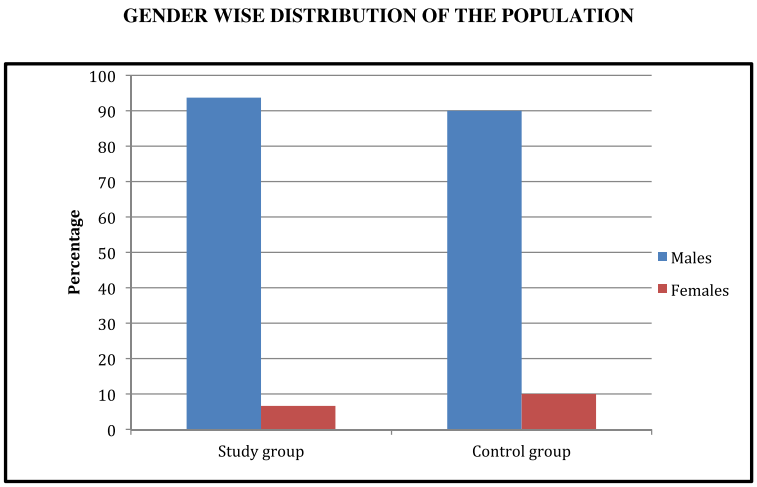

Fig. 4 Genderwise distribution of the study population. Gives a description of the percentage distribution of the study population in the two study groups based on the gender. It is observed that, about 93.3% of the males and 6.7% of the females in the study population belonged to the OSF Group. The majority of the population in the OSF Group were males (93.3%)

Table 2 Association of gender with 5A/5A,5A/6A,6A/6A in study and control groups

Group	Gender	5A/5A		5A/6A		6A/6A	
		Present	Absent	Present	Absent	Present	Absent
Study group	Males	3	25	5	23	20	8
	Females	0	2	0	2	2	0
	Total	3	27	5	25	22	8
Control group	Males	0	18	2	18	18	2
	Females	0	2	0	2	0	2
	Total	0	20	0	20	0	20
Chi square value		0.238		0.429		0.779	
p value		0.626		0.513		0.377	

The female population included in the study group was 6.7% and in the control group was 10%. The frequency of the 5A/5A, 5A/6A genotype in the study population was present only in males
On computing the Chi-square test there was no significant difference present between the gender and genotypes

difference between the type of habit and polymorphisms. Hence, this finding does not highlight the association of the type of habit-history in a patient suffering from OSF with polymorphism (Fig. 6).

To identify the risk factors associated with OSF we considered other parameters which included duration, quantity and frequency of chewing and the stages of OSMF. There was no significant association of the duration and frequency of chewing and the stages of OSMF with polymorphisms (Fig. 7). However, the association of the quantity of the products consumed showed a significant difference with the 5A/5A genotypes. The study group that showed the presence of the 5A/5A genotype, consumed less than 5 packets of tobacco per day Tables 3 and 4.

Considering the above findings of the study group we correlated each of the variables in the study group to the genotypes. On computing the chi-square test of association for duration, frequency, stages of OSF and genotypes, the association of duration of the habit and stage of OSF was significant with the genotypes Table 5. Thus we conclude that polymorphism

Age–groups	5A/5A	5A/6A	6A/6A
19–34 years	3	4	25
35–50 years	0	2	12
51–67 years	0	1	3
P value	0.40	0.793	0.811

