Original Article

Expression of cyclooxygenase 2 in oral submucous fibrosis: An immunohistochemical pilot study

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Abstract

Introduction: Oral submucous fibrosis (OSF) is associated with inflammatory changes in at least some stages of the disease. Prostaglandin is one of the main inflammatory mediators and its production is controlled by various enzymes such as cyclooxygenase (COX). The genetic and pharmacological data strongly indicate that COX-2 should be investigated as a potential target for the prevention and treatment of OSF.

Methodology: The study group comprised histologically confirmed specimens ($n = 10$ each) of early OSF, moderate OSF, advanced OSF and normal oral mucosa for comparison. Immunohistochemistry was performed with avidin–biotin technique and evaluated with scoring methods.

Results: The difference in percentage of expression in normal tissue and OSF was statistically highly significant ($P < 0.001$). Positive COX-2 exhibited cytoplasmic staining. One-way analysis of variances test was performed to evaluate COX-2 expression in different grades of OSF. Cytoplasmic staining assessed in terms of intensity, percentage of expression and Q Score did not show any statistical difference (percentage of expression F = 0.029, $P = 0.971$; Q Score F = 0.154, $P = 0.858$).

Conclusions: Our study indicates that COX-2 may be an important marker of disease progression and might be a reliable prognostic indicator.

Keywords: Cyclooxygenase-2, immunohistochemistry, oral submucous fibrosis, salivary expression

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INTRODUCTION

Oral submucous fibrosis (OSF) is chronic, progressive, precancerous condition with high chance of malignant transformation. Numerous biological pathways are involved in pathogenesis of submucous fibrosis and its transition to cancer. Precise molecular mechanisms deserve exploration. Inflammation is observed in some stage of OSF and may have a role in disease progression and malignant transformation.[1]

Prostaglandin-endoperoxide synthase, commonly called as cyclooxygenase (COX), is the key regulatory enzyme in tissue inflammation and is present in two isoforms COX-1 and COX-2. COX-2 is an inducible form of COX, and its overexpression has been shown to promote tumorigenesis by activation of carcinogens, cytokines,

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neoangiogenesis, stimulating progression and inhibiting apoptosis.[2] Researchers have found that molecular changes in oral premalignant condition are preceded by alteration in COX gene expression.[3]

Upregulation of COX-2 has been shown in oral potentially malignant lesions and oral squamous cell carcinoma.[4-8] It can be a prognostic predictor and molecular target, thus it needs to be evaluated. The aim of the study was to assess the immunohistochemical expression of COX-2 enzyme in normal mucosa and submucous fibrosis and further deliberate difference in histological grades of submucous fibrosis.

METHODOLOGY

Formalin-fixed, paraffin-embedded OSF tissue blocks were obtained from departmental archives. 5-μm sections of samples were stained with routine hematoxylin and eosin and analyzed under light microscopy. The stained sections were analyzed by three oral pathologists without prior knowledge of clinical data to histologically grade the submucous fibrosis and dysplasia according to the WHO 2005[9,10] In the cases of disagreement, the pathologists discussed the findings and performed the final evaluation. Ten samples of early OSF (EOSF), moderate OSF (MOSF) and advanced OSF (AOSF) each were randomly selected. Ten samples of normal mucosa were processed.

Immunohistochemistry procedure

Immunohistochemistry (IHC) was performed with avidin–biotin technique and the 5-μm sections were placed on positively charged slides. Sections were deparaffinized, rehydrated and quenched. IHC staining was done with commercially prepared antibodies for COX-2 in Autostainer Intelipath (monoclonal antibodies from mice, MACH 1 Mouse Probe, Biocare medicals USA). Antigen retrieval was done using ethylenediamminetetