ORIGINAL ARTICLE

Immunohistochemical expression of CD34 and basic fibroblast growth factor (bFGF) in oral submucous fibrosis

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ABSTRACT

Background: Oral submucous fibrosis (OSMF) is an insidious chronic fibrotic condition that involves the oral mucosa and occasionally the pharynx and esophagus. Vascularity in OSMF has always been a matter of debate. The prevailing concept is that epithelial atrophy occurs due to lack of perfusion but the recent data challenges this concept. Therefore, the present study was conducted to evaluate the immunoreactivity of CD34 and basic fibroblast growth factor (bFGF) in different histological grades of OSMF. This might further shed light to the role of microvasculature in OSMF, so that the epithelial atrophy and resultant malignant transformation seen in the advanced stages might be elucidated. **Materials and Methods:** A total of 30 cases of OSMF were included in the study and mean vascular density (MVD) was calculated using CD34 and bFGF. Five cases of OSMF with dysplasia and 2 cases of OSMF turning malignant were added during the course of the study. **Results:** Mean vascular density was found to decrease significantly as the diseases advanced. Furthermore, vascularity increased significantly in cases of OSMF turning towards malignancy. **Conclusion:** Our study supports the concept of epithelial atrophy aftermath of lack of perfusion. There is reduced vascularity as the disease advances and this denies the systemic absorption of carcinogens, which affects the already compromised epithelium. Consequently, liberation of angiogenic factors occurs because of malignant transformation, which explains the neoangiogenesis and increased vascularity in OSMF turning towards malignancy. Further studies are required to identify the mechanism leading to carcinogenesis in the atrophied epithelium aftermath of fibrosis and decreased vascularity.

Keywords: bFGF, CD34, oral submucous fibrosis

INTRODUCTION

Oral submucous fibrosis (OSMF), first described in 1950, is a chronic precancerous condition that predominantly affects people of South-East Asian origin. The majority of patients present with an intolerance to spicy food and rigidity of lips, buccal mucosa, tongue and palate leading to varying degrees of limitation of opening of mouth and tongue movements. It is characterized by mucosal rigidity of varying intensity with fibroelastic changes of juxtaepithelial layer that affects most parts of the oral cavity, pharynx and the upper third of the esophagus.[1]

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Characteristic histopathologic features of OSMF include epithelial atrophy with loss of rete ridges with the advancement of the disease. The atrophy of the epithelium is explained to be the aftermath of the stromal changes, which will undergo progressive hyalinization, decrease in vascularity and cellularity with resultant tissue ischemia.

The conventionally believed concept is that the epithelial atrophy in OSMF is due to lack of perfusion, caused by decreased vascularity of subjacent connective tissue stroma. Recent studies have challenged this prevailing concept stating that the vascularity does not decrease with the advancement of the disease.[2-4] There is a paucity of good case control studies on the integrity and viability of mucosal vasculature in OSMF.

The degree of vascularity in OSMF remains a debated topic. Although angiogenesis cannot be measured directly, quantification of microvasculature can be done by the assessment of mean vasculature density (MVD) using endothelial markers such as CD34, CD31 and CD105.

CD34 (human hematopoietic progenitor cell antigen) is a 110-kDa transmembrane surface glycoprotein of unknown functions. It is considered to be an important marker for tissue vascularisation and represents microvascular density in the tissue. CD34 is expressed on hematopoietic stem cells, the endothelium, the interstitial cells of Cajal and dendritic cells present in the dermis, around blood vessels and in the nerve sheath.[2] Immunohistochemically, CD34 is primarily expressed on small or newly formed vessels and endothelial cells of endothelial tumors where endothelial cells of small and large vessels in normal and tumor tissue have been reported to be stained with equal intensity.[5]

