Original Research Article

A Study on Role of Cystatin C (CST3) GENE and its Susceptibility to Oral Submucous Fibrosis

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Abstract

Background &Objectives: OralSubmucous Fibrosis (OSF) is a chronic disorder characterized by fibrosis of the mucosa lining the upper digestive tract involving the oral cavity, oro- and hypopharynx and the upper third of the oesophagus. The alkaloids from areca nut are the most important chemical constituents biologically, in producing this lesion.Arecoline a product of areca nut was found to elevate Cystatin C mRNA (CST3) and protein expression in a dose-dependent manner. Cystatin C is a 13 kDa non-glycosylated, basic protein belonging to the cystatin family. It is consistently and dramatically upregulated in a variety of fibrotic diseases. However, little is known about the correlation between cystatin C and its role in OSF. **Aim**: The aim of the study was to assess the cystatin C gene profile in areca nut chewers with oral submucous fibrosis (cases) and without oral submucous fibrosis (controls). **Methods:**20 cases of OSF and 20 age and sex matched controls were categorized 4 groups 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 Odds ratio to assess the frequency and association of the alleles in the case-control group. **Results:**Significant difference (p value <0.10) obtained between the case and control subjects. **Conclusion:**The results of this study show that cystatin C gene profile was significantly higher in study groups compared to controls.

Keywords: Areca nut; Cystatin C (CST 3); oral submucous fibrosis; genetic polymorphism

Introduction

Oral Submucous Fibrosis (OSF) is a chronic disorder characterized by fibrosis of the mucosa lining the upper digestive tract involving the oral cavity, oro- and hypopharynx and the upper third of the oesophagus. The alkaloids from areca nut are the most important chemical constituents biologically, in producing this lesion. Arecoline a product of areca nut was found to elevate Cystatin C mRNA (CST3) and protein expression in a dose-dependent manner. Cystatin C expression was significantly higher in OSF specimens and expressed mainly by fibroblasts, endothelial cells, and inflammatory cells. Cross-links between the molecules are essential for the tensile strength of collagen fibres. These areas are resistant to attack by collagenases but can be attacked by a number of other serine and cysteine proteinases. CST3 encoding a cysteine proteinase inhibitor might contribute to the stabilization of collagen fibrils in OSMF. It has been well established by researchers that virtually all oral cancers are preceded by visible clinical changes in the oral mucosa usually in the form of white or red patch,[1] to which tobacco use is one of the most important risk factors for the development of these oral mucosal lesions including oral potentially malignant disorders (OPMDs)and cancer[2].Prevention and early detection of such oral potentially malignant disorders (OPMDs) have the potential of not only decreasing the incidence but also improving the survival of those who develop oral cancer.

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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 0.2-2.3% 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[4].Oral submucous fibrosis (OSF) is a chronic, insidious oral mucosal condition. The hallmark of the disease is submucosal fibrosis that affects the oral cavity and progressively involves the pharynx and the upper esophagus. It is characterized by juxta-epithelial inflammatory reaction followed by chronic change in the fibro- elasticity of the lamina propria and is associated with epithelial atrophy[5].It is usually considered to be a result of persistent direct contact of the quid mixture with oral tissues resulting in continuous irritation and mucosal breach by various components, including biologicallyactive alkaloids (arecoline, arecaidine, arecolidine, guvacoline, guvacine, flavonoids (tannins and catechins) and copper[6]. This leads to burning sensation in the oral cavity, blanching, and stiffening of oral mucosa and oropharynx, ultimately resulting in restricted mouth opening.Collagen fibers form three-dimensional scaffolding by combining cross-linked collagen molecules with other extracellular matrix components. The terminal regions of each collagen molecule consist of terminal peptides, which contain the sites of intra- and intermolecular cross-links. These areas are resistant to attack by collagenases but can be attacked by a number of other serine and cysteine proteinases. CST3 (Cystatin C) encoding a cysteine proteinase inhibitor might contribute to the stabilization of collagen fibrils. Because Collagenase, CST3, and Lysyloxidase have been well documented to be involved in the collagen biosynthesis and degradation, they are hypothesized to contribute to the pathogenesis of OSF by increasing collagen synthesis and reducing collagen

degradation. Cystatin C, in turn, inhibited the lysosomal cysteine proteases like Cathepsin B and H, resulting in decreased degradation of collagen[9].This suggests that cystatin C may play an important role in the pathogenesis of areca quid chewing-associated OSF. On the basis of these observations, the present work was undertaken to identify the in situ localization of cystatin C expression in OSF specimens. More specifically, we have therefore measured the relative levels of cystatin C in OSF compared with normal subjects and the effects of arecoline, a major areca nut alkaloid, on cystatin C in normal subjects.

Aims & Objectives

1. To assess the Cystatin C gene profile in patients who were areca nut chewers with oral submucous fibrosis (cases).
2. To assess the Cystatin C gene profile in areca nut chewers without oral submucous fibrosis (controls).
3. To evaluate the cystatin C gene profiles in cases as compared to control.

Materials and Methods

Study Design Source of Data: Study was conducted on the patients visiting the Department of Dental Sciences, Ayaan Institute of Medical Sciences, Hyderabad, Telangana, India. Prior to the collection of clinical samples ethical clearance was obtained from the institution review board.This case-control study group included 40 participants of which 20 patients who chewed arecanut and suffered from OSF and 20 controls who chewed arecanut but were apparently healthy individuals devoid of OSF.

Method of Collection of Data

Inclusion Criteria: 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

- Patients who have been previously treated or under the course of treatment of OSF,
- Chronic systemic conditions (heart, kidneys, liver, or infectious diseases including AIDS)
- Undergoing antibiotic or steroid treatment,
- Pregnant and lactating mothers,
- Systemic disorders associated with psychological morbidity, and
- Patients unwilling to participate in the study

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.

Methodology:

1. **Sample Collection:**Peripheral Blood Mononuclear Cell (PBMC) samples were collected from consenting subjects after obtaining their informed consent. EDTA (Ethylene diamine tetraacetic acid) coated vacutainers were used for the collection of 2 ml PBMC from antecubitalvein . The collection tubes were coded and transported to the laboratory in -20ºC cryobox and stored at -20ºC deep freezer.
2. **DNA (De-oxy ribo Nucleic Acid)isolation:**DNA was isolated using a rapid non-enzymatic method, after optimization, genomic DNA was isolated using the following protocol.
3. **DNA quantification:** DNA quantification and purity was assayed using EPOCH nano drop spectro-photometeric analyzer.
4. **Primer design:** FP 5´-TGGGAGGGACGAGGCGTTCC-3´ RP 5´-TCCATGGGGCCTCCCACCTG-3´
5. **Polymerase Chain Reaction Protocol: This protocol serves as guideline for primer reactions.**

Composition of Taq DNA polymerase 2x Master Mix RED (1.5mM $MgCl_2$ final concentration)

Tris - HCL pH 8.5,$(NH_4)_2SO_4$.3mM $MgCL_2$, 0.2% 0.4 mM of each Dntp 0.2 units /µl cybergreen Inert red dye and stabilizer

T E Buffer preparation:10 mMTris-Cl, pH 7.5 1 mM EDTA,

Make from 1M stock of Tris-Cl (pH 7.5) and 500 mM stock of EDTA (pH 8.0).

10ml 1M Tris-Cl pH 7.5 per L - 2ml stock of 500mM EDTA (pH 8.0).

1M Tris (crystallized free base): Tris(hydroxymethyl) amino methane FW 121.4 g/mol 60.57 g in 0.5L mq water pH to 7.5 using HCl in 0.5M EDTA

Results

The present study was taken up with the intention to study the association and correlation of single nucleotide polymorphism in subjects who had the habit of chewing tobacco and manifested with OSF and those who did not manifest OSF.

Total of 40 subjects were included into the study, of which 20 subjects formed study group and the other 20 formed control group.

Table 6: Gender wise distribution of the study population

Group	Gender	No.	Percentage (%)
Study group (OSF)	Male	19	95
	Female	1	5
	Total	20	100%
Control group	Male	17	85
	Female	3	15
	Total	20	100%

Table 6 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 95 % were male in the study population (OSF Group) and (85 %) were male in control group. The majority of the population in the OSF Group were males (95%).This distribution highlights the fact that OSF has a predominantly male predilection.

Table 7: Age wise distribution of the study population

Age -groups	Study group	Control group
20-30 Years	13	14
31-40Years	05	11
41-50 Years	0	02
51-60Years	02	03
Mean	28	33.12
Standard deviation	5.1	6.3
Mean age of total population	32.50 years	

Table 7 describes the population distribution according to age. The age range of the study population was 20-60 years. The mean age of study group was 28 years and that of control group was 33.12 years. As it was observed the mean age of the entire population was 32.50 years.Most of the individuals, in the study group belonged to the age group of 20-30 years which apparently coincides with the age

distribution worldwide and also coincides with other previously done studies[13].

Table 8: Percentage Distribution of the subjects based on the type of habit

Type of Habit	Study group (%)	Control group (%)
Gutka	**39**	24.3
Betel	26	21.2
Paan	29.3	**34.0**
Betel +paan	32.1	19.8
Mean	9.2	7.8
Standard deviation	1.88	2.1

Table 8 describes the population distribution based on the type of habit.Majority of the population were gutka chewers (39%) followed by paan (29.3 %), and majority of the population in control group were paan chewers(34.0%) followed by gutka(24.3 %).

Table 9: Percentage Distribution of groups based on quantity of habit

No. of packets/day	Study group (%)	Control group (%)
0-5/day	24	44
6-10/day	**71**	**52**
>10/day	14	9

Table 9 shows the distribution of the population based on the number of times of chewing of habits. Within the study group 71 % of patients consumed 6-10pacs/day and 52% consumed 6-10 pacs/day in control group. From this table it can be said that irrespective of the group, entire population chewed tobacco products 6-10packs/day. This could be said that the population was influenced by advanced marketing of the product.

Table 10: Percentage Distribution of groups based on duration of habit

Duration of habit	Study group (%)	Control group (%)
0-5 Years	7	6
6-10 Years	21	8
11-15 Years	3	11
> 15 Years	4	8
Mean	10.8	12.33
Standard deviation	4.11	5.81

Table 10 shows the distribution of population based on the duration of habit. Majority of the study patients (21 %) belonged to the category that chewed products for 6-10 years and (7%) belonged to the category where they chewed products for 0-5 years of duration in controls. The mean of the study population was 10.8 years and that of control group was 12.33 years, standard deviation of study population was 4.11 , and that of controls is 5.81.

Table 11: Percentage Distribution of subjects based on the frequency of chewing habits

Frequency ofchewing	Study group (%)	Control group (%)
0-5 times	**53**	32.1
6-10 times	27.2	**42.2**
>10times	24.3	24.3

Table 11 shows the distribution of the population based on the number of times of chewing of habits. Within the study group 53% of subjects consumed the products less than 5times/day whereas in control group 42.2 % consumed 6-10times /day.

Table 12: Percentage distribution of population based on the stages of OSF

Stages of OSF	Study Group (%)
Stage 1	22
Stage 2	28
Stage 3	**43**
Stage 4	12
Mean	6.8

The majority of individuals suffering from OSF belonged to the stage 3, 2 respectively. Thus the above table shows 43% of the individuals in the study belonged to the moderate and severe stages of OSF .

Table 13: DNA Quantittation Case Group

Sample Read#	260/280	ng/µL	Sample Read#	260/280	ng/µL
1	1.902	169.592	16	1.922	86.952
2	1.926	110.193	17	1.903	145.943
3	1.904	151.708	18	1.887	136.41
4	1.882	169.561	19	1.906	258.481
5	1.876	17.737	20	1.728	21.207
6	1.546	41.833	21	1.869	134.141
7	1.921	31.656	22	1.632	138.351
8	1.5	1.681	23	1.773	93.06
9	1.686	116.883	24	1.994	52.374
10	1.398	120.487	25	1.896	218.225
11	1.507	67.656	26	1.793	16.717
12	1.996	38.377	27	1.851	123.336
13	1.967	24.477	28	1.894	67.627

14	1.834	30.55	29	1.95	139.825
15	1.823	45.818	30	1.938	21.735

The ratio of the absorbance at 260 and 280 nm ($A_{260/280}$) is used to assess the purity of nucleic acids. For pure DNA, $A_{260/280}$ is widely considered ~1.8.

Table 14: DNA Quantittation Control Group

Sample Read	260/280	ng/µL	Sample Read	260/280	ng/µL
1	1.701	12.781	16	1.944	104.459
2	1.886	74.433	17	1.768	46.853
3	1.92	17.153	18	1.593	49.439
4	1.897	143.553	19	1.782	70.07
5	1.965	97.407	20	1.673	115.538
6	1.56	21.624	21	1.266	9.969
7	1.901	128.644	22	1.612	27.823
8	1.776	115.4	23	1.856	36.548
9	1.926	131.342	24	1.61	43.986
10	1.836	50.409	25	1.736	1.736
11	1.498	20.024	26	1.825	55.061
12	1.884	119.885	27	0.867	18.144
13	1.934	236.299	28	1.997	30.825
14	1.844	122.728	29	1.441	44.394
15	3.273	7.313	30	1.849	62.563

Data Analysis

Table 15: Results of Pearson Chi-Square Test Done to Assess the Association of Gender in Case and Controls With Polymorphism

Study Group	Haplotype AA	Haplotype AB	Haplotype BB	Total No.Of subjects
Male	9	8	2	20
Female	1	0	0	
Control Group	**Haplotype AA**	**Haplotype AB**	**Haplotype BB**	**Total No.Of subjects**
Male	9	6	2	20
Female	0	0	3	
Pearson chi-square value	0.812			

The female population included in the study group was 5 % hence Pearson chi-square was not performed, and in the control group was 15 %. The frequency of the AA/AB/BB haplotype alleles in the study population wa 9,8 and 2 respectively. On computing the Chi-square test there was no significant difference present between the gender and genotypes.

Table 16: Association of Age Groups with Polymorphism

Age-Study Groups	Haplotype AA	Haplotype AB	Haplotype BB	Total No.Of subjects
20-30 Years	10	3	0	13
31-40Years	5	0	0	5
41-50 Years	0	0	0	0
51-60Years	0	1	1	2
p value	**0.189**			
Age -Control Groups	**Haplotype AA**	**Haplotype AB**	**Haplotype BB**	**Total No.Of subjects**
20-30 Years	12	2	1	14
31-40Years	7	1	2	11
41-50 Years	0	1	2	02
51-60Years	1	1	0	03
p value	**0.128**			

No significant association was present between the age-groups between the case- control group and AA/AB/BB genotypes.

Table 17: Association of Type of Habit With Polymorphism

No significant difference was noted with the type of habit between the case-control group and AA/AB/BB genotypes

Age-Study Groups	Haplotype AA	Haplotype AB	Haplotype BB	Total No.Of Subjects
Gutka	6	4	2	**12**
Betel	4	1	1	6
Paan	0	3	0	3
Betel+paan	1	4	9	14
p value	0.182			
Age Control Groups	**Haplotype AA**	**Haplotype AB**	**Haplotype BB**	**Total No.Of Subjects**
Gutka	4	5	2	11

Betel	2	2	0	4
Paan	0	2	0	**2**
Betel+paan	4	1	3	8
p value	0.155			

Table 18: Association of Quantity of the Product with Polymorphism

Study Group	Haplotype AA	Haplotype AB	Haplotype BB	Total No.Of subjects
0-5packs/day	3	2	0	5
6-10packs /day	10	8	3	21
>10/day	4	0	0	4
P Value	0.346			
Control Group	**HAPLOTYPE AA**	**Haplotype AB**	**Haplotype BB**	**Total No.Of subjects**
0-5packs/day	6	6	0	12
6-10packs /day	11	5	0	16
>10/day	0	0	2	2
P Value	0.000*			

The association of the quantity of the product consumed was statistically significant with the AA/AB/BB haplotypes in control group with P value =0.000*, but there was no significant association of the quantity of the product consumed in study group.

Table 19: Association of Duration of the Habit with Polymorphism

Study Group	Haplotype AA	Haplotype AB	Haplotype BB	Total No.Of subjects
0-5 Years	3	1	0	4
6-10 Years	4	5	2	11
11-15 Years	0	2	0	2
>15Years	2	1	0	3
P value	0.407			
Control Group	**Haplotype AA**	**Haplotype AB**	**Haplotype BB**	**Total No.Of subjects**
0-5 Years	0	0	1	1
6-10 Years	4	0	0	4
11-15 Years	7	3	1	11
>15Years	3	1	0	4
P value	0.800			

No significant association was present between the age-groups between the case- control group and AA/AB/BB genotypes.

Table 20:Association of Frequency of the Habit with Polymorphism

Study Group	Haplotype AA	Haplotype AB	Haplotype BB	Total No.Of subjects
0-5 times	6	4	1	11
6-10 times	3	1	2	6
>10times	2	1	0	3
P value	0.269			
Control Group	**Haplotype AA**	**Haplotype AB**	**Haplotype BB**	**Total No.Of subjects**
0-5 times	4	3	0	7
6-10 times	8	1	0	9
>10times	2	0	2	4
P value	0.000*			

The association of the frequency of the product consumed was statistically significant with the AA/AB/BB genotypes in control group with **P value =0.000*,** but there was no significant association of the duration of the product consumed in study group.

Table 21: Association of Stages of OSF with Polymorphism

Stages of OSF	Haplotype AA	Haplotype AB	Haplotype BB	Total No.Of subjects
Stage 1	1	2	1	4
Stage 2	2	1	2	5
Stage 3	0	7	2	9
Stage 4	2	0	0	2
P value	0.411			

No significant association was noted with the frequency, duration and stages of the habit and AA/AB/BB polymorphism**.**

Discussion

Oral Submucous Fibrosis (OSF) is a chronic disorder characterized by fibrosis of the mucosa lining the upper digestive tract involving the oral cavity, oro- and hypopharynx and the upper third of the oesophagus. The alkaloids from areca nut are the most important chemical constituents biologically; in producing this lesion.Areca-

nut/betel-leaf/tobacco chewing habits are widely prevalent in many parts of Asia and in migrant communities arising there from. Many betel-quid products in different parts of the world are not actually chewed; rather, they are placed in the mouth or applied to the oral cavity and remain in contact with the oral mucosa. The synergistic effect on the carcinogenic potency of tobacco/betel quid in oral cancer by alcohol consumption is well-documented in the literature.

OSF is widely recognized as condition precancerous to oral cancer. The malignant transformation rate of OSF ranges from 1.2 to 23% worldwide. 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. In 2002, the statistics for OSF from the Indian continent alone was about 5 million people (0.5% of the population of India)[5].Cystatin C is a low moleclar weight (13.4 kDa) protein that functions as an inhibitor of various cysteine proteases in the bloodstream. It inhibits both endogenous proteases, such as lysosomalcathepsins, and proteases of parasites and microorganisms. Cystatin C consists of 120 amino acid residues encoded by a 7.3 kb gene located in chromosome 20. CST3 or Cystatin C is consistently and dramatically upregulated in a variety of fibrotic diseases[10]

Arecoline a product of areca nut was found to elevate this Cystatin C mRNA (CST3) and protein expression in a dose-dependent manner. Cystatin C expression was significantly higher in OSF specimens and expressed mainly by fibroblasts, endothelial cells, and inflammatory cells. Cross-links between the molecules are essential for the tensile strength of collagen fibres. These areas are resistant to attack by collagenases but can be attacked by a number of other serine and cysteine proteinases. CST3 encoding a cysteine proteinase inhibitor might contribute to the stabilization of collagen fibrils in OSMF[6].It is essential to evaluate the OSF risk associated with polymorphisms of collagen- related genes in low- and high-exposure groups separately to examine whether the high-risk genotypes are consistent or not. Because several genes are involved in the metabolism and cross-linking of collagens, therefore, it is not only important to evaluate the combined effects of multiple genes as well as to evaluate the main effect of single gene[9].In this present we have tried to assess the cystatin C gene single nucleotide polymorphism in subjects who had habit of chewing arecanut/tobacco and had OSF (Case), and also in the subjects who had habit of chewing arecanut/tobacco and had no OSF (Control).To the best of our knowledge, this is the first study to examine the effect of single nucleotide polymorphism of Cystatin C3 gene in OSF. We evaluated single nucleotide gene polymorphism of collagen-related gene cystatin C that is situated on chromosome 20 and its main effect on case and control subjects. Our case-control study included 40 individuals out of which 20 patients were suffering from OSF in the case group and 20 in the control group.In a study done by, Chung-Jung Chiu et al[9] and Rothman KJ et al,[10] all of their control subjects were betel quid chewers. It is unusual in epidemiological studies to exclude controls that have not had the exposure of interest. Because there was no confirmed case of OSF among nonchewers of betel quid, betel quid chewers were considered the source population of OSF, rather than the entire nondiseased population, which the controls would usually represent[9,10].Total number of males included was 36 and 4 females. The male predominance of OSF cases were found similar to other studies which were done elsewhere in India, where the male: female ratios were 2.2:1,3:1,2:1, 4:1, 5,3:1[12].However, in this study, and above mentioned studies males were found to be dominating, as they were using gutkha and other related products more frequently because of easy availability in all the places and changing life trends, where as females being more conscious about their health and esthetic value, probably felt uncomfortable to ask the vendors in getting the gutkha products.We made an attempt to assess the effect of collagen- related gene Cystatin C in subjects who suffered from OSF after chewing tobacco/ betel products; we also have tried to define the putative high-risk genotypes of the genes as those associated with the highest risk of OSF.India has seen a marked increase in the occurrence of OSF in recent years especially in states of Bihar, Madhya Pradesh, Gujarat and Maharashtra. In India, production of the nut has risen nearly threefold and may reflect the commercialization of areca products since the early 1980s[19]In our study the distribution of the individuals was based on the mean age which was 32.50 years. It was noted that the age group between 20-30 years were in majority in the study group, which was similar to studies by Shah N et al, [7] R Rajendran et al[13] and Soma gupta et al[14]In our study we correlated each of the variables in demographics to the haplotypes. We assessed the association of the haplotypes with gender predilection. The frequency of the AA/AB/BB haplotype alleles in the study population was present 95 % in males. 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 on performing Chi-square test.People less than 35 years were 5 times more likely to develop OSMF as compared to the older population. Interestingly this finding also coincides with the WHO criteria IARC monographs on oral cancer. In a study conducted by PC Gupta et.al it was interesting to note that younger age subjects developed OSF even with a short duration of areca-exposure. It was also found that an increase in the prevalence of OSF, especially in the younger age groups, directly attributable to the use of areca nut products was observed. This could lead to an increase in the incidence of oral cancer in the future[15].As OSF is a well recognized potentially malignant disorder of the oral mucosa the malignant potential of OSF in 1956, was first described by Paymaster and this has been estimated to be 7-30% recently and pathogenesis is thought to be multi-factorial. One of the main pathogenesis is the chewing of areca-nut[5].Thus, we analysed the association of the type of habits in the population with the haplotypes. Nevertheless in our study there was no significant difference between the type of habit and polymorphisms. Thus, this finding does not peak the association of the type of habit-history in a patient suffering from OSF with polymorphism. However, allele AA was higher in subjects who consumed gutka.The other risk factors associated with OSF were identified and considered, which includes the duration, quantity and frequency of chewing.MAority of the study group patients (21 %) belonged to the category that chewed products for 6-10 years and (7%) belonged to the category where they chewed products for 0-5 years of duration in controls. The mean of the study population was 10.8 years and that of control group was 12.33 years, standard deviation of study population was 4.11, and that of controls is 5.81.However there was no significant association of the duration with polymorphisms.In the present study group 71 % of patients consumed 6-10pacs/day and 52% consumed 6-10 pacs/day in control group. This seems that irrespective of the group , entire population chewed tobacco products 6-10 packs/day and within the study group. The analysis of the severity of the disease progression with polymorphism was evaluated in association of each stage of OSF with the Cystatin C haplotypes. Based on the clinical staging by Chandramani B More et al,[10] classification system for the surgical management of OSF, we distributed the study group into 4 stages. The majority of the subjects belonged to the stage 2and 3, yet there was no significant difference between the individual stages of OSF and polymorphisms.The association of the frequency of AA/AB or BB haplotypes single nucleotide polymorphism (SNP) at promoter region on chromosome 20 was also analysed.The frequency of haplotype AA is 50%, AB 40% , BB 10% was of study population while in control population it was AA is 45%, AB 30% , BB 25% respectively. On statistical analysis with Odds ratio for alleles AA/AB and AA/BB p values were p = 0.090 and p=0.128 which was significant (p=<0.10).With these mentioned statistics we can say that cystatin C gene profile was significantly higher in study group s compared to controls. Findings of our study was similar to study done by Chung-Jung Chiu et al and Chung-Hung Tsai et al[8]. Lee *et al*[17] used arecoline, a major areca nut alkaloid, to explore whether

expression of Twist could be changed dose-dependently in human primary buccal mucosal fibroblasts (BMFs).Collagen gel contraction and migration capability in arecoline-stimulated BMFs and primary OSMF-derived fibroblasts (OSMFs) with twist knockdown was presented. They observed that the treatment of arecoline dose-dependently increased twist expression transcript and protein levels in BMFs.Based on Chiu's study[9] and other studies, the genotypes associated with the highest OSMF risk in the lower-exposure group were CC of COL1A1, AA of COL1A2, TT of collagenase-1, CC of TGF-β1, AA of LOX and AA of CST3. On the other hand, TT of COL1A1, BB of COL1A2, AA of collagenase-1, CC of TGF-β1, GG of LOX and AA of CST3 genes led to the highest risk to OSMF in the high-exposure group. There was a consistent relationship between genotype distribution of TGF-β1 and CST3 genes and the risk of OSMF in both low- and high-exposure groups, while the other four genes showed inconsistency.In a most recent study by Jeneffar Roselin Christopher et al, in a total of 50 patients with 25 in each group (Group I—periodontally healthy, Group II—Stage III/IV periodontitis) .Cystatin C, CSTC protein level and *CST3* gene expression were analyzed using enzyme-linked immunosorbent assay (ELISA) and real-time polymerase chain reaction, respectively. Elevated concentrations of CSTC protein and *CST3* gene expression were observed in Group II in comparison with Group I, which was considered statistically significant ($p < 0.001$). Further, a highly significant ($p < 0.001$) positive correlation was witnessed between CystatinC ,CSTC protein and *CST3* gene in both groups. In addition, the overall correlation between Cystatin C,CSTC protein, *CST3* gene, and clinical parameters was positive and highly significant ($p < 0.001$)[18]. Nevertheless,further studies are required with a larger sample size to culminate the role of the polymorphisms in the cystatin C3 gene with OSF A follow up of long term is a must in these patients 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.

Conclusion

The study was conducted in the Department of Dental Sciences, Ayaan Institute of Medical Sciences, Hyderabad. The study included 40 subjects. After obtaining ethical clearance and consent from the patients, blood samples were collected from 20 cases of OSF and 20 controls. DNA was isolated and PCR based restriction analysis was done.The prevalence of OSF and the rate of malignant transformation are different among countries. Quitting betel nut chewing is the best strategy to prevent OSF and potential malignancy. Regardless of the strategy, clinical diagnosis and treatment are still based on conservative methods. The treatment must improve the elasticity of the oral mucosa and mouth opening distance. This ensures that patients have normal oral functions like speaking and eating to improves the patient's quality of life and provides an adequate nutritional intake.High-quality clinical studies are needed to help clinicians to develop and apply molecular biomarkers and to formulate standard treatment guidelines for OSF.The following conclusions were drawn from the results:

1. A long-term follow up of these patients is a must to see the prognosis and their susceptibility to malignancy in case subjects and that for controls to develop OSF.
2. 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.

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