

Senescent Fibroblast in Oral Submucous Fibrosis Aids in Disease Progression and Malignant Transformation

Shyamala Karnam, Girish H C, Vaidhehi N. Nayak

Department of Oral Pathology and Microbiology, Rajarajeswari Dental College and Hospital, Mysore Road, Bengaluru, Karnataka, India

Abstract

Aim: To study the role of senescent fibroblasts (SFs) and its secretory phenotypes promoting fibrosis and malignancy in oral submucous fibrosis (OSMF).

Material Methods: A total of 20 cases of OSMF and 20 normal oral mucosal tissues were taken for the study. The tissue sections were stained for IHC-immunohistochemistry with senescent cell marker p^{16INK4a}. The supernatant solution of the transport medium was studied for leached out senescent associated secretory proteins (SASP): matrix metalloproteinases 9 (MMP9), interleukins 6 (IL6), vascular endothelial growth factor (VEGF). Tissues were studied for malignant transformation with p53 and Ki67. Fibrosis in the OSMF was tested with lxyloxidase (LOX). The Statistical Package for the Social Sciences (SSPS) statistical software was used to analyze the data.

Results: IHC staining for p^{16INK4a} showed positivity in the connective tissue of OSMF cases which was statistically significant. Antibody assay using enzyme-linked immunosorbent assay (ELISA) showed elevated levels of secretoproteins IL6, MMP9, VEGF in OSMF cases. LOX enzyme levels were also significantly increased in OSMF cases. Proliferative markers Ki67 and p53 were positive in IHC staining in the epithelium of OSMF.

Conclusion: This study confirms the presence of SF and its secreto phenotypes in OSMF and showed increased LOX expression which is implicated in fibrosis. These findings suggest that SF may contribute to fibrosis in OSMF. The study also confirms the malignant transformation of the overlying epithelium as shown by p53 and Ki67 positivity.

Keywords: Oral Submucous fibrosis, p^{16INK4a}, senescence associated secretophenotypes, senescent fibroblasts

Address for correspondence: Dr. Shyamala Karnam, Professor, Department of Oral and Maxillofacial Pathology, Rajarajeswari Dental College and Hospital, No 14, Ramohalli Cross, Kumbalgodu, Mysore Road, Bangalore - 560 060, Karnataka, India.

E-mail: shyamalakprasanna@gmail.com

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INTRODUCTION

Oral submucous fibrosis (OSMF) is a chronic, progressive, debilitating disease and potentially malignant condition of the oro-pharyngeal region. It is characterized by generalized sub mucosal fibrosis. Chronic habit of chewing “areca nut” has been considered as the main causative factor.^[1] In spite of so many registered OSMF patients in India, we lack

proper supportive literature to understand the pathogenesis of this particular condition from our part of the world.

Various factors contribute to the pathogenesis of OSMF which include: growth factors, cytokines, chemical mediators, immunomodulators, and enzymes. The interplay between the mediators and the fibroblast cell brings about

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increased fibrosis of the extracellular matrix.^[2] Also the accumulation of senescent fibroblast (SF) is proved to bring about fibrosis by secretion of senescence associated proteins. Studies have also indicated that SF causes neoplastic transformation of epithelial cells.^[3] The SFs and their secretory phenotypes (SASP) have been shown to play a role in lung fibrosis and deletion of the same has shown to improve pulmonary health.^[4] As OSMF is a premalignant condition with characteristic features of fibrosis, the study attempts to demonstrate the presence of SF and evaluate its malignant potential. This should give us an insight into the potential use of senolytics in the treatment of OSMF.

Aim: Role of senescent fibroblasts in oral submucous fibrosis.

Objectives

1. To demonstrate accumulation of SFs and their secretory proteins through elevated immunohistochemical (IHC) expression of P16INK4a and increased IL-6, VEGF and MMP9 levels by enzyme linked immunosorbent assay (ELISA) respectively.
2. To ascertain the role of SASP on fibrosis by comparing and correlating the levels of SASP with LOX.
3. To assess levels of the immunohistochemical expression of Ki67 and p53 expression in OSMF for neoplastic transformation.

MATERIALS AND METHODS

Nearly 22 patients with clinically confirmed OSMF in the test group and 20 normal oral mucosal tissues in the control were included in the study.

Inclusion criteria: Clinically confirmed OSMF.

Exclusion criteria:

1. Localized scleroderma/any other collagen disorders.
2. Previously biopsied/surgical site
3. History of vesiculobullous lesions with scarring
4. Diabetes mellitus type I and II

Tissue collection and processing:

A sufficiently large biopsy (5–6 mm) in diameter and (2–3 mm) depth from each patient from the most representative area was made under local anesthesia, following informed patient consent. The biopsy sample was divided and one portion was taken into 10% neutral buffered formaline for routine processing under graded alcohol and clearing agent and subjected to hematoxylin and eosin staining for histopathological confirmation of diagnosis. The other portion was taken in Dulbecco's modified Eagle's medium (DMEM) for ELISA. Normal

oral mucosal tissue from opercular tissue from prophylactic third molar area was collected for control.

Routinely processed tissues were immunohistochemically stained for the demonstration of P¹⁶INK^{4a} (for senescent cells in the nucleus and cytoplasm), Ki67 (for young fibroblasts in the nucleus), and p53 (to demonstrate the malignant potential of the OSMF epithelium). Intensity of brown staining was assessed in the tissues by counting the number of cells stained per 1 mm²⁺ field and scored as 0 if negative (fewer than 10% cells positive), + (10 – 25%) if mild, ++ (25–50% cells positive) if moderate, and +++ (>50% of cells positive) if high expression.

Enzyme linked immunosorbent assay

ELISA (sandwich) was performed on the tissues stored in DMEM to demonstrate IL-6, VEGF, MMP-9 (Sigma Aldrich)(fibrosis), and lysyl oxidase (fine touch) (for fibrosis). Detection levels for the molecules were pg/ml. All the observations were tabulated and subjected to statistical analysis

RESULTS

Statistical Analysis: Statistical package for social sciences [SPSS] for Windows, Version 22.0. Released 2013. Armonk, NY: IBM Corp., was used to perform statistical analyses.

Descriptive Statistics: Descriptive analysis of all the explanatory and outcome parameters was done using mean and standard deviation for quantitative variables, frequency, and proportions for categorical variables.

Inferential Statistics: Mann Whitney test was used to compare the mean values of different study parameters between case and control groups.

Chi-square test was used to compare the expression of P16INK4a, Ki67, and P53 markers in connective tissue and epithelium between OSMF and normal mucosa.

Spearman's correlation test was used to assess the correlation between P16INK4a [CT] and P53 [epithelium] among the case group.

Kruskal Wallis test followed by Mann Whitney's post hoc test was used to compare the mean values of different ELISA markers based on the P16INK4a [CT] and P53 [epithelium] expression among the case group.

The level of significance [P-value] was set at $P < 0.05$.

1. Immunohistochemistry

p16INK4A stained positively in OSMF cases:

Immunohistochemical expression of *p16INK4A* in OSMF was compared with normal mucosa in both epithelium and connective tissue [Table 1, Figure 1]. OSMF showed 100% positive expression for *p16INK4A* in the connective tissue region of which 25% showed mild [Photomicrograph 1], 60% moderate [Photomicrograph 2], and 15% showed high expression [Photomicrograph 3] for the same. Around 60% of normal mucosa was positive for *p16INK4A*, all showing mild expression in the connective tissue region. The epithelium in both OSMF and normal mucosa showed negative expression. The Chi-square test demonstrated a significant difference between OSMF and normal mucosa at $P < 0.001$.

Ki67 was positive in OSMF epithelium and negative in connective tissue

Immunohistochemical expression of Ki67 in OSMF was compared with normal mucosa in both epithelium and connective tissue [Table 2, Figure 2]. About 100% positive expression of Ki67 was observed in the epithelium of OSMF with 50% mild [Photomicrograph 4] and 50% moderate intensity [Photomicrograph 5]. Rest of the areas understudy (normal mucosa and connective tissue of OSMF) stained negative for Ki67. The Chi-square test demonstrated a significant difference between OSMF and normal mucosa at $P < 0.001$.

Photomicrograph 4: Photomicrograph showing mild immunohistochemical expression of Ki67 in OSMF

P53 stained positive in OSMF epithelium

Immunohistochemical expression of P53 in epithelium of OSMF samples was compared with normal mucosa [Table 3, Figure 3]. About 100% of the OSMF showed positive expression for p53 in

the epithelium with 55% mild [Photomicrograph 6], 40% moderate [Photomicrograph 7], and 5% high expression [Photomicrograph 8]. Rest of the areas understudy (normal mucosa and connective tissue of

Table 1: Comparison of expression of P16INK4a in connective tissue and epithelium between OSMF and normal mucosa using Chi-square test

Variable	Expression	OSMF		Normal Mucosa		χ^2	P
		n	%	n	%		
Connective tissue	No expression	0	0%	8	40%	25.882	<0.001*
	Mild	5	25%	12	60%		
	Moderate	12	60%	0	0%		
	Severe	3	15%	0	0%		
Epithelium	No expression	20	100%	20	100%		

*Statistically significant

Table 2: Comparison of expression of Ki67 in connective tissue and epithelium between OSMF and normal mucosa using Chi-square test

Variable	Expression	OSMF		Normal Mucosa		χ^2	P
		n	%	n	%		
Epithelium	No expression	0	0%	20	100%	40.000	<0.001*
	Mild	10	50%	0	0%		
	Moderate	10	50%	0	0%		
	Severe	0	0%	0	0%		
Connective tissue	No expression	20	100%	20	100%		

*Statistically significant

Table 3: Comparison of expression of P53 in connective tissue and epithelium between OSMF and normal mucosa using Chi-square test

Variable	Expression	OSMF		Normal Mucosa		χ^2	P
		n	%	n	%		
Epithelium	No expression	0	0%	20	100%	40.000	<0.001*
	Mild	11	55%	0	0%		
	Moderate	8	40%	0	0%		
	Severe	1	5%	0	0%		
Connective tissue	No expression	20	100%	20	100%		

*Statistically significant

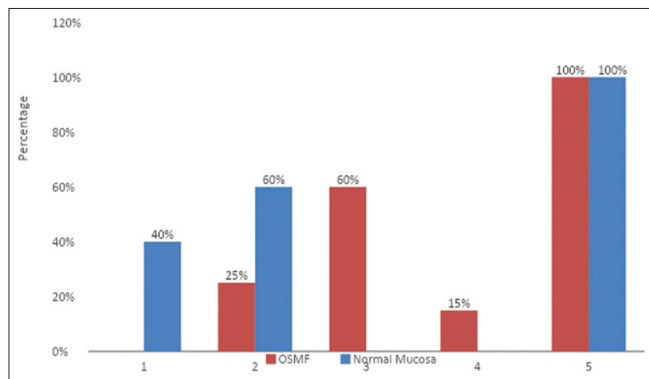


Figure 1: Comparison of expression of P16INK4a in connective tissue and epithelium between OSMF and normal mucosa using Chi-square test

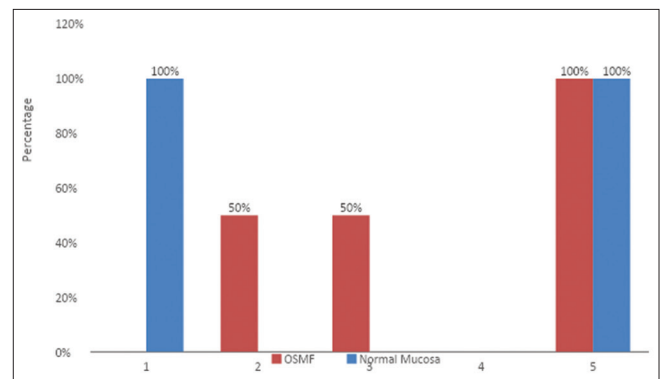
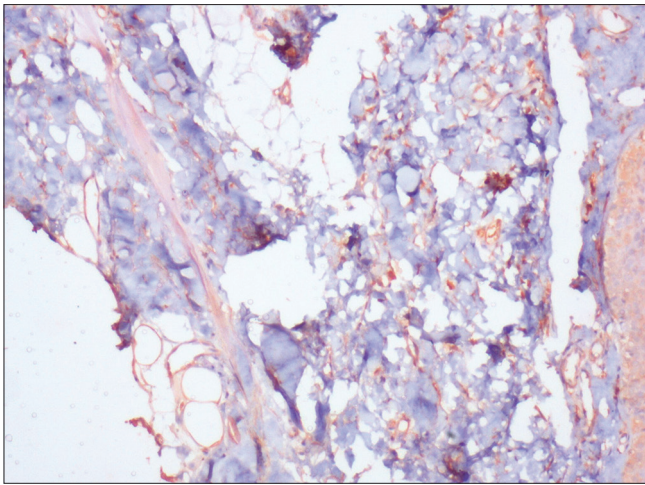
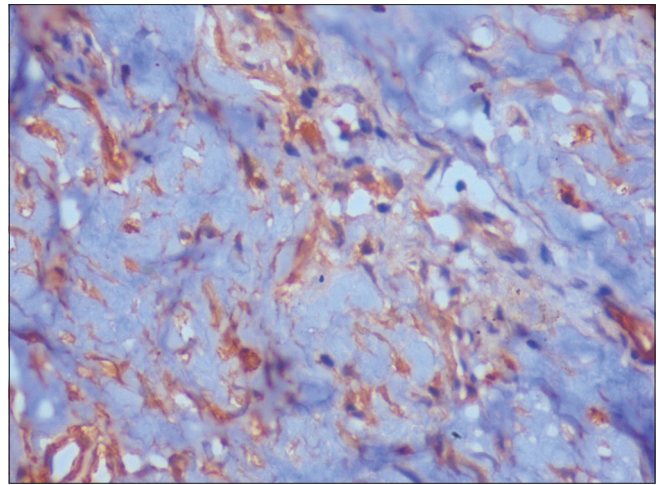


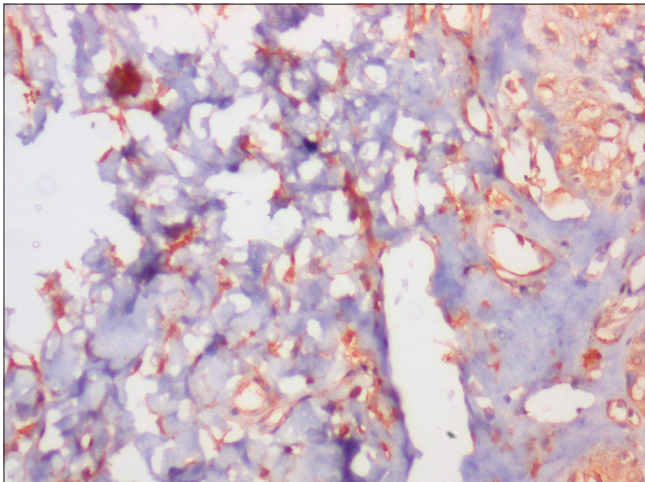
Figure 2: Comparison of expression of Ki67 in connective tissue and epithelium between OSMF and normal mucosa using Chi-square test



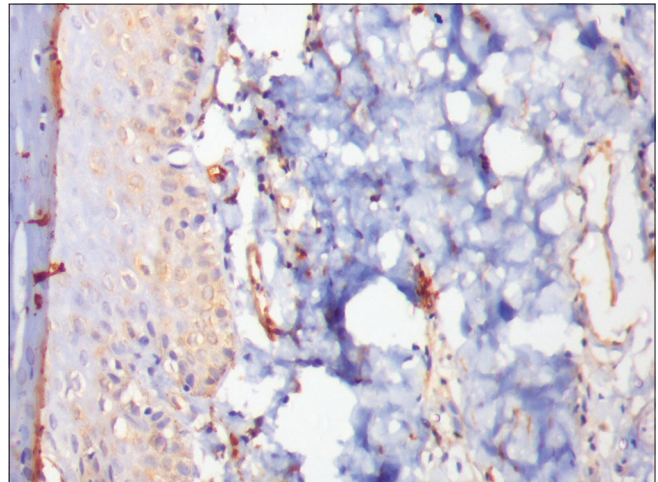
Photomicrograph 1: Photomicrograph showing mild immunohistochemical expression of *p16INK4A* in OSMF



Photomicrograph 2: Photomicrograph showing moderate immunohistochemical expression of *p16INK4A* in OSMF



Photomicrograph 3: Photomicrograph showing high immunohistochemical expression of *p16INK4A* in OSMF



Photomicrograph 4: Photomicrograph showing mild immunohistochemical expression of Ki67 in OSMF

OSMF) were negative for p53. The Chi-square test demonstrated a significant difference between OSMF and control groups at $P < 0.001$.

No correlation was found between *p16INK4A* and p53

An attempt was made to correlate immunohistochemical expression of *p16INK4A* in connective tissue with p53 expression in the epithelium of OSMF using Spearman's correlation test [Table 4, Figure 4]. The OSMF with mild *p16INK4A* expression showed mild p53 expression in 20% of the OSMF and high expression in 5% of the OSMF. The OSMF with moderate *p16INK4A* expression showed moderate p53 expression in 35% of the OSMF and mild expression in 25% of the OSMF. Essentially only 20% of mild expression and 35% of moderate expression have correlated between *p16INK4A* [CT] and P53 [epithelium] among the case group, indicating that there is a weak positive correlation between these two IHC markers. However, the

observed correlation for the expression between these two IHC markers was not statistically significant [$P = 0.73$].

Enzyme linked immunosorbent assay

Elevated levels of IL6, MMP9, VEGF, and LOX were observed in OSMF cases

ELISA values of IL6, LOX, MMP9, and VEGF were compared between OSMF and normal mucosa through Mann Whitney test [Table 5]. Mean value of IL6 in OSMF was 0.073 ± 0.020 and in normal mucosa it was 0.051 ± 0.071 , and the difference was statistically significant with the $P < 0.001$ [Figure 5]. Similarly mean values of LOX [Figure 6] in OSMF 3.999 ± 1.905 and in normal mucosa 2.383 ± 1.434 , MMP9 [Figure 7] in OSMF 6102.100 ± 2885.358 and in normal mucosa 1610.385 ± 592.050 , and VEGF [Figure 8] in OSMF 2433.155 ± 2076.738 and in normal mucosa 306.589 ± 570.394 ; when compared showed statistically

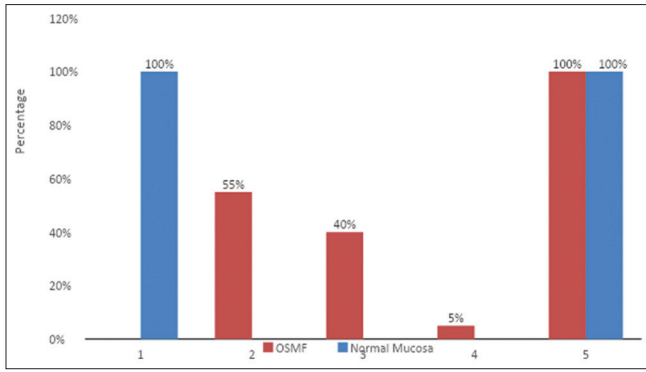


Figure 3: Expression of P53 in epithelium and connective tissue between OSMF and normal mucosa

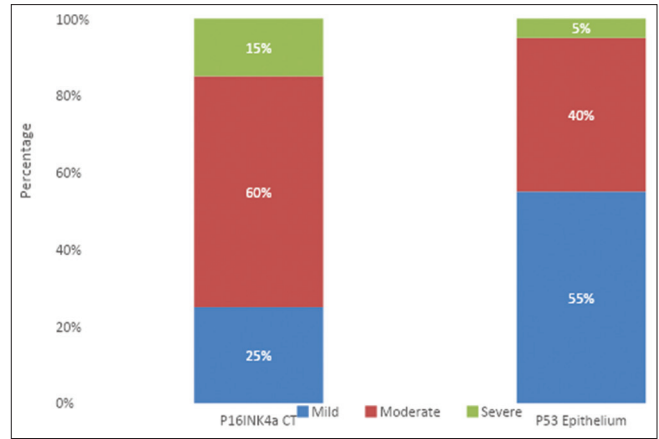


Figure 4: Correlation between P16INK4a (connective tissue) and P53 (epithelium) among case group

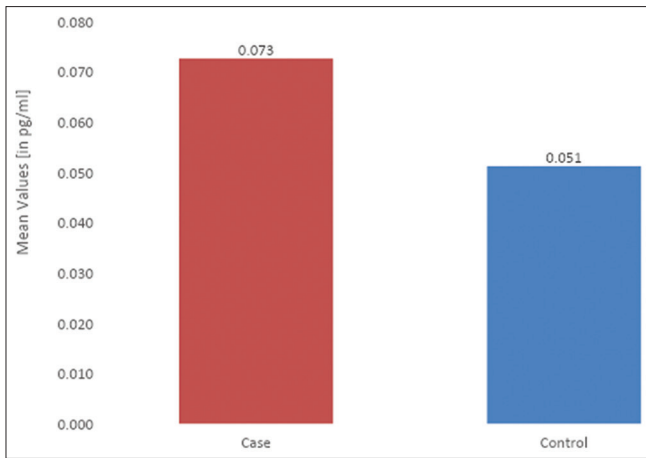


Figure 5: Comparison of mean values of IL6 between cases and controls

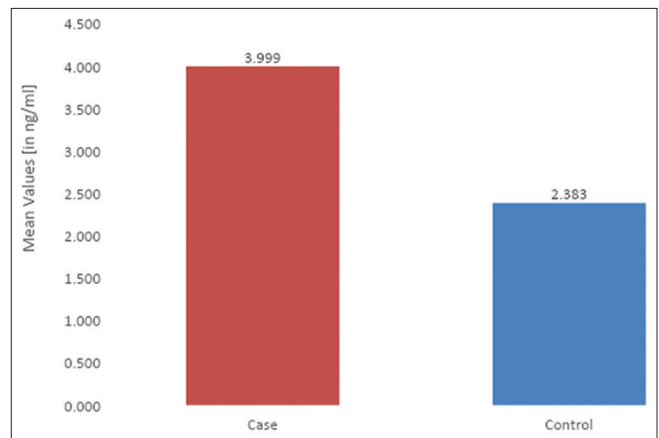


Figure 6: Comparison of mean values of LOX between cases and controls

Table 4: Correlation between P16INK4a [CT] and P53 [epithelium] among case group using Spearman's correlation test

P16INK4a CT	P53 Epithelium			Total
	Mild	Moderate	Severe	
Mild	4	0	1	5
	20.0%	0.0%	5.0%	25.0%
Moderate	5	7	0	12
	25.0%	35.0%	0.0%	60.0%
Severe	2	1	0	3
	10.0%	5.0%	0.0%	15.0%
Total	11	8	1	20
	55.0%	40.0%	5.0%	100.0%

$\rho=0.28$, $P=0.73$; The correlation coefficients are denoted by "rho"; Minus sign denotes negative correlation; Correlation coefficient range; 1.0: no correlation; 1.01-0.20: very weak correlation; 0.21-0.40: weak correlation; 0.41-0.60: moderate correlation; 0.61-0.80: strong correlation; 0.81-1.00: very strong correlation

significant difference with P values 0.02, <0.001 , and <0.001 , respectively.

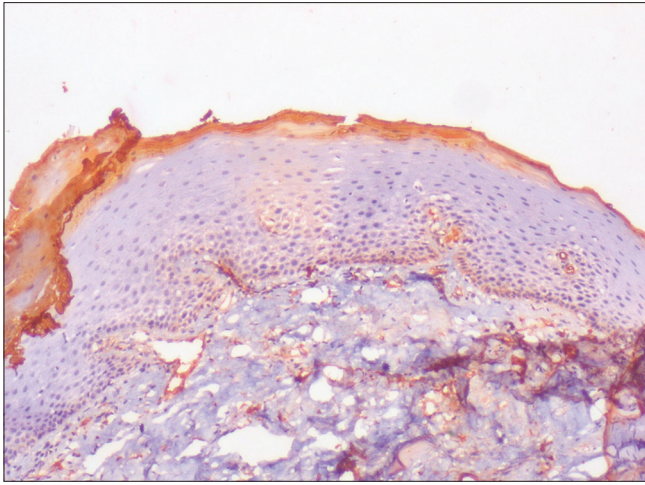
Comparison of IL6 levels and p16INK4A expression was not statistically significant

The test results demonstrate the comparison of mean IL6 levels based on *p16INK4A* expression among the

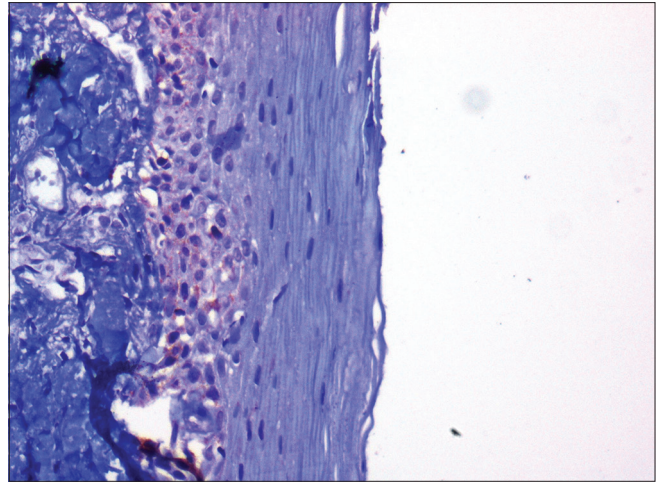
OSMF groups [Table 6]. The mean IL6 level in mild *p16INK4A* expression was 0.077 ± 0.012 , for moderate expression it was 0.076 ± 0.022 , and for severe expression it showed 0.051 ± 0.008 . This difference in the mean IL6 levels based on *p16INK4A* expression was statistically significant at $P = 0.04$. The multiple comparisons between the different expressions of *p16INK4A* reveal that the mean IL6 levels in severe expression was significantly lesser as compared to both mild and moderate expression at $P = 0.03$. However, the mean IL6 levels did not significantly differ between mild and moderate expression of IL6.

Comparison of MMP9 levels and p16INK4A expression was not statistically significant

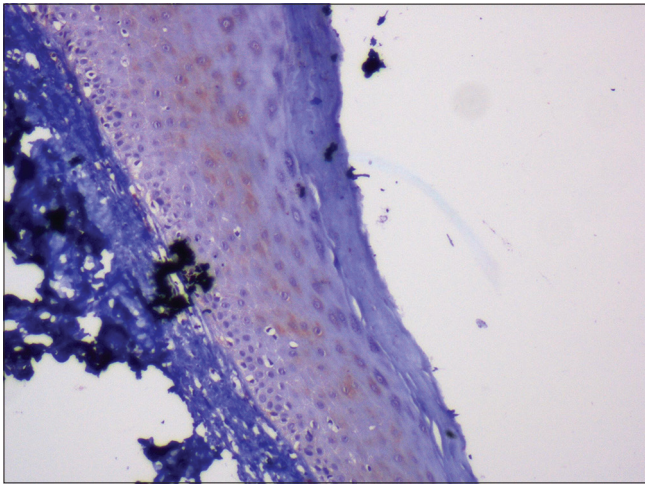
The mean MMP9 level in mild *p16INK4A* expression was 8655.200 ± 873.876 , for moderate expression it was 4823.517 ± 2743.277 , and for severe expression it showed 6390.333 ± 3707.591 . This difference in the mean MMP9 levels based on *p16INK4A* expression was statistically significant at $P = 0.03$. The multiple comparisons between



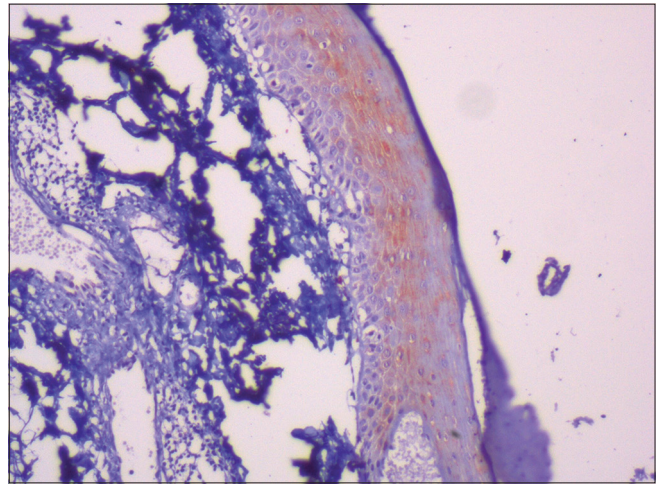
Photomicrograph 5: Photomicrograph showing moderate immunohistochemical expression of Ki67 in OSMF



Photomicrograph 6: Photomicrograph showing mild immunohistochemical expression of p53 in OSMF



Photomicrograph 7: Photomicrograph showing moderate immunohistochemical expression of p53 in OSMF



Photomicrograph 8: Photomicrograph showing high immunohistochemical expression of p53 in OSMF

Table 5: Comparison of mean values of different study parameters between case and control groups using Mann Whitney test

Parameters	Groups	n	Mean	SD	Mean Diff	P
IL6 [pg/ml]	Case	22	0.073	0.020	0.021	<0.001*
	Control	19	0.051	0.071		
LOX [ng/ml]	Case	22	3.999	1.905	1.616	0.02*
	Control	13	2.383	1.434		
MMP [pg/ml]	Case	22	6102.100	2885.358	4491.715	<0.001*
	Control	13	1610.385	592.050		
VEGF [pg/ml]	Case	20	2433.155	2076.738	2126.566	<0.001*
	Control	20	306.589	570.394		

*Statistically significant

the different expressions of *p16INK4A* reveal that the mean MMP9 levels in moderate expression was significantly lesser as compared to both mild and severe expression at $P = 0.06$. However, the mean MMP9 levels did not significantly differ between mild and severe expressions of MMP9.

However, the mean comparison of VEGF and LOX levels between different intensities of expression of p16INK4A did not show any significant differences [Table 6].

Comparison of IL6 levels with p53 expression was not statistically significant

The test results demonstrate the comparison of mean IL6 levels based on p53 expression among the case groups [Table 7a and b]. The mean IL6 level in mild p53 expression was 0.080 ± 0.024 , for moderate expression it was 0.062 ± 0.008 , and for severe expression it showed 0.072 . This difference in the mean IL6 levels based on p53 expression was statistically significant at $P = 0.04$. The multiple comparisons between the different expressions of p53 reveal that the mean IL6 levels in moderate expression was significantly lesser as compared to both mild and severe expressions at $P = 0.03$. However, the mean IL6 levels did not significantly differ between mild and moderate expression of IL6.

Comparison of MMP9 levels with p53 expression was not statistically significant

The mean MMP9 level in mild p53 expression was 6909.555 ± 2926.044, for moderate expression it was 4336.138 ± 2254.107 and for severe expression it showed 9635.000. This difference in the mean MMP9 levels based on p53 expression was statistically significant at P = 0.04. The multiple comparisons between the different expressions of p53 reveal that the mean MMP9 levels in moderate expression was significantly lesser as compared to both mild and severe expression at P = 0.04. However,

the mean MMP9 levels did not significantly differ between mild and severe expressions of MMP9.

However, the mean comparison of VEGF and LOX levels between different intensities of expression of p53 did not show any significant differences [Table 7a and b]

DISCUSSION

Senescent cells are terminal nondividing cells known to aid in tumor suppression, wound healing, embryogenesis, etc. Accumulation of senescent cells is also known to promote

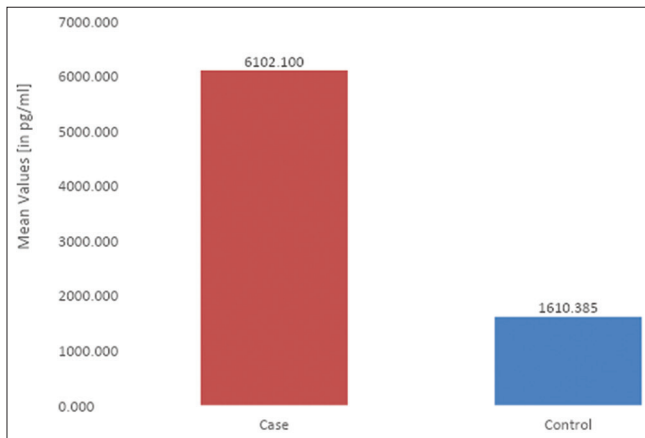


Figure 7: Comparison of mean values of MMP9 between cases and controls

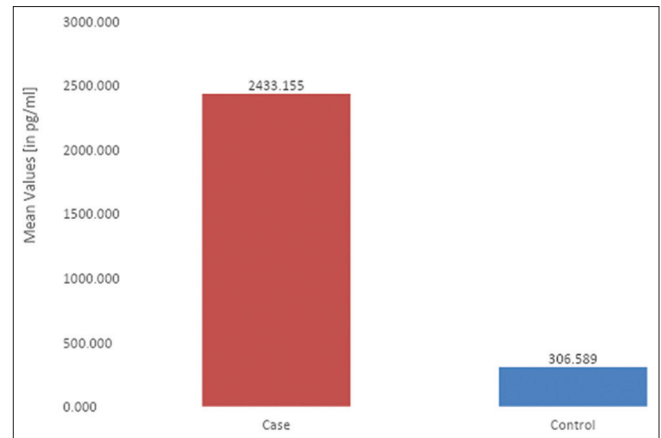


Figure 8: Comparison of mean values of VEGF between cases and controls

Table 6: Comparison of mean values of different ELISA markers based on the P16INK4a [CT] expression among the case group using Kruskal Wallis test followed by Mann Whitney's post hoc test

Parameters	Expression	n	Mean	SD	Min	Max	P ^a	Sig. Diff	P ^b
IL6 [pg/ml]	Mild	5	0.077	0.012	0.059	0.091	0.04*	Mild vs moderate expression	0.53
	Moderate	12	0.076	0.022	0.049	0.134		Mild vs severe expression	0.03*
	Severe	3	0.051	0.008	0.042	0.057		Moderate vs severe expression	0.03*
LOX [ng/ml]	Mild	5	4.646	1.998	2.322	7.126	0.71	Mild vs moderate expression	-
	Moderate	12	3.749	1.840	1.731	7.685		Mild vs severe expression	-
	Severe	3	4.069	2.682	2.163	7.136		Moderate vs severe expression	-
MMP [pg/ml]	Mild	5	8655.200	873.876	7380	9635	0.03*	Mild vs moderate expression	0.006*
	Moderate	12	4823.517	2743.277	333.1	8545		Mild vs severe expression	0.46
	Severe	3	6390.333	3707.591	2985	10340		Moderate vs severe expression	0.47
VEGF [pg/ml]	Mild	4	2835.275	2558.145	635.1	6528	0.82	Mild vs moderate expression	-
	Moderate	11	1992.455	1924.264	116.2	6764		Mild vs severe expression	-
	Severe	3	3027.000	3192.165	1080	6711		Moderate vs severe expression	-

*Statistically significant, ^aP-value derived by Kruskal Wallis test, ^bP-value derived by Mann Whitney post hoc test

Table 7a: Comparison of mean values of different ELISA markers based on the P53 [epithelium] expression among the case group using Kruskal Wallis test followed by Mann Whitney's post hoc test

Parameters	Expression	n	Mean	SD	Min	Max	P ^a	Sig. Diff	P ^b
IL6 [pg/ml]	Mild	11	0.080	0.024	0.042	0.134	0.04*	Mild vs moderate expression	0.03*
	Moderate	8	0.062	0.008	0.049	0.074		Mild vs severe expression	0.47
	Severe	1	0.072	-	0.072	0.072		Moderate vs severe expression	0.24
LOX [ng/ml]	Mild	11	4.618	2.058	1.991	7.685	0.25	Mild vs moderate expression	-
	Moderate	8	3.413	1.618	1.731	6.421		Mild vs severe expression	-
	Severe	1	2.322	-	2.322	2.322		Moderate vs severe expression	-
MMP [pg/ml]	Mild	11	6909.555	2926.044	333.1	10340	0.04*	Mild vs moderate expression	0.04*
	Moderate	8	4336.138	2254.107	996.1	8545		Mild vs severe expression	0.19
	Severe	1	9635.000	-	9635	9635		Moderate vs severe expression	0.12

*Statistically significant

Table 7b: Comparison of mean values of VEGF [pg/ml] ELISA parameter based on the P53 [epithelium] expression among the case group using Mann Whitney test

Parameters	Expression	n	Mean	SD	Mean Diff	P
VEGF [pg/ml]	Mild	11	3077.700	2494.088	1865.643	0.12
	Moderate	7	1212.057	781.991		

tumorigenesis. Senescent cells have a paracrine effect on its tissue microenvironment by secreting senescence associated secretory proteins (SASP) consisting of cytokines, growth factors, chemokines, extracellular matrix components, etc. The effect of senescent cells and its SASP varies in different biological scenarios.^[5] The present study explores the role of SFs in fibrosis and malignant transformation of OSMF.

In the present study, we have demonstrated the presence of SF by positive *p16INK4A* staining in the connective tissue of OSMF cases. Studies by several authors have established that most senescence cells express P^{16INK4a}, which helps to differentiate quiescent or terminally differentiated cells which do not express P^{16INK4a}.^[6] There was negative staining for Ki67 in the connective tissue of OSMF. This feature along with positive staining for P^{16INK4a} further supports the senescent cell nature which is nonproliferative. This is similar to a study by Lawless *et al.*^[7] which said that negative expression of Ki67 is a marker in itself for senescent cells with high expression of positive senescent markers, such as p21 and SA b-Gal. It is also argued that absence of Ki67 staining in the mesenchyme of OSMF is against the idea of replicative senescence and OSMF can be thought to be the result of cellular senescence induced by stress such as oxidative damage, areca nut, reactive oxygen species, etc.

The senescence associated secretoproteins (SASP) IL6, MMP9, VEGF were also demonstrated to be increased in OSMF as compared to normal mucosa using ELISA method.^[8] Sheetal C (2014) in their immunohistochemical study on MMP9 and VEGF have shown increased expression in OSMF also positive correlation with increase in severity of the disease, which supports our findings.^[9] Similar studies have shown increased MMP-9 gene expression and protein to be elevated in both human and experimental lung fibrosis.^[10,11]

In contrast to our findings, a study by Pitiyage *et al.*^[12] have found that the cytokines were below detectable to low levels in OSMF cases. Since their study was *in vitro*, the role of tissue microenvironment and nature of cultured fibroblast versus *in vivo* fibroblast must be the determining factor. It is stated that SASP needs more than 5 days to be released in culture plates after senescence induction and the proteins are not secreted at the same time. Hence the discrepancy

can be explained. Severity of habit may also play a role in cytokine release.

In the present study the mean value of IL6 in OSMF was 0.073 ± 0.020 and in normal mucosa it was 0.051 ± 0.071 and the difference was statistically significant with the *P* value <0.001 . Similar findings were observed by CH Tsai *et al.*^[13] in their *in vitro* study wherein IL6 levels were significantly increased in OSMF specimen as compared to normal buccal mucosal fibroblast ($P < 0.05$).^[14]

The above SASP also correlated with LOX levels confirming their role in fibrosis. However, when the individual senescent proteins were correlated with *p16INK4A* the statistical significance was not found. Likewise, with SASP secretome and p53 protein no statistically significant correlation was found. These findings suggest that the amount of SASP secretome does not correlate neither to the number of senescent cells present nor to the degree of neoplastic change. Since the study did not consider grading the severity of OSMF cases, correlation could not be established.

The study showed 100% positivity for Ki67 and p53 protein in the epithelium of OSMF cases indicating neoplastic transformation of epithelium at a molecular level. Similarly findings by Manjunath *et al.*^[15] have shown positive correlation with p53 mutation and OSMF cases confirming to malignant transformation. Anura *et al.*^[16] too in their study have suggested that wild type p53 causes atrophy and fibrosis in OSMF, and mutated p53 causes dysplastic changes in OSMF.

CONCLUSION

Although the pathogenesis of OSMF is researched extensively, study on the role of SF in OSMF is very limited. The study by Pitiyage *et al.* have said that accumulation of SF improves fibrosis in OSMF cases.^[8] Whereas many studies have stated that senescent cells increase fibrosis. In this study, the presence of SF has been proved by *p16INK4A* staining and its secreto phenotypes were demonstrated by ELISA. The study also showed increased LOX expression which is implicated in fibrosis. These findings suggest that SF may contribute to fibrosis in OSMF. The study also confirms the malignant transformation of the overlying epithelium as shown by p53 and Ki67 positivity.

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Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form the patient(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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Conflicts of interest

There are no conflicts of interest.

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