Original Article

Clinicopathological Correlation of Cyclooxygenase 2 Expression in Oral Submucous Fibrosis: An Immunohistochemical Study

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ABSTRACT

Background: Oral submucous fibrosis (OSMF) has a high prevalence in Southeast Asia with increased malignant transformation rates. Numerous biomarkers are currently being investigated to predict the disease prognosis and for early detection of malignant changes. **Materials and Methods:** A prospective study was conducted comprising 40 subjects with clinically and biopsy-proven OSMF being included in the study as experimental group (n = 28) and patients with no tobacco/betel nut habit, who underwent surgical removal of third molar, being included as control group (n = 12). About 5-µm sections from formalin-fixed paraffin-embedded tissue blocks were obtained for immunohistochemical (IHC) study. The expression of cyclooxygenase 2 (COX 2) was evaluated in the experimental group and compared in morphologically normal oral epithelium. The intensity of stain was assessed at different levels of epithelium (basal, stratum spinosum, superficial level) and connective tissue. **Results:** Based on IHC expression of COX 2, all the patients of the control group were negative for COX 2, and among the OSMF group, 19 patients (67.9%) were positive and 9 patients (32.1%) were found to be negative for COX 2. The association of COX2 expression on comparison of controls with OSMF was found to be statistically significant (χ^2 =21.955; P = 0.000). On comparison of immune expression of COX 2 in different clinical stages based on functional staging, we found significant association of COX 2 expression with the stage of OSMF (χ^2 = 7.368; P = 0.025). **Conclusion:** The significant expression of COX 2 in different clinical stages of OSMF when compared with normal shows the role of COX 2 in the pathogenesis of OSMF and could serve as a potent biomarker for assessing the disease progression.

Keywords: *Cyclooxygenase 2, immunohistochemistry, malignant transformation, oral submucous fibrosis*

Introduction

Oral submucous fibrosis (OSMF) is a predominant disease of Southeast Asia, related to areca nut chewing habit.[1,2] This debilitating chronic disorder is characterized by fibrosis of the mucosa lining the upper digestive tract and has one of the highest potential to undergo malignant transformation among various oral potentially malignant disorders (OPMDs).[3] The increased prevalence as a consequence of areca nut usage in various formulations in India, morbidity associated with the disease, and risk of malignant

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transformation make OSMF a public health priority. The prognosis of different clinical stages of OSMF seems to be varied, thus making the mechanism of malignant transformation highly unpredictable.

Immunohistochemical (IHC) analysis consists of using monoclonal or polyclonal antibodies to detect specific antigens secreted by cells of interest in tissue samples and has been proven to be a valuable tool in assessing the disease behavior.[4] Over the recent past, attempts have been made to establish a potent diagnostic biomarker to explore the carcinogenesis underlying OSMF through IHC analysis.[5] The array of diagnostic markers studied using IHC in OSMF includes cell proliferation markers such as Ki 67 and cyclin D1,[5,6] tumor suppressor genes p53, p63, and p16,[6,7] transcription markers c-Jun, β-catenin, growth factor receptors c-Met and insulin-like growth factor II mRNA-binding protein 3 (IMP3),[5] epithelial to mesenchymal transition markers,[8] oncoproteins such as Bcl2,[9] apoptotic markers such as caspase 8, caspase 3,[10,11] cancer stem cell markers such as CD (cluster differentiation) 44,[12] pan endothelial markers associated with tumor angiogenesis CD34, CD105, growth factor markers such as basic fibroblast growth factor,[13,14] transforming growth factor-β1,TGF-β2,[15] cytokeratin markers such as CK 19,[16] and inflammatory markers such as cyclooxygenase (COX) 2.[17]

