

# Epithelial Atrophy, Fibrosis and Vascularity Correlation with Epithelial Dysplasia in Oral Submucous Fibrosis, a Prospective Study

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## Abstract

**Background:** The pathogenesis of oral submucous fibrosis (OSF) still remains conflicting and has been linked to alterations in epithelial thickness, fibrosis, and vascularity. Although changes in these individual parameters have been extensively studied in relation to epithelial dysplasia their combined relation with dysplasia has not been studied much. Any such relation, if present, may further help in understanding this disease process. Therefore, the aim of this study was to assess the relationship between epithelial thickness, fibrosis, and vascularity with dysplasia in OSF. **Materials and Methods:** The study consisted of 30 OSF patients. Incisional biopsy was taken from the most fibrosed area of the buccal mucosa. Hematoxylin–Eosin-stained slides were assessed for epithelial thickness, fibrosis, and vascularity using image analysis software. The slides were also assessed for epithelial dysplasia. Relationship of epithelial atrophy, fibrosis, and vascularity with dysplasia was assessed using one-way ANOVA. Pearson's correlation coefficient was used for evaluating the relationship between epithelial thickness, fibrosis, and vascularity. **Results:** Epithelial dysplasia was found in all patients. Eleven patients had mild (36.67%), thirteen had moderate (43.33%), and six had severe (20%) dysplasia. None of the parameters were found to have a significant relationship with dysplasia. However, moderate and positive correlation was found between epithelial thickness and fibrosis. This relation was statistically significant. **Conclusion:** Positive correlation between epithelial thickness and fibrosis in present study therefore contradicts the hypothesis of fibrosis induced epithelial atrophy. As dysplasia is influenced by multiple factors therefore habits and burning sensation needs to be incorporated in future studies assessing dysplasia in OSF.

**Keywords:** Epithelial atrophy, epithelial dysplasia, fibrosis, oral submucous fibrosis, vascularity

## INTRODUCTION

Oral submucous fibrosis (OSF), a potentially malignant disorder of the oral mucosa, is predominantly seen in South Asian countries. Although primarily a collagen disorder, OSF has been linked to dysplasia of the overlying epithelium, the pathophysiology of which still remains unclear.<sup>[1]</sup>

The sequence of events in this disease process includes lymphocytic infiltration in a very early stage, followed by fibrosis in the subepithelial zone in an early stage. The fibrosis in the intermediate stage then extends into the muscle layer and leads to constriction of the blood vessel. This ultimately leads

to a marked reduction of vascularity in an advanced stage.<sup>[2]</sup> It has been hypothesized that reduced vascularity creates hypoxic environment, which then results in atrophy of the epithelium. The compromised epithelium thus allows the perfusion of the carcinogens, which over the course of time, undergoes dysplastic changes and subsequently leads to malignant transformation.<sup>[3]</sup>

Epithelial dysplasia, which is the most important predictor of malignancy, is usually associated with hyperplastic epithelium.

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However, grading epithelial dysplasia in an atrophic epithelium of OSF is one of the trickiest things and still remains a challenge. The malignant transformation in the dysplastic epithelium in OSF has been found to be 7%–13%.<sup>[4]</sup> With an increase in the incidence of the OSF cases, there is also an increased likelihood of malignant transformation in the dysplastic epithelium.

Epithelium in the past was considered atrophic based on a reduction in the number of cell layers and loss of rete ridges.<sup>[5]</sup> Epithelial thickness has been widely studied in the literature, including morphometric and cellular changes.<sup>[6,7]</sup> Although the thickness of the epithelium has been assessed in relation to grades of OSF earlier,<sup>[8]</sup> its relation with dysplastic epithelium has still not been studied.

Fibrosis in submucosa leads to a marked reduction in mouth opening and therefore results in significant morbidity in OSF patients. To our knowledge, there are only two studies in the literature, which correlated the relationship of fibrosis and epithelial dysplasia. Both the studies were found to have varied results, with non significant relationship between epithelial dysplasia and degree of fibrosis to epithelial dysplasia significantly increasing with increase in fibrosis.<sup>[1,9]</sup>

Vascularity in OSF, though, has been widely studied in the literature using hematoxylin and eosin (H and E) stain and molecular markers, its role in OSF is still a matter of debate.<sup>[10-13]</sup> It has been found that there is increased vascularity in an early stage of OSF followed by a decrease in vascularity in later stages.<sup>[10]</sup> However, this concept differs from the other studies, which stated that the mean vascularity density (MVD) i.e., the number of vessels is more or less similar to the normal oral mucosa, thus denying the concept of ischemia-induced epithelial atrophy.<sup>[7,14]</sup>

The above-mentioned parameters, i.e., epithelial atrophy, fibrosis, and vascularity though has been studied individually at histopathological and molecular levels but there combined relationship with epithelial dysplasia has still not been assessed much. Thus these parameters which are substantially affected in OSF when assessed together in relation to epithelial dysplasia may provide an insight into the pathogenesis of epithelial dysplasia in OSF. Therefore the aim of the study was to assess these parameters in relation to epithelial dysplasia in OSF.