It is also known that the growth factors such as basic fibroblastic growth factor (bFGF), transforming growth factor-β (TGF-β), platelet derived growth factor (PDGF) and hypoxia inducible factor (HIF) may play important roles in maintaining the vascularity of underlying connective tissue of OSMF. Fibroblast growth factors (FGFs) are heparin binding proteins and interact with cell surface associated heparan sulfate proteoglycans, which is essential for FGF signal transduction. FGF family comprises of 22 members, all of which are structurally related signaling molecules that bind to FGF receptors (FGFRs). They help in angiogenesis by promoting the proliferation and organization of endothelial cells into tube like structures. Of all FGFs, bFGF has angiogenic properties and is highly mitogenic for a variety of cells. bFGF may either directly stimulate endothelial cell proliferation or facilitate VEGF-endothelial cell interaction through the modulation of endothelial cell integrin or VEGF-receptor expression.[6]

Since there is a lack of literature focusing on angiogenesis in OSMF, we conducted the present study with an aim to determine and quantify the expression of CD34 and bFGF in OSMF and also to compare the mean vascular density in different histological grades of OSMF by immunohistochemistry (IHC) in formalin-fixed paraffin-embedded tissues. Furthermore, we tried to find the association between the expression of CD34 and bFGF in different histological grades of OSMF and to correlate epithelial cell layer thickness with the advancing stage of OSMF.

This might further shed light on the role of microvasculature in OSMF, so that epithelial atrophy and resultant malignant transformation seen in advanced stages might be elucidated.

MATERIALS AND METHODS

Patients and tissue samples

The study sample included a total of 40 cases: 10 cases of normal mucosa and 30 cases of OSMF. The OSMF cases were obtained from the embedded tissue blocks retrieved from the archives of the Department of Oral Pathology and Microbiology of our institution. Ten biopsy specimens of oral mucosa (gingiva) of healthy individuals undergoing impacted tooth removal were taken as controls. All healthy individuals signed consent forms; approval of the ethical committee was obtained. All healthy individuals were non areca nut or betel quid users and demonstrated normal mucosa at the site of biopsy specimen removal.

Histological grading was done according to Pindborg and Sirsat classification (1966).[7] Of the 30 blocks, 11 cases were early, 17 were moderately advanced and 2 were advanced oral submucous fibrosis cases [Figure 1]. Five cases of OSMF with dysplasia and 2 cases of OSMF turning malignant were added during the course of the study. All 47 cases were considered for immunohistochemical staining with CD34 and bFGF.

Immunohistochemistry

On Poly-L Lysine-coated slides, 5-μm thick sections were taken. The slides were then incubated overnight at 50°C in a hot air oven for proper adhesion of sections. The slides were de-paraffinized in three changes of xylene for 10 min each and then hydrated through graded alcohols. Antigen retrieval was performed by heating specimens immersed in EDTA buffer in a pressure cooker (Prestige, 2.5 liters). The heating consisted of three whistles followed by immediate cooling. The slides were then allowed to cool at room temperature for 20 min. Endogenous peroxidase was blocked in Novocastra™ peroxidase for 30 min followed by two changes in Tris buffer saline (TBS, pH 7.4). Sections were then incubated with protein block (Novocastra, Leica Biosystems Newcastle Ltd, UK) for 10 min to reduce nonspecific antibody binding followed by incubation with primary mouse monoclonal antibody from tissue culture supernatant diluted in PBS for CD34 and bFGF (BioGenex, USA) for 90 min. Sections were then treated with Novocastra™ post primary for 30 min. After thorough washing with TBS (pH 7.4), sections were treated with Novolink™ polymer for 30 min at room

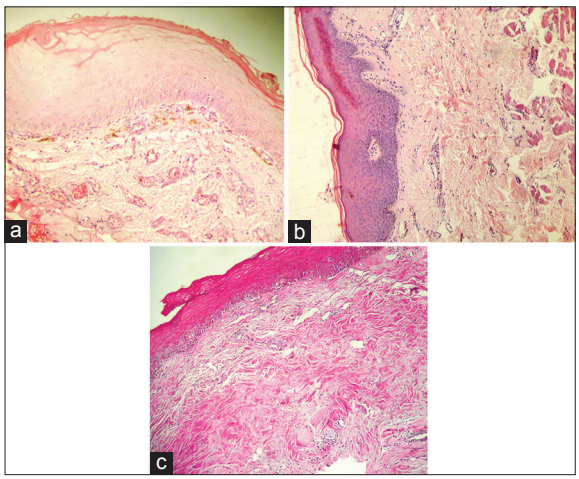

Figure 1: Photomicrographs of histological sections of (a) Early OSMF, (b) Moderately advanced OSMF (c) Advanced OSMF (H&E stain, ×100)

temperature. After three washes with TBS, substrate DAB (3,30-diaminobenzidine tetrahydrochloride) was applied to the sections for 5-10 min in the dark. Sections were counterstained with Harris hematoxylin, dehydrated with ethanol and xylene; and then mounted permanently with DPX. Negative control sections were processed by omitting primary antibody. Skeletal muscle tissue known to be immunoreactive for bFGF was used as an internal positive control for bFGF and positive staining by endothelium lined vascular spaces in pyogenic granuloma tissue were taken as external positive control for CD34.

Scoring and statistical analysis

For evaluation of CD34 and bFGF expression in OSMF, the slides were examined under a compound microscope at x400 magnification by two observers simultaneously using a double-headed microscope. The three most vascularised areas were selected under high power and the number of blood vessels for CD34 and bFGF expression in the OSMF tissue was evaluated independently by two observers [Figures 2 and 3]. Brown-stained endothelial cells (cytoplasmic expression), individually or in clusters that were clearly separate from adjacent microvessels, tumor cells or other connective tissue elements were counted as a single countable microvessel. Vessels with muscular walls were not counted. Vessel lumen and red cells, though often present, were not used to define a microvessel.[8] To evaluate the immunohistochemical expression of CD 34 and bFGF, mean of the three fields was taken as the final score.

Statistical analysis was performed using ANOVA followed by Bonferonni test and *P*-value less than 0.05 was considered significant. For statistical analysis, SPSS software was used.

RESULTS

Subjects

In the normal group, there were 7 males and 3 females; in the OSMF group, there were 19 males and 11 females; in the OSMF with dysplasia group, there were 4 females and one male and in the OSMF turning malignant group there was 1 male and 1 female. The mean age for controls was 30.6 years and for OSMF cases it was 42.5 years. Of the total cases, 56.7% cases had a habit of areca nut chewing, 40% used betel quid with tobacco and 3.3% cases used commercial products.

Histological evaluation

The study group consisted of 11 cases of early OSMF (stage 2), 17 moderately advanced (stage 3), 2 advanced cases of OSMF (stage 4) and 10 healthy subjects (controls). In addition, 5 cases of OSMF with dysplasia (OSMF-D) and 2 cases of OSMF turning malignant (OSMF-M) were included during the course of the study [Figure 4]. Histological grading was done according to Pindborg and Sirsat criteria (1966). Both carcinoma cases were graded as moderately differentiated squamous cell carcinoma.

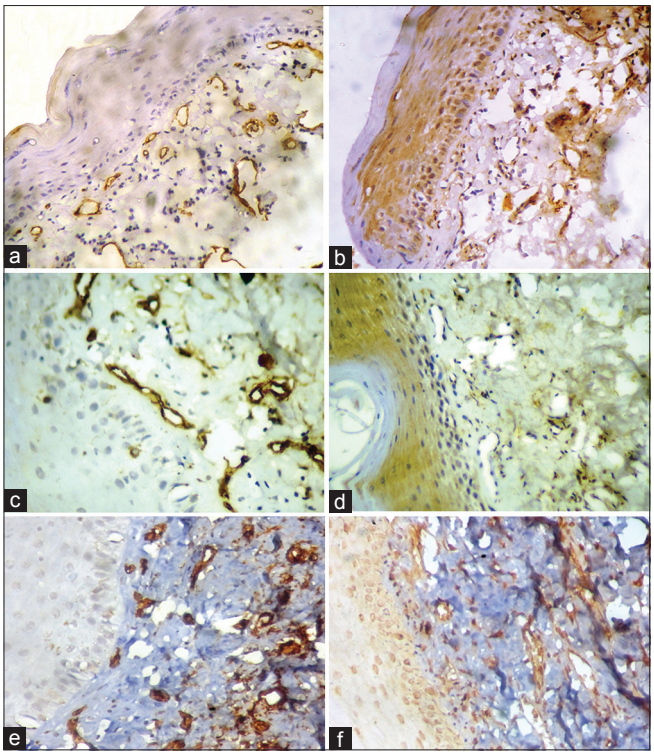

Figure 2: Immunohistochemical staining for CD34 and bFGF. (a) and (b) Early OSMF demonstrating CD34 and bFGF expression, respectively. (c) and (d) Moderately advanced OSMF demonstrating CD34 and bFGF expression, respectively. (e) and (f) Advanced OSMF demonstrating CD34 and bFGF expression, respectively (IHC stain, ×100)

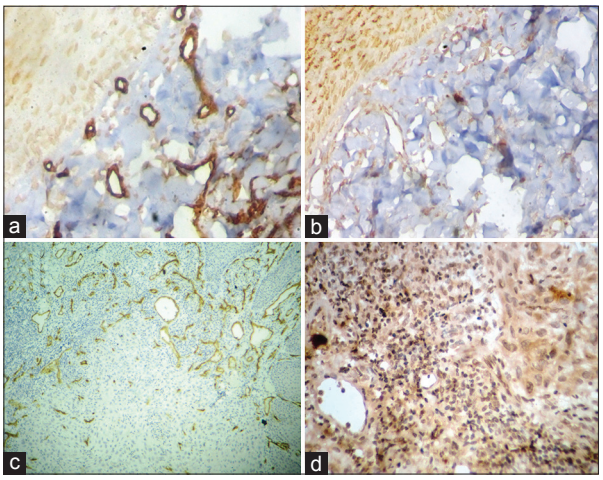

Figure 3: Immunohistochemical staining for CD34 and bFGF. (a) and (b) OSMF with dysplasia demonstrating CD34 and bFGF expression, respectively. (c) and (d) OSMF turning malignant demonstrating CD34 and bFGF expression, respectively (IHC stain, ×100)

Type of surface keratinization (30 cases and 10 controls)

Five cases of healthy controls were non keratinized and 5 were parakeratinized. Twenty-two cases of OSMF showed parakeratinization, 4 were orthokeratinized and 4 had mixed type of epithelium.

Quantitative results of angiogenesis

The number of blood vessels was demonstrated by CD34 and bFGF in 10 healthy controls and 37 cases including 30 cases of OSMF without dysplasia, 5 cases of OSMF with dysplasia and 2 cases of OSMF turning malignant [Figure 5].

Mean vascular density (MVD) was assessed in 30 cases of OSMF compared with controls. MVD in controls, stage 2 OSMF, stage 3 OSMF and stage 4 OSMF was 42.08, 20.48, 17.40 and 14.85, respectively, using CD34. As evidenced by bFGF expression, MVD in controls, stage 2 OSMF, stage 3 OSMF and stage 4 OSMF was 32.50, 15.55, 13.88 and 14.50, respectively.

MVD was also assessed by including 5 cases of OSMF with dysplasia and 2 cases of OSMF turning towards malignancy. MVD in controls, stage 2 OSMF, stage 3 OSMF, stage 4 OSMF, OSMF with dysplasia and OSMF turning malignant was 42.08, 20.48, 17.40, 14.85, 22.04 and 42.30 respectively as evidenced by CD34 expression. Using bFGF, MVD in controls, stage 2 OSMF, stage 3 OSMF, stage 4 OSMF, OSMF with dysplasia and OSMF turning malignant was 32.50, 15.55, 13.88, 14.50, 10.94 and 33.85, respectively. The difference in MVD between cases and controls was statistically significant (*P* value 0.000).

Epithelial cell layer thickness (ET)

ET (calculated in number of cells under high power field) in healthy controls was in the range of 20-37 with mean ET of 32.90. OSMF cases showed mean ET of 16.10. Since mean ET of controls was 32.90, it was decided to take half of this mean value as cut off value to label epithelium atrophic.[2] Loss of rete ridges was considered as additional criteria. This correlation of ET amongst OSMF cases and healthy controls was statistically significant (*P* value 0.000) as in stage 2 OSMF, mean ET was 15.45, in stage 3 OSMF 17.29 and in stage 4, it was 9.50 [Figure 6]. The correlation of ET amongst different stages of OSMF and healthy controls was also statistically significant. For the evaluation of keratinization and epithelial thickness correlation, 5 cases of OSMF with dysplasia and 2 cases of OSMF turning malignant were excluded.

DISCUSSION

OSMF is an insidious chronic fibrotic condition that involves the oral mucosa and occasionally the pharynx and upper portion of the esophagus.[9] As the disease advances, subepithelial and submucosal fibrosis leads to stiffness of oral mucosa and the deeper tissues, with progressive limitation in the opening of the mouth and protrusion of the tongue thus causing difficulty in eating, swallowing and phonation. Epithelial hypoplasia and atrophy is marked in advanced stages of the disease.

Vascularity in OSMF has always been a matter of debate. The prevailing concept is that with advancement in the stage of OSMF, vascularity decreases resulting in epithelial atrophy

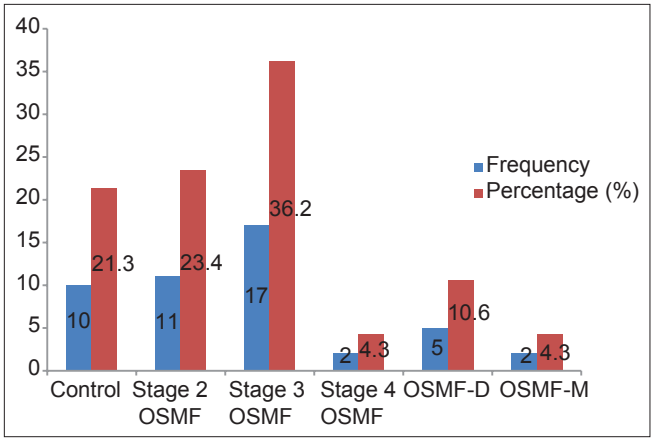

Figure 4: Percentage distribution of cases

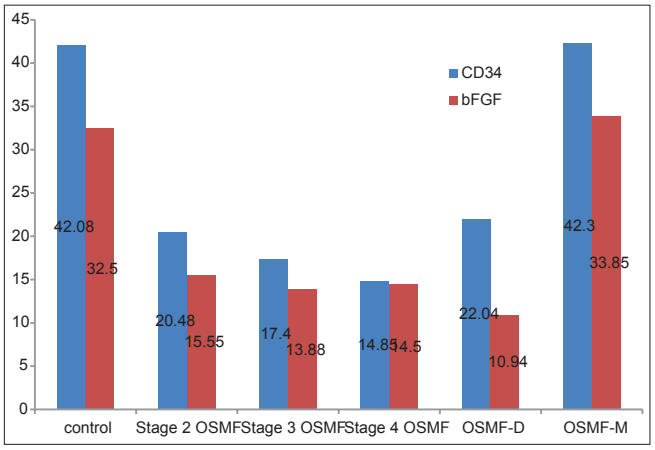

Figure 5: Correlation of MVD amongst different stages of OSMF, OSMF with dysplasia, OSMF turning malignant and healthy controls (using CD34 and bFGF)

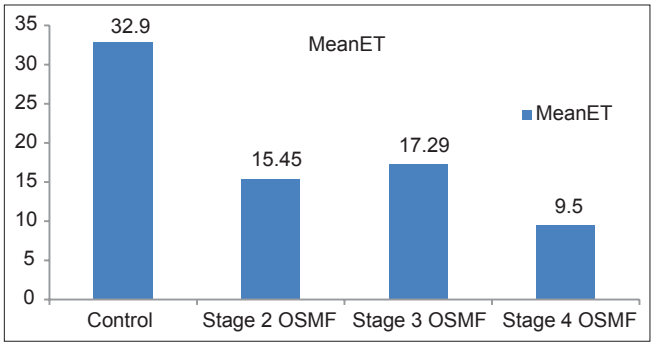

Figure 6: Correlation of ET amongst different stages of OSMF cases and healthy controls

because of lack of perfusion. However, the recent literature suggests that there is no significant decrease in the vascularity with advancing stage.[2-4] Vascularity cannot be measured directly but it can be quantified indirectly using angiogenic markers. Thus, the present study was conducted to assess the vascularity in OSMF using two angiogenic markers viz. CD34 and bFGF.

Mean vascular density is the mean value of microvessel count, obtained using a specific objective magnification with known field diameter on a selected microscope in a limited number of fields (three or four), subjectively selected from the vascularised areas (hot spots).[2]

In the present study, CD34 detected a greater number of microvessels than bFGF. Therefore, it is suggested that CD34 expression is a more reliable method of quantifying tumor vasculature. In previous studies, it was shown that CD34 is a better marker than vWf and factor VIII-RA.[10,11]

Fang *et al.*, Singh *et al*. and Debnath *et al.,* morphometrically analyzed the mucosal vascularity in OSMF and concluded that microvessel hyperplasia occurs in early stages of OSMF and is markedly decreased in stage III and stage IV. In our study, where MVD was correlated with different stages of OSMF, we observed a statistically significant decrease in MVD as compared to controls. MVD in OSMF as evidenced by CD34 showed a stepwise decrease as OSMF advanced from stage II to stage IV. With bFGF, the mean value for MVD also decreased as the stage progressed. These findings are consistent with the studies of Fang *et al.,* Singh *et al.* and Debnath *et al.*[12-14] We had noted a slight increase of MVD in stage IV as compared to stage III by bFGF. This may be due to a smaller sample size in advanced stage.

On contrary to this, Desai *et al.,* who assessed MVD immunohistochemically, found MVD to be increasing as the disease progressed.[2] Rajendran *et al.,* also found MVD to be more or less same in the different groups studied by image analysis.[3] They attributed the presence of increased vascularity in their observation as an adaptive response to compensate for transient ischemia/hypoxia leading to predominance of collagen. Desai *et al.,* in their demonstration of increased vascularity by the presence of CD34 positive cells, hypothesized that they play an important role in tumor proliferation once the malignant transformation takes place.[2]

When MVD of total OSMF cases was compared with controls we had a statistical significant decrease in MVD, which was contrary to the findings of Desai *et al.,* who found increased MVD compared to controls.

It was found that correlation of epithelial thickness (ET) amongst OSMF cases and healthy controls was statistically significant. Also, a statistically significant decrease in ET was found when correlated with the advancing stages of OSMF and healthy controls. Our results are similar to the findings of Desai *et al.*[2]

Although it was not a part of our study, a significant decrease of MVD seen in our samples prompted us to include 5 cases of OSMF with dysplasia (OSMF-D) and 2 cases of OSMF turning malignant (OSMF-M). We observed a significant increase in MVD by CD34 expression in OSMF-D and OSMF-M and an increased MVD in OSMF-M with bFGF. There was a significant correlation when the MVD of different stages of OSMF, OSMF-D and OSMF-M was compared with that of controls.

In most studies, a higher intratumoral MVD density has usually been correlated with adverse histoprognostic features.[15-18] Pazouki *et al.,* and Carlile *et al.,* have also reported that a significant increase in vascularity occurs during transition from normal tissue to dysplastic state to early cancer.[19,20]

We speculate that the decreased vascularity seen in advanced stages of OSMF will result in transient ischemia/hypoxia and this hypoxia may play a role in the progression of fibrosis in OSMF by the increased production of extracellular matrix (ECM). *In vitro* studies in renal fibrosis have shown that hypoxia stimulates fibroblast proliferation and increased expression of mRNAs for ECM proteins (collagen-I, collagen-III and fibronectin), TIMP-1 and TIMP-3 but suppressed levels of MMP-1 mRNA, consistent with increased accumulation of ECM.[21] Similar results are shown by *in vivo* studies. Tilakratne *et al.,* also noted increased expression of HIF-1α in fibroblasts and epithelial cells in OSMF and have highlighted the importance of hypoxia in OSMF.[22]

It is believed that this progressive hyalinization of stroma and consequent lack of nutrition to the epithelium will lead to ischemic atrophy of the epithelium, as evidenced by reports in the previous literature.[23] However, this observation is not consistent with the views of Rajendran *et al.,* who proposed the possible role of iNOS in the atrophy of epithelium in OSMF. According to their study, the lack of cellularity and hypoplasia is due to cytotoxic and genotoxic effects exerted on keratinocytes and stromal cells.

The present study supports the view put forward by Tilakratne *et al.,* that the dense fibrosis and decreased vascularity of the corium, in the presence of an altered cytokine activity, creates a unique environment for carcinogens from both tobacco and areca nut to act on the epithelium.[24] It could be assumed that carcinogens from areca nut accumulate over a long period of time either on or immediately below the epithelium, allowing the carcinogens to act for a longer duration before it diffuses into deeper tissues. Less vascularity may deny the quick absorption of carcinogens into the systemic circulation.

Thus, it is prudent to believe that the accumulation of carcinogens in addition to stromal fibrosis and lack of vascularity affects the already compromised epithelium. As the epithelium acquires dysplastic features and invades the connective tissue, the tumor cells create an environment to flourish. There is liberation of angiogenic factors, which result in neoangiogenesis and thus proliferation of tumor cells. This explains an increased MVD as OSMF turns towards malignancy.

Desai *et al.,* had observed a decreased or complete absence of CD34- positive stromal cells, especially in the juxtaepithelial location in OSMF, against the uniform distribution of CD34-positive stromal cells in normal mucosa.[2] In our study also, there was complete loss of CD34 positive stromal cells juxtaepithelially and within the stroma. However, contrary to their observation of uniform distribution of stromal fibroblasts in normal mucosa, our study did not show any CD34 stromal fibroblasts. This may be due to the methodological differences, including the sensitivity of endothelial markers.

We observed that bFGF immunoreactivity was increased in fibroblasts and in endothelial cells in early OSMF cases and the expression of bFGF in stroma increased notably in advanced fibrosis. This is consistent with the study of Bishen *et al.*[6]

The observation of decreased vascularity in different stages of OSMF and the significant relationship of ET with the stages of OSMF reiterates the prevailing concept that lack of perfusion leads to epithelial atrophy, which may later undergo dysplastic changes and turn malignant as evidenced by increased vascularity in OSMF-D and OSMF-M in the present study.

More detailed studies with larger sample size should be undertaken to clarify the individual mechanisms operating at the different stages of OSMF, in OSMF with dysplasia and OSMF turning malignant so that we can provide the therapeutic interventions at the appropriate time.

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