Among the various markers, inflammatory markers play a key role in OSMF because constant areca nut chewing results in trauma and injury to the oral mucosa, which results in inflammation of epithelial cells resulting in the production of cytokines.[18] Changes mediated by inflammation play a major part in various molecular pathways involved in the disease progression and transformation to oral squamous cell carcinoma (OSCC).[3] COX plays an important part in the synthesis of prostaglandins (PGs) from arachidonic acid in the inflammatory pathway. The two isoforms that exist for cyclooxygenase include COX 1 and COX 2, which mainly differ in the arrangement of amino acids.[19] Among the two isoforms, COX 2 is the inducible isoform and generally more expressed in the presence of inflammation and tumor progression.[20] COX 2 is regulated by growth factors and different cytokines such as IL1β, IL6, and TNFα[21] and hence is overexpressed during inflammation. Substantial evidence proves the role of inflammation in the progression of cancer. Inflammation may play a role in tumor initiation by triggering the production of reactive oxygen species, responsible for DNA damage and tumor promotion, where inflammation may induce secretion of growth factors such as epithelial growth factor and fibroblast growth factor. Thus, inflammation triggers an uncoordinated proliferation of the initiated tumor cells.[22,23] Studies have shown increased expression of COX 2 in oral cancer; and COX 2 expression has been linked to tumor vascularization, metastasis, and prognosis in oral cancer.[24,25]

Previous studies have shown higher expression of COX 2 in OSCC and have shown to be a promising inflammatory biomarker in grading of cancerous lesions.[26,27] However, the current literature on the role of COX 2 in OSMF is sparse; the evaluation of which can prove to be valuable for assessing risk stratification in different clinical stages of OSMF. Hence, the current study aims to explore the immunoexpression and clinicopathological correlation of COX 2 in OSMF, which may serve as a potent biomarker for early identification of malignant transformation.

Materials and Methods

The prospective study was conducted at the Department of Oral Medicine and Radiology from June 2018 till January 2019. Institutional Ethics Committee approval (IEC No. CSP/17/AUG/60/239) was obtained before the commencement of the study. The study comprised 40 subjects with clinically and biopsy-proven OSMF being included in the study as experimental group (n = 28) and patients with no tobacco/betel nut habit, who underwent surgical removal of third molar, were included as control group (n = 12).

The clinical details such as age, gender, habits, frequency of use, and mouth opening were recorded. The experimental group patients were graded based on functional staging by Khanna and Andrade.[28] Patients diagnosed with other OPMDs, positive for betel nut habits in different formulations with no clinical manifestations of OSMF, were excluded from the study. Informed consent was obtained from all patients of both the groups included in the study, before collecting the tissues. About 5-μm sections from formalin-fixed paraffin-embedded tissue blocks were obtained for the IHC study.

Immunohistochemistry

IHC was performed with an avidin-biotin technique using 5-μm sections on slides coated with 3-aminopropyltriethoxysilane (APES). The sections were deparaffinized using xylene and rehydrated in absolute alcohol. Endogenous peroxidase activity quenched by immersing the sections in 0.03% hydrogen peroxide in distilled water for 10 min followed by phosphate-buffered saline (PBS) wash. Sections were pre-incubated with power block (BioGenex) for 10 min and then incubated with primary antibody against COX 2 (BioGenex, Fremont, CA, USA, anti-COX 2, Cat no. ANA32-5M, Clone COX 2/3320R, rabbit monoclonal antibody) at room temperature for

90 min, and COX 2 expression was observed using the SuperSensitive™ Polymer-HRP IHC Detection System (Super Sensitive™ Polymer-HRP IHC Detection system (QD400-60KEN), BioGenex, Fremont, CA, USA). Sections were counterstained with hematoxylin, dehydrated, and mounted in dibutylphthalate polystyrene xylene. The expression of COX 2 marker was evaluated and compared in morphologically normal oral epithelium. Number of positive cells in the epithelium and connective tissue (CT) inflammatory cells were counted in 10 high power fields (40×) and percentage positivity was calculated. Counting was done using the software ProgRes CapturePro v2.8.8 to visualize the microscopic field in a computer monitor. The scoring was done based on Sappayatosok *et al.*,[29] where 0 denoted no stained cells; 1 referred to less than 25% of cells showing positivity; 2 referred to 25–50% of cells showing positivity; 3 referred to 50–75% of cells showing positivity; and 4 referred to greater than 75% of cells showing positivity. The intensity of stain was assessed at the different levels of epithelium (basal, stratum spinosum, superficial level) and CT. The immunohistochemically stained tissue sections were scored by an oral pathologist blinded to the clinical parameters.

Statistical analysis

All the data were analyzed using SPSS Statistical Software (IBM Corporation version 16).