## MATERIALS AND METHODS

The study protocol was independently reviewed and approved by the institutional ethical board. The present study is a co-relational and prospective research. Thirty consequent OSF patients reporting to the Oral Medicine unit and willing to be a part of this study were recruited by the principal investigator. Thirty tissue samples were also taken and served as a control group from the normal mucosa of patients undergoing disimpaction surgeries. Patient's recruitment was done from January 2016 to December 2017. Informed consent

was obtained from all individuals (patients and controls) subjected to the study. The WHO declaration of Helsinki and its subsequent amendments were followed in both the test and the control group.

The inclusion criteria of the study were restricted mouth opening, burning sensation in the oral cavity, presence of palpable fibrotic bands, blanching of the oral mucosa, areca nut, and tobacco chewing habit and histopathological report of OSF. For final inclusion, the patients were needed to satisfy at least three of the six inclusion criteria.

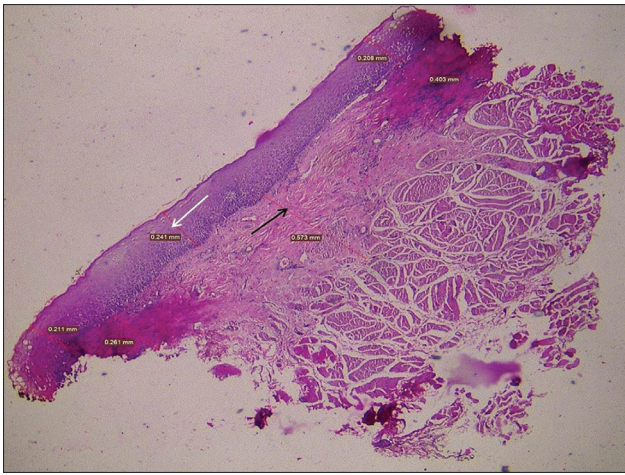
The exclusion criteria were any history of previous treatment, restricted mouth opening due to odontogenic infection, trauma, impacted third molars, any major systemic illness, patients having temporomandibular joint disorders, any concurrent oral lesions, any history of maxillofacial surgery, and benign or malignant tumor of the jaw.

The recruited patients were staged according to Lai *et al.* classification<sup>[15]</sup> into Group A (interincisal opening of more than 35 mm), Group B (interincisal opening between 30 and 35 mm), Group C (interincisal opening between 20 and 30 mm), and Group D (interincisal opening <20 mm).

Incisional biopsy was taken from the most fibrosed area (obtained by palpating the fibrous bands vertically with fingers) of the buccal mucosa. The excised tissue was fixed in 10% neutral buffered formalin and embedded in paraffin box. Four-micrometer-thick sections were cut, deparaffinized, and stained with H and E for histological examination. The H and E slides were assessed by two oral pathologists having >5 years of experience in evaluating the histopathology of OSF patients. Any difference in the slide assessment was taken to a third senior oral pathologist and resolved. The oral pathologists were blinded to the age, gender of the patients, and grade of the disease.

Quantitative assessment of epithelial thickness, fibrosis, and vascularity was done using image analysis software (Leica application suite, LES core version 3.8) of Leica research microscope (Model Number DM1000 LED, Leica Microsystems GmbH Ernst–Leitz–Straie 17-37 | 35578 Wetzlar [Germany]).