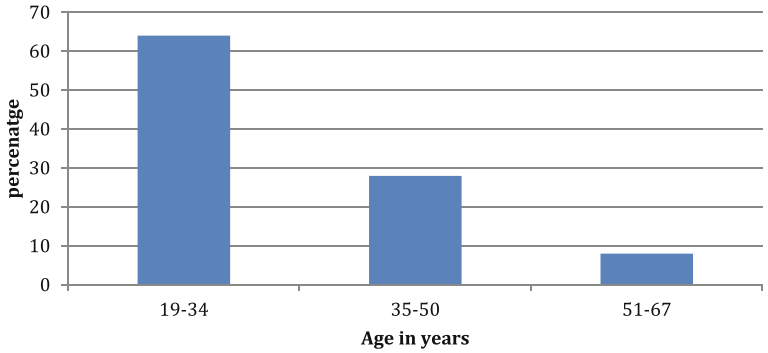

Fig. 5 Describes the population distribution according to age. The age range of the study population was 19–67 years. As it was observed the mean age of the entire population was 33.71 years. Most of the individuals, 64% in the study group belonged to the age group of 19–34 years. which apparently coincides with the age distribution worldwide in terms of propensity to PML. Association of Age groups with 5A/5A, 5A/6A, 6A/6A in. No significant association was present between the age-groups and polymorphism

Type of habit	Genes_Present		
	5A/5A	5A/6A	6A/6A
Betel quid	0	0	4
Betel quid + tobacco	2	3	17
Betel quid + tobacco + smoking + alcohol	1	2	1
No habit	0	2	18
p value	0.21	0.15	0.01

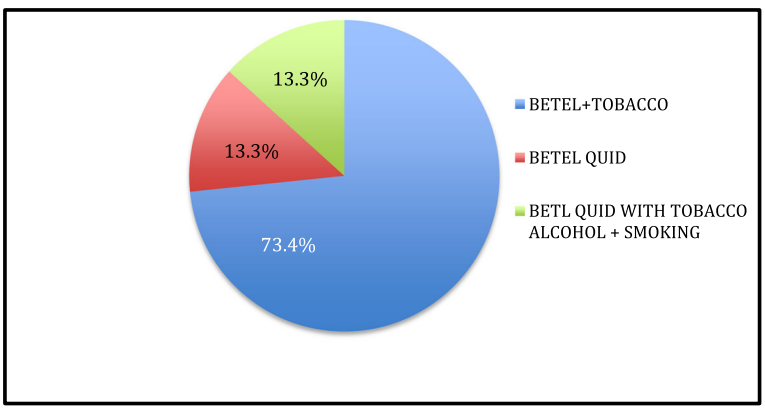

Fig. 6 Describes the population distribution based on the type of habit. Majority of the population were betel quid chewers with tobacco (73.4%). Thus the synergistic effect by combining tobacco with betel quid, alcohol and smoking was also considered to assess their association in developing OSF and their correlation with polymorphism. Association of type of habit with polymorphism. No significant difference was noted with the type of habit and 5A/5A, 5A/6A(polymorphism)

Group Of Osmf	Frequency	Percentage(%)
GROUP I	3	10.0
GROUP II	10	33.3
GROUP III	8	26.7
GROUP IV	9	30.0

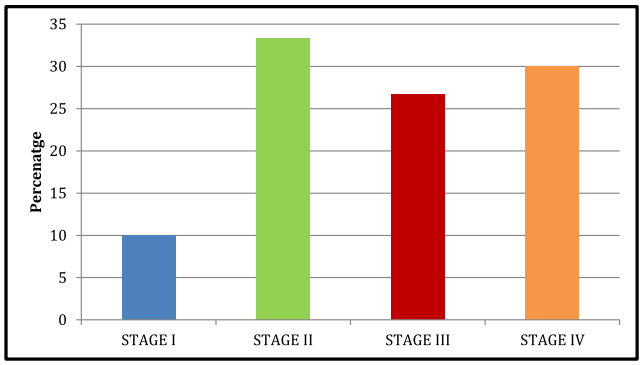

Fig. 7 The majority of individuals suffering from OSF belonged to the Group IV,III respectively. Thus the above graph shows 56.7% of the individuals in the study belonged to the moderate and severe stages of OSF Association of Duration of the habit with stages of OSF were statistically significant $p = 0.01$ and $p = 0.04$ with 5A/5A, 5A/6A,6A/6A genotypes. Thus the association of duration of the habit and stage of OSF was significant with the genotypes

can be associated with a prolonged habit history causing an increase in the disease severity but the possibility of progression or risk markers susceptible for malignancy can be evaluated by further increasing the sample size in the study group. This is also important in the view that the individual mechanisms operating at various stages of the disease initial, intermediate and advanced need further study in order to establish an association with polymorphisms and to propose appropriate therapeutic interventions [29].