Results

A total of 40 patients were included in the study (n = 28—OSMF group; n = 12—control group). The age of the patients in both experimental and control groups varied from 21 to 68 years with a median age of 42 years. According to classification based on gender, 22 males and 6 females were included in the OSMF group; 9 males and 3 females were included in the control group.

Table 1 shows the clinical and histopathological parameters assessed in COX 2-positive and -negative group of OSMF patients. Based on IHC expression of COX 2, all the patients of the control group were negative for COX 2, and among the OSMF group, 19 patients (67.9%) were positive and 9 patients (32.1%) were found to be negative for COX 2. The association of COX 2 expression with OSMF compared with normal patients was found to be statistically significant (χ^2 =21.955; P = 0.000), as shown in Tables 2 and 3.

On comparison of immune expression of COX 2 in different clinical stages based on functional staging, we found a very significant association of COX 2 expression with different clinical stages of OSMF (χ^2 = 7.368; P = 0.025), as shown in Table 4 and Graph 1. Among the patients who had the habit of using pan, 71.4% (n = 10); maava, 75% (n = 3); gutka, 50% (n = 1); and betel nut, 62.5% (n = 5) were found to show COX

Table 1: Clinical histopathological features in COX 2-positive and -negative groups of OSMF patients

Criteria	Total (n=28)	COX 2-negative (n=9)	COX 2-positive (n=19)
Age (years)			
< 42	19	7 (36.8%)	12 (63.2%)
>42	9	2 (22.2%)	7 (77.8%)
Gender			
Male	22	6 (27.3%)	16 (72.7%)
Female	6	3 (50%)	3 (50%)
Clinical stage			
Stage I	6	3 (50%)	3 (50%)
Stage II	12	6 (50%)	6 (50%)
Stage III	10	0 (0%)	10 (100%)
Habits			
Pan	14	4 (28.6%)	10 (71.4%)
Betel nut	8	3 (37.5%)	5 (62.5%)
Maava	4	1 (25%)	3 (75%)
Gutka	2	1 (50%)	1 (50%)
Epithelial nature			
Normal	4	0 (0%)	4(100%)
Mild dysplasia	10	2 (20%)	8 (80%)
Moderate dysplasia	1	0 (0%)	1 (100%)
Atrophic	13	7 (53.8%)	6 (46.2%)
Inflammation			
Mild	19	7 (36.8%)	12 (63.2%)
Moderate	6	2 (33.3%)	4 (66.7%)
Severe	3	0 (0%)	3 (100%)
Fibrosis			
Negligible	1	0 (0%)	1 (100%)
Mild	13	4 (30.8%)	9 (69.2%)
Moderate	5	1 (20%)	4 (80%)
Intense	9	4 (44.4%)	5 (55.6%)
Vascularity			
Enlarged and increased	12	4 (33.3%)	8 (66.7%)
Minimum	13	9 (69.2%)	4 (30.8%)
Enlarged	2	2 (25%)	6 (75%)
Increased	1	0 (0%)	1 (100%)

Table 2: Comparison of COX 2 expression between normal patients and different clinical stages of OSMF patients

Clinical presentation	COX 2-negative	COX 2-positive	Total
Normal	12 (100%)	0 (0%)	12
OSMF stage I	3 (50%)	3 (50%)	6
OSMF stage II	6 (50%)	6 (50%)	12
OSMF stage III	0 (0%)	10 (100%)	10

p value = 0.0000; χ^2= 21.955

2 expression; however, we did not find any statistical association. The histopathological parameters which were assessed included nature of epithelium, degree of inflammation, level of fibrosis, and vascularity. Most of the samples showed atrophic epithelium (n = 13) and COX 2 expression was observed in 43.2% (n = 6) samples. Statistical significance (P-value = 0.035; χ^2= 8.598) was observed on comparing the nature of epithelium, classified as normal, mild dysplasia, moderate dysplasia, and atrophic as shown in Table 5. Among the samples, based on inflammation, 63.2% (n = 12) of mild, 66.7% (n = 4) of moderate, and 100% (n = 3) of severe inflammation showed positive immunoexpression for COX 2. However, there was no statistically significant association identified with COX 2 expression based on the degree of inflammation.

Table 3: Comparison of COX 2 expression between normal patients and OSMF patients

Clinical presentation	COX 2-negative	COX 2-positive	Total
Normal	12 (100%)	0	12
OSMF	9 (32.1%)	19 (67.9%)	28

P-value = 0.0000; χ^2= 15.510

Table 4: COX 2 expression in different clinical stages of OSMF

Clinical stage	COX 2-negative	COX 2-positive	Total
Stage I	3 (50%)	3 (50%)	6
Stage II	6 (50%)	6 (50%)	12
Stage III	0 (0%)	10 (100%)	10

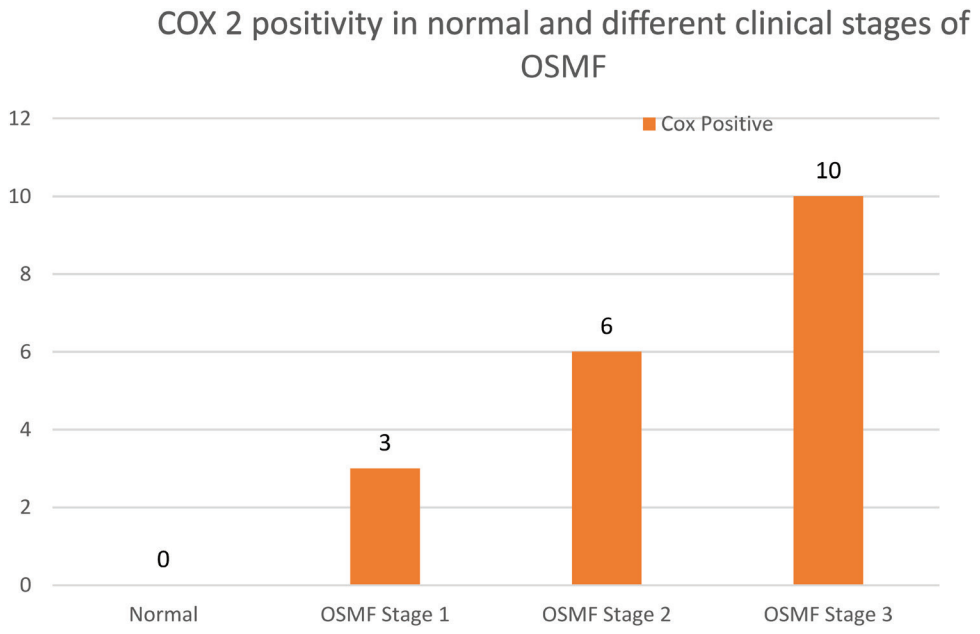
P-value = 0.025; χ^2= 7.368

The vascularity was found to be increased and enlarged in majority of the samples (n = 12), in which COX 2 positivity was found in 66.7% (n = 8) of the samples. Based on vascularity, on comparison between normal and OSMF, the results were found to be statistically significant (P-value = 0.014; χ^2= 10.628), as shown in Table 6. Based on fibrosis, 69.2% (n = 9) of mild fibrosis, 80% (n = 4) of moderate fibrosis, and 55.6% (n = 5) of severe fibrosis showed positive immunoexpression for COX 2, which was found to be statistically significant (P-value = 0.004; χ^2= 13.075), as shown in Table 7. The comparison of COX 2 expression in epithelium and CT was assessed, and there was no significant difference observed. The clinical and histopathological parameters were analyzed using univariate and multivariate analyses, which showed expression of COX 2, epithelial nature, and vascularity together as a statistically significant model, with disease progression in OSMF as shown in Table 8.

The expression of COX 2 is depicted in Figures 1–4. Figure 1 shows the normal oral epithelium with complete absence of COX 2 expression. Figure 2 shows the OSMF in stage 1 with sporadic epithelial and inflammatory cell positivity for COX2. Figure 3 shows OSMF in stage 2 with COX 2-positive epithelial cells in the stratum basale part of epithelium. Figure 4 shows OSMF in stage 3 with intense COX 2 cytoplasmic positivity in more than 80% of the epithelial cells.

Discussion

In this study, COX 2 expression has been evaluated in different stages of OSMF using IHC to serve as a



Graph 1: Graphical representation of COX 2 expression in normal and OSMF patients

Table 5: COX 2 expression based on nature of the epithelium

Epithelial nature	COX 2-negative	COX 2-positive	Total
Normal	12 (75%)	4 (25%)	16
Mild dysplasia	2 (20%)	8 (80%)	10
Moderate dysplasia	0 (0%)	1 (100%)	1
Atrophic	7 (53.8%)	6 (46.2%)	13

P-value = 0.035; χ^2= 8.598

Table 6: COX 2 expression based on differences in vascularity

Vascularity	COX 2-negative	COX 2-positive	Total
Minimum	12 (100%)	0 (0%)	12
Enlarged	3 (50%)	3 (50%)	6
Enlarged and increased	6 (50%)	6 (50%)	12
Increased	0 (0%)	10 (100%)	10

P-value = 0.014; χ^2= 10.628

Table 7: COX 2 expression based on severity of fibrosis

Fibrosis	COX 2-negative	COX 2-positive	Total
No fibrosis	12 (92.3%)	1 (7.7%)	13
Mild fibrosis	4 (30.8%)	9 (69.2%)	13
Moderate fibrosis	1 (20%)	4 (80%)	5
Intense fibrosis	4 (44.4%)	5 (55.6%)	9

P-value = 0.004; χ^2= 13.075

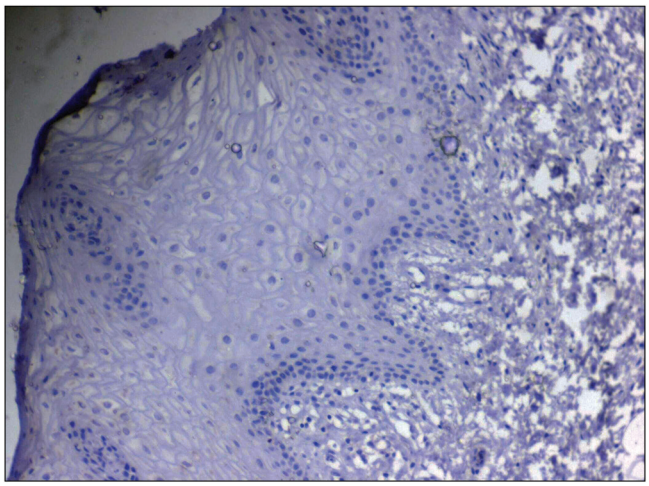


Figure 1: Normal oral epithelium with complete absence of COX 2 expression, IHC, 20×

potential biomarker in assessing the progress of the disease. Arachidonic acid is released by the activation of phospholipase A2 from membrane phospholipids and is subsequently transformed by the enzyme COX to PGs and thromboxane. In particular, the COX pathway holds a greater clinical relevance because it is the major target for anti-inflammatory, analgesic, and antipyretic effects of non-steroidal anti-inflammatory drugs. It was found that COX exists in two distinct isozymes (COX-1

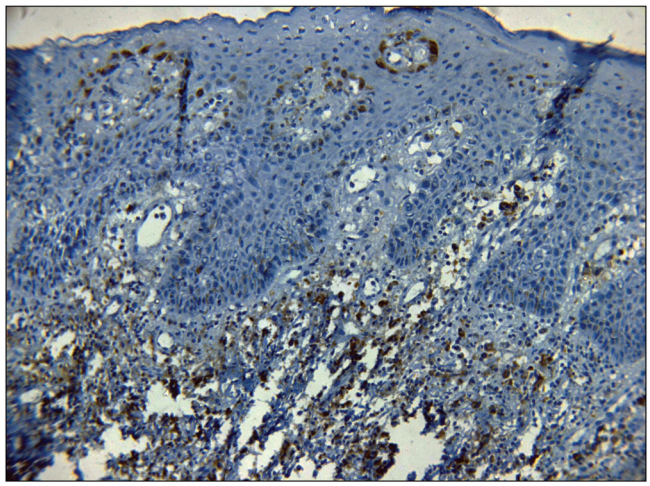


Figure 2: Clinical stage 1 OSMF showing sporadic epithelial and inflammatory cell positivity for COX2. IHC, 20×

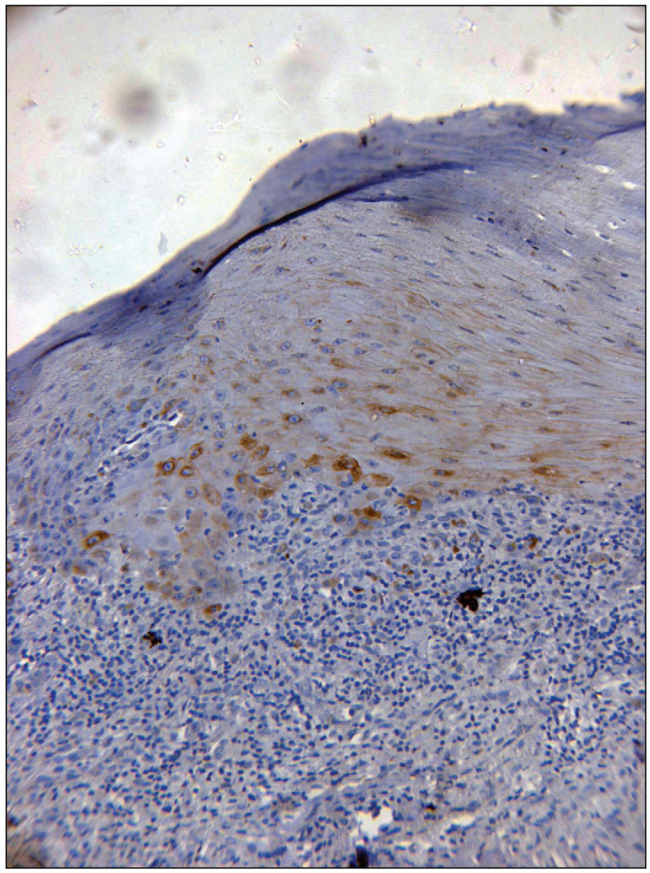


Figure 3: Clinical stage 2 OSMF showing COX 2-positive epithelial cells in the stratum basale part of epithelium. IHC, 20×

and COX-2), of which COX-2 is primarily responsible for inflammation.[19] This proinflammatory enzyme is involved in the alteration of cell adhesion, inhibition of apoptosis, alteration of the response to growth regulatory signals and is found to play an important role in tumorigenesis of head and neck cancer.[30]

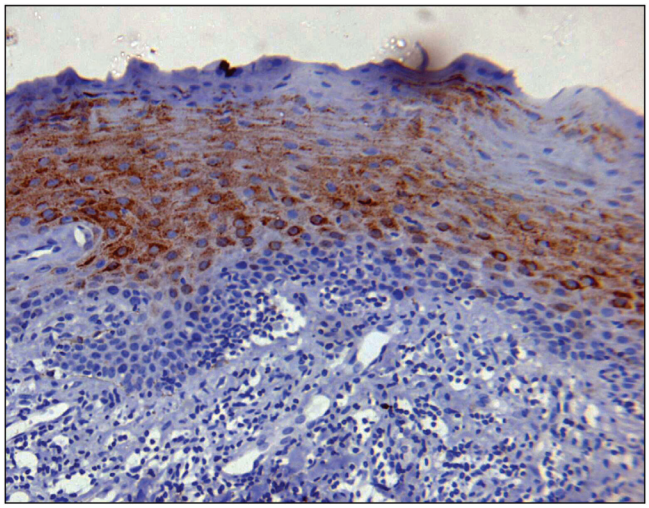


Figure 4: Clinical stage 3 OSMF showing intense COX 2 cytoplasmic positivity in more than 80% of the epithelial cells. IHC, 20×

Constant areca nut chewing in OSMF triggers inflammatory response leading to cascade of molecular changes resulting in OSMF. Such exogenous carcinogens causing aberrant and persistent tissue inflammation have been suggested to induce the progression of cancer and tissue fibrosis.[31,32] As the severity of inflammation increases, T cells and macrophages activate cytokines, growth factors, and procollagen genes, resulting in further progression of fibrosis, leading to progressive reduction of mouth opening in the advanced stages.[33] Thus inflammatory changes activated by areca nut chewing promote proinflammatory enzyme COX 2, which plays a crucial role in the progression of OSMF and cancer.

In a study comparing normal, precancerous, and oral cancer samples, it was found that COX 2 expression varied according to the severity and was intensely seen in oral cancer followed by precancerous samples.[26] *In-vitro* study using primary gingival keratinocytes stimulated by areca nut extract showed an upregulation of COX 2 expression and suggested that COX 2 contributed to the pathogenesis of OSMF and oral cancer.[34]

The immunoexpression of COX 2 in the present study was significantly increased in the OSMF group, when compared with controls, with none of the normal patients showing positivity to COX 2, the results of which were in correlation with a similar study.[17] Similar correlation with the present study was observed in a study done by Tsai *et al.*,[35] in which COX 2 expression was upregulated in OSMF specimens compared with normal buccal mucosa, with strong immunostaining for COX-2 being detected in epithelial cells, fibroblasts, and

Table 8: Univariate and multivariate analyses

	Univariate		Multivariate	
Parameter	***F*-value**	***P*-value**	***F*-value**	***P*-value**
Gender	0.013	0.912		
Age	2.826	0.101		
Epithelial nature	541.661	0.000*	23.790	0.000
Inflammation	0.491	0.616		
Vascularity	11.891	0.000*	23.471	0.000
COX 2 expression	24.067	0.000*	24.067	0.000

inflammatory cells. Statistically significant expression of COX 2 was observed on comparison with normal, OSMF, and OSCC, with increased expression of advanced stage of OSMF in a study done by Singh *et al*.[36] The current study showed significant positivity for COX 2 with respect to different clinical stages of OSMF. The association of COX 2 with histopathological parameters such as degree of fibrosis and vascularity showed statistically significant difference. The study done by Rangaswamy *et al.*[17] showed no significant correlation with histopathological grading of OSMF. Though, in the current study, correlation of COX 2 with histopathological grading was not done, it was observed that the COX 2 expression significantly increased with the increase in severity of fibrosis. Hence, it could be stated that COX 2 role in the inflammatory pathway adds to progressive fibrotic changes, leading to further reduction of mouth opening with advanced stages of OSMF. On assessing the relation between different grades of inflammation, no significant difference was found. The probable association of degree of inflammation with COX 2 expression can be further evaluated with a larger sample size. However, significant association was evident based on the vascularity and expression of COX 2, which shows that increased vascularity triggered during inflammation plays a substantial role in disease progression. In an attempt to find the association of dysplasia connected with OSMF with COX 2 expression, it was found to be statistically insignificant, probably attributed to the lower sample size.[17] However, in the present study on comparison with normal, atrophic, mild, and moderately dysplastic epithelium, statistically significant difference was noted with the expression of COX 2. Evaluation of clinical and histopathological parameters, using univariate and multivariate analyses, showed that COX 2 expression along with nature of the epithelium and vascularity together as a model would help in identification of disease progression in OSMF. Further correlation of different grades of dysplasia and vascularity with the expression of COX 2 is worthwhile to be explored with adequate sample size for added validation regarding its predictability in early detection of malignant transformation.

Conclusion

The significant COX 2 expression in different clinical stages of the present study represents the possible role of COX 2 in the pathogenesis and progression of OSMF. Hence, it could serve as a valuable biomarker to assess the stage of disease presentation and predict malignant transformation. Future clinical trials using COX 2 intervention and long-term follow-up could further establish the role of COX 2 in the prevention of disease progression.

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Conflicts of interest

There are no conflicts of interest.

Authors contributions

CVD was involved in study conception, literature search, data acquisition, manuscript preparation; SS and RA were involved in study conception and manuscript review; VS was involved in data analysis and manuscript preparation; HS was involved in data collection, data acquisition; and VR was involved in study conception, statistical analysis, manuscript editing, and review. All the authors approved the final version of manuscript for publication.

Ethical policy and institutional review board statement

The research project was approved by the Institutional Ethics Committee, SRIHER (DU) Ref No. CSP/17/AUG/60/239.

Patient declaration of consent

The authors certify that they have obtained the appropriate patient consent.

Data availability statement

The data set used in the current study is available on request from Divyambika (cvdivyambika@sriramachandra.edu.in).

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