The thickness of epithelium was measured from the superficial layer of the epithelium till the basal layer and the fibrosis was calculated from the basal layer of epithelium till the end of the fibrous layer.<sup>[1,6]</sup> These measurements were taken under  $\times 4$  [Figure 1]. Vascularity was assessed under  $\times 40$  & was calculated by counting the number of blood vessels in the connective tissue layer lying immediately below the epithelium<sup>[10]</sup> [Figure 2]. Epithelial thickness and vascularity were measured in both tests and the control group. Since fibrosis is seen in OSF patients and not in healthy individuals, it was measured only in the test group. The stained slides of insufficient thickness at this stage were excluded from the study. Photomicrographs of the images were captured with the help of a camera fitted onto the microscope, which were then displayed on the computer monitor. The image analysis



**Figure 1:** Epithelial thickness (white arrow) measured quantitatively from superficial layer of the epithelium till the basal layer. Thickness of fibrosis (black arrow) measured from the basal layer of the epithelium till the muscle layer (H and E;  $\times 4$ )

software had an inbuilt measurement tool for the quantitative assessment of epithelial thickness, fibrosis, and vascularity. Measurements were taken from three different fields, i.e., extreme left, right and from the center of the stained slides. Data obtained were then transported to excel sheets for calculation of average values of each parameter.

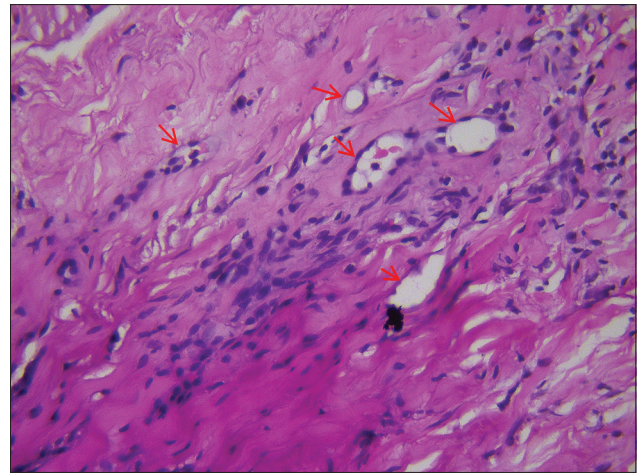
The presence or absence of epithelial dysplasia was assessed using the World Health Organization criteria of histological typing of cancer and precancer of the oral mucosa. The dysplastic features present till the parabasal layers (lower third) were considered as mild epithelial dysplasia; the changes up to the middle third were considered as moderate epithelial dysplasia, and the changes involving greater than two-thirds of the epithelium were considered as severe epithelial dysplasia.<sup>[16]</sup> The relationship between epithelial atrophy, the thickness of the fibrous layer, and vascularity was then correlated with epithelial dysplasia.

### Data analysis

Data obtained were compiled on an MS Office Excel Sheet (v 2010) and was subjected to statistical analysis. IBM SPSS 20.0 (IBM SPSS Statistics for Windows, Armonk, NY: IBM Corp) software was used for analysis. Descriptive statistics like age and gender of the subjects were recorded. Comparison of the mean of epithelial thickness and vascularity between OSF patients and the control group was evaluated using *t*-test. The relationship between epithelial dysplasia, thickness of fibrosis, and vascularity with three grades of epithelial dysplasia, i.e., mild, moderate, and severe was assessed using one-way ANOVA. Correlation of epithelial thickness, fibrosis, and vascularity was done using the Pearson correlation coefficient test.

## RESULTS

The study consisted of 30 OSF patients and 30 healthy individuals as control group. The age of the OSF patients ranged



**Figure 2:** Number of blood vessels were counted in the connective tissue lying immediately below the basal layer of epithelium. Measurements were taken from three different fields and a mean was calculated (H and E;  $\times 40$ )

from 16 to 46 years, with a mean of 30.27 years  $\pm$  8.71. There were 26 males and 4 females. The mean epithelial thickness in the control group was 1.20 mm  $\pm$  0.32 and was 0.46  $\pm$  0.38 in OSF patients. Comparison between mean epithelial thickness between cases and controls done using *t*-test revealed a high statistically significant difference ( $P < 0.01$ ) with higher mean in controls as compared to cases. However, there was a statistically nonsignificant difference in the mean vascularity between cases and controls ( $P > 0.05$ ) [Table 1].

Eighteen patients (60%) belonged to Group C OSF, five patients (16.67%) belonged to Group B, four patients (13.33%) belonged to Group A and three (10%) belonged to Group D. The relationship of the disease grading and epithelial dysplasia evaluated using one-way ANOVA revealed a nonsignificant relationship [Table 2].

Epithelial dysplasia was present in all OSF patients. Eleven patients (36.67%) had mild dysplasia, 13 (43.33%) had moderate dysplasia and six (20%) had severe epithelial dysplasia. A nonsignificant relationship was found between the three parameters (epithelial thickness, fibrosis, and vascularity) and epithelial dysplasia using one-way ANOVA [Table 3].

Pearson correlation showed moderate and positive correlation between epithelium thickness and fibrosis [Table 4]. This relationship was found to be statistically significant. There was a slight and positive correlation between vascularity and fibrosis and vascularity and epithelium thickness.

## DISCUSSION

Many theories have been put forward explaining dysplastic changes in OSF epithelium. Grading dysplasia in OSF presents a major challenge to oral pathologists because of the atrophic epithelium. The pathogenesis of this disease is still conflicting. Some researchers believe that with the advancement of fibrosis, there is a decrease in vascularity. This process results in ischemic changes and ultimately causes atrophy of the overlying

**Table 1: Comparison of epithelial thickness and vascularity between cases and controls using *t*-test**

	Number of patients in each group	Mean±SD	P
Epithelial thickness			
Cases	30	0.46±0.38	0.00**
Controls	30	1.20±0.32	
Vascularity			
Cases	30	3.89±2.24	0.84
Controls	30	3.76±2.88	

Since the *P* value was less than 0.01 the results were highly significant for epithelial thickness for cases and control group. *P* value of greater 0.05 indicates a non significant difference for vascularity between cases and control group. SD: Standard deviation

**Table 2: Relation of epithelial dysplasia with grade of oral submucous fibrosis using one-way ANOVA**

Grade of OSF	Grades of epithelial dysplasia				P
	Mild	Moderate	Severe	Total	
A	2	2	0	4	0.66
B	1	3	1	5	
C	6	8	4	18	
D	2	0	1	3	
Total	11	13	6	30	

OSF: Oral submucous fibrosis

epithelium. The potential carcinogens accumulate in the atrophic epithelium and gradually result in malignant transformation.<sup>[1]</sup> Contrastingly other researchers are of the views that mean vascular density comparatively increases or more or less is the same when compared with normal oral mucosa, thus denying the concept of fibrosis induced vasoconstriction.<sup>[7,14]</sup>

The question addressed in the study was whether dysplasia seen in OSF is associated with changes in epithelial thickness, fibrosis, and vascularity.

A nonsignificant relation was found between the grade of OSF and epithelial dysplasia. Grading in the present study was done based on maximum inter incisal opening rather than histopathological grade. This could be the reason for nonsignificant results between different grades of OSF and epithelial dysplasia.

The most striking feature in the present study was the presence of epithelial dysplasia in all OSF patients. Eleven (36.7%) patients had mild, 13 (43.3%) had moderate, and six (20%) patients had severe dysplasia. These findings were in contrast to the earlier studies. Pindborg *et al.*<sup>[2,17]</sup> have indicated the occurrence of epithelial dysplasia in the range of 7%–23%. Desai *et al.*<sup>[7]</sup> did not find dysplastic features in any of their OSF patients. Jayasooriya *et al.*<sup>[11]</sup> found dysplasia in 46/107 (43%) of the patients. Thus, there are mixed results in the literature regarding the prevalence of dysplasia in OSF.

An increased incidence of epithelial dysplasia in the present study could be because of the increased prevalence of mixed

habits among the patients. Alarmingly, the high incidence of epithelial dysplasia in this study indicates a high malignant transformation rate in OSF. Particularly because OSF affects a younger population in whom the dysplastic cells get a longer duration to change into malignancy. This aspect could be explored in future studies.

### Epithelial thickness

The mean epithelial thickness was 0.46 mm ± 0.38 in this study and was least in patients with severe dysplasia. However, a statistically nonsignificant relation was found between epithelial thickness and epithelial dysplasia. The earlier studies in the literature considered epithelium to be atrophic when there was a decrease in the number of cell rows and reduction in width or loss of rete pegs.<sup>[5,18]</sup> Singh *et al.*,<sup>[8]</sup> for the first time, did the morphometric analysis of epithelium thickness in OSF patients and found that thickness did not consistently decrease with increasing grades of the disease. Although epithelium thickness has been studied in relation to the grade of the disease, its relation with epithelial dysplasia has still not been assessed. To the best of our knowledge, this is the first study where epithelial thickness and epithelial dysplasia association is evaluated.

### Fibrosis

The mean thickness of fibrosis found in our study was 1.62 mm ± 1.12. The present study did not find any correlation between fibrosis and dysplasia. There are two studies in the literature which has assessed the relationship between fibrosis and epithelial dysplasia. Illeperuma *et al.*<sup>[9]</sup> found no statistically significant correlation between the degree of fibrosis and different grades of epithelial dysplasia. Contrastingly, Jayasooriya *et al.*<sup>[11]</sup> found significant increase in the incidence of epithelial dysplasia with an increase in the thickness of fibrosis. Interestingly both these studies were done in Sri Lankan population.