Chaudhary and colleagues in their study found that the 5A allele might play an important role in the susceptibility to HNC, as individuals with 5A/5A genotype had nearly two fold risk of HNSCC (OR = 1.94) when compared to controls [28]. However, Tu and colleagues found that the 5A/5A genotype was associated with the risk of oral submucous fibrosis but not OSCC [9].

In a study conducted by Choudhary et al the frequency of 5A/6A or 5A/5A promoter genotypes having 5A alleles was associated with MMP-3 single nucleotide polymorphism (SNP) in OSF (5A allele frequency = 0.15, p = 0.01, OR = 2.26, 95% CI = 1.22–4.20). Similar difference was also found in MMP-3 genotype distribution in HNSCC (5A allele frequency = 0.13, $p = 0.03$, OR = 1.94, 95% CI 1.06–3.51) as compared to controls (5A allele frequency = 0.07) [30]. Hence we evaluated the association of the genotypes in MMP-3 gene -1171 5A > 6A polymorphism in OSF patients and healthy individuals (controls) devoid of any habits. The entire population included in the study comprised of 50 individuals comprising of 30 cases and 20 controls. The total number of individuals exhibiting polymorphisms were 10.

Table 3 Distribution of the subjects based on the quantity of the products consumed

Quantity	Frequency	Percentage(%)
Less than 5 packs/day	17	56.7
More than 5 packs/day	13	43.3

56.7% in the study population chewed less than 5 packs 43. 3% chewed more than 5packs

Table 4 Association of quantity of the habit with polymorphism

Quantity (No of packets per day)	5A/5A	5A/6A	6A/6A
Less than five	3	4	10
More than 5	0	1	12
Controls	0	2	18
p- value	0.045	0.37	0.02

The association of the quantity of the product consumed was statistically significant with the 5A/5A polymorphism $P = 0.04$

Table 5 Results of chi- square to evaluate association the variables in the study group to the genotypes

	OSF Group			Genotypes		
	Duration		Stage	5A/5A	5A/6A	6A/6A
Chi-Square	19.800	8.253	15.200	38.720	25.920	18.000
df	4	8	4	1	1	1
Asymp. Sig.	.001	0.40	.004	.000	.000	.000

Association of Duration of the habit with stages of OSF were statistically significant $p = 0.01$ and $p = 0.04$ with 5A/5A, 5A/6A,6A/6A genotypes
Thus the association of duration of the habit and stage of OSF was significant with the genotypes

In the OSF group the homozygous 5A > 5A genotypes were seen in 3 individuals and was not present in controls. The heterozygous 5A > 6A genotypes were seen in five individuals and in two controls. The 6A/6A genotype was distributed evenly in the study and control group. The presence of the 5A/6A genotype in the control group may suggest that these patients could be at a relative risk of developing OSF either due to the effects of environmental factors or the possibility of higher exposure of such individuals to the environmental factors since birth that must have resulted in the polymorphism.

In our study the association of the -1171 5A > 6A polymorphisms in the promoter region of MMP-3 gene was not significant in the study and control group. On analysing the different genotypes it was noted that the polymorphic association of these genotypes was not statistically significant as the proportion of the population (50) with the 5A/5A and 5A/6A was in the ratio of 40:10, suggesting that the polymorphism is incoherent considering small sample size (Fig. 8).

Further, we also analysed the association of the frequency of 5A/6A or 5A/5A promoter genotypes having 5A alleles with MMP-3 single nucleotide polymorphism (SNP) in OSF and found that the frequency of the homozygous 5A allele and 6A allele present in the OSF group was more than controls. This result is similar to the study conducted by Choudhary et.al, which showed a significant difference in 5A allele frequency between OSF and HNSCC as compared with controls. The study concluded that the 5A genotype had a more than two fold risk for OSF (OR = 2.26) and little less in HNSCC (OR = 1.94) in relation to the control group [28].