### Vascularity

In this study, the MVD was found to be 3.89 mm ± 2.24, which was not significant with dysplasia. Vascularity in OSF has been widely studied in the literature, not only using H and E stain but also by using molecular markers.<sup>[10-13]</sup> However, there are conflicting results in the literature regarding vascularity in OSF. Tekade *et al.*<sup>[19]</sup> in their study found microvessel hyperplasia in the early stage of OSF and decrease in middle and late stages of the disease. Rajendran *et al.*<sup>[14]</sup> found that MVD was more or less similar to controls. These findings were not statistically significant. They also found that the mean vascular percentage area and mean vascular luminal diameter showed a significant increasing trend with the OSF progression. The study speculated that the usual tissue reaction resulting from ischemia/hypoxia does not seem to operate OSF. The mean vascular dilatation noted was assumed to be an adaptive response to compensate for the transient ischemia likely to occur during the disease process when collagenization predominates.

Contrary to the earlier findings, Pandiar and Shameena<sup>[11]</sup> revealed a statistically significant decrease in MVD in OSF

**Table 3: Comparison of epithelial thickness, fibrosis, and vascularity between grades of epithelial dysplasia using one-way ANOVA**

Epithelial dysplasia	n	Epithelial thickness			Fibrosis			Vascularity		
		Mean±SD	95% CI	P	Mean±SD	95% CI	P	Mean±SD	95% CI	P
Mild	11	0.46±0.43	0.17-0.75	0.74	1.59±0.67	1.14-2.04	0.30	3.42±2.89	1.48-5.37	0.42
Moderate	13	0.52±0.38	0.29-0.74		1.90±1.43	1.05-2.77		4.51±1.93	3.34-5.68	
Severe	6	0.37±0.35	0.00-0.73		1.04±0.94	0.06-2.02		3.39±1.26	2.07-4.71	
Total	30	0.46±0.38	0.32-0.61		1.62±1.12	1.20-2.04		3.89±2.24	3.05-4.72	

CI: Confidence interval, SD: Standard deviation

**Table 4: Correlation between epithelial thickness and fibrosis using Pearson correlation coefficient**

	Pearson's correlation (r) and P value	Fibrosis
Epithelium thickness	r	0.58
	P	0.001**

A moderate and positive correlation between epithelial thickness & fibrosis ( $r = 0.58$ ,  $P < 0.01$ \*\*). This correlation was statistically significant

patients in comparison to controls. Interestingly, there was a significant increase in MVD in OSF with dysplasia and OSF turning into malignancy. However, the numbers of patients in both the groups were less, i.e., 5 and 2, respectively. Since all the patients in the present study had epithelial dysplasia, vascularity could not be compared with OSF patients without epithelial dysplasia.

There is a significant increase in vascularity during the transition from normal tissue to the dysplastic state to early cancer, thus making MVD a more reliable parameter for assessing vascularity.<sup>[20,21]</sup> Since a higher MVD is correlated with adverse histoprognostic features, vascularity in the present study was assessed using MVD alone.<sup>[21,22]</sup>

When the relationship of epithelial thickness, fibrosis, and vascularity was assessed with each other a moderate and positive correlation was found between epithelial thickness and fibrosis. This relation was found to be statistically significant. This finding, therefore, denies the concept of fibrosis induced atrophy of overlying epithelium. There was a slight and positive correlation of vascularity with epithelial thickness and fibrosis. The values were statistically nonsignificant.

The limitations of this study were that there was sparse literature on the relation between these parameters and dysplasia. A comparative discussion of our results, therefore, could not be done. Furthermore, in the present study, we used H and E stained slides for assessing fibrosis. However, special stains like Trichrome Mallory's stain, which is used for analyzing connective tissues, should have been performed in the study.

## CONCLUSION

To conclude, epithelial thickness, fibrosis, and vascularity did not have a significant relationship with epithelial dysplasia. However, a positive relation was found between epithelial thickness and

fibrosis, which contradicts the theory of fibrosis induced atrophy of epithelium. Since epithelial dysplasia is influenced by multiple other parameters like habit involved and its duration, mouth opening, histopathological grade, visual analogue scale for oral burning these needs to be incorporated in future studies with a larger sample size. One of the major challenges an oral pathologist faces is grading dysplasia in an atrophic epithelium therefore, future studies also need to work on establishing guidelines for grading dysplasia that will help the treating physician and histopathologist in the assessment and management of OSF.

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## Conflicts of interest

There are no conflicts of interest

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