Group		5A/5A n(%)	5A/6A n(%)	6A/6A n(%)
Study group	Present	3(10.0)	5(16.7)	22(73.3)
	Absent	27(90.0)	25(83.3)	8(26.7)
Control group	Present	0(0.0)	2(10.0)	18(90.0)
	Absent	20(100.0)	18(90.0)	2(10.0)
Chi square		2.12	0.443	2.083
p value		0.145	0.50	0.149

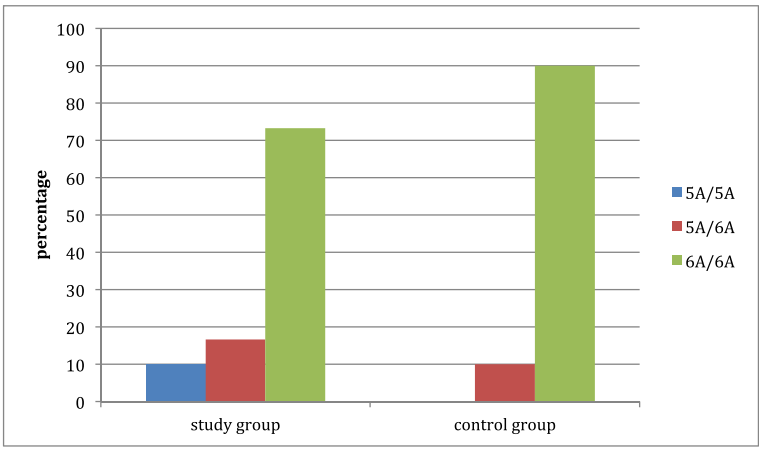

Fig. 8 Results of chi- square to evaluate association the variables in the study group to the genotypes

In accordance to the above study, on computing the fisher's exact test we found a statistical significant association of the 5A allele and 6A allele with the study and control group. The frequency of the 5A allele in OSF cases was 84.6%and the frequency of the 6A allele was 56.32%. Thus, it was noted that the frequency of 5A genotype is higher in OSF when compared with controls (Fig. 9).

This finding is also in accordance to the study conducted by Tu et al. on MMP3 functional promoter polymorphism in male areca users showing an association with the risk of OSF. The study also concluded that the genotypic linkage among the collagen-related genes, MMPs, TIMPs, and cytokines related to OSF formation requires more information to specify the risk of OSF in areca chewers [9].

Conclusion

Further study in the field is required with a larger sample size to highlight the role of the polymorphisms in the -1171 5A > 6A promoter of MMP3-gene with OSF and also their presence in healthy individuals. A long term follow up of these patients is mandatory to see the prognosis and their susceptibility to malignancy. Hence, the positive outcome of an association with polymorphisms would result in the development of potential diagnostic and therapeutic possibilities in potentially malignant and malignant lesions.

The following were the observed limitations and a few recommendations:

1. With respect to the categorization of study groups, tobacco /betel quid chewers with OSF were included; but to establish the role of polymorphism, inclusion of patients who had the tobacco habit but did not suffer from OSF could have been considered as well.
2. The possible errors during the chemical solvent preparation, the standardisation and method optimisation contribute to limitations in the analysis.
3. The logistics of transporting serum samples, maintaining a temperature of 4°c and carrying out the processing in a distant laboratory from

ALLELE FREQUENCY	5A	6A	ODDS RATIO (OR)	95%CI	Fisher's exact test (one-tailed)	Fisher's exact test (two - tailed)	Pearson chi square test
STUDY GROUP	**11**	49	4.27	0.84-41.37	**p= 0.046***	p=0.07	p=0.052
CONTROLS	2	38					
TOTAL	13	87					
FREQUENCY IN OSF	**84.6%**	56.3%					

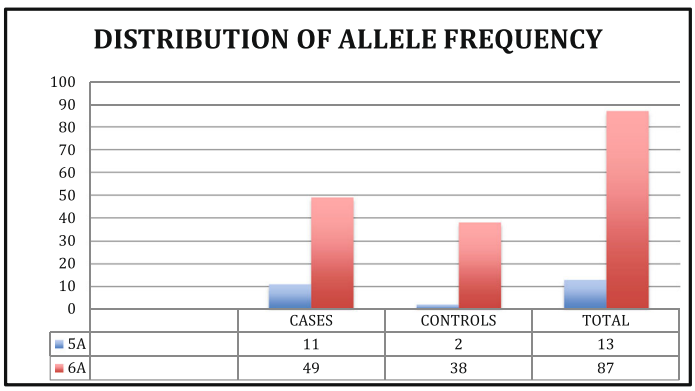

Fig. 9 The above table shows the total number of alleles distributed in the case control group. Total number of 5A alleles were 13 and the 6A alleles were 87. On computing the Fisher's exact test one-tailed there was a significant association of the 5A alleles and 6A alleles with the cases and control group, $P = 0.046$. The frequency of the 5A allele and the 6A allele in the OSF group was greater than that in the control group. However the frequency of 5A allele in OSF was higher when compared to controls

the site of sample collection were possible limitations.
4. The reliability of the history of tobacco habit, with respect to the product, the duration and the frequency as elicited from the patient, is questionable.
5. The evidences supporting the relationship between OSF formation and MMPs expression are uncertain. Because the increased expression of TIMP-1 and its inhibitory activity toward MMP activity may underlie the pathogenesis of OSF, it is important to know whether the OSF subjects carrying 5A alleles in MMP3 had the genotypic repression of TIMP-1 [30].

Since OSF is considered as a high-risk pre-cancerous condition, knowledge of genetic susceptibility to malignant transformation is of immense importance, especially in developing cancer predictive markers for OSF patients [10].

Abbreviations
bFGF: Basic Fibroblastic growth factor; BQ: Betel quid; CTGF: Connective tissue growth factor; DNA: Deoxyribonucleic acid; ECM: Extracellular matrix; FGF: Fibroblast growth factor; HNSCC: Head and Neck squamous cell carcinoma; IF: Interferon; IL: Interleukin; LOX: Lysyl Oxidase; MMPs: Matrix Metalloproteinases; OL: Oral Leukoplakia; OPL: Oral Premalignant lesions; OSF /OSMF: Oral submucous fibrosis; PAI: Plasminogen activation inhibitor; SNP: Single- nucleotide polymorphism; TGFβ: Transforming growth factor beta; TIMPs: Tissue inhibitor of metalloproteinases; TNF-α: Tumor necrosis factor

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Availability of data and materials
The in-vitro study has been done in the biotechnology department of PC Jabin College Hubli.
The details of the materials used and the data collected have been included in the manuscript.
Data of the patients are collected in patients file in SDM College of Dental sciences.

Authors' contributions
PP carried out the molecular genetic studies, participated in the sequence alignment and drafted the manuscript. SH participated with the DNA isolation and PCR procedures. AP,KB and SH participated in the design of the study. MM performed the statistical analysis. PP, AS analysed the data of the study and participated in its design and coordination and helped to draft the manuscript. AP and KB approved the final version to be published. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Consent for publication
I have read and understood the consent form and the information provided to me. I was free to ask any questions and they have been answered. I exercise my free power of choice and hereby give my consent to be interviewed and examined by the investigators. I agree to cooperate for any investigations considered necessary for my treatment. I consent to the taking and publication of any photographs in the course of this procedure for the purpose of advancing health education. I understand that my identity will remain confidential.
The above consent form states the identity of the patient will remain confidential. Hence the images or the written data in the manuscript does not reveal the patients identity and does not breach his /her terms of privacy.

Ethics approval and consent to participate
IRB No.2013/P/OM/16. At the institutional review board meeting held on 15-11-2013,the study titled "A Study On Genetic Polymorphism In Matrix Metalloproteinases-3 In Oral Submucous Fibrosis Patients And In Controls." Was presented and discussed. The Committee has decided to approve and grant ethical clearance for the study to be carried out by the Principal Investigator and Dr. Atul P Sattur as guide at SDM College of Dental Sciences and Hospital,Sattur,Dharwad.
All the members voted for the proposed study and none of the members voted against the proposed study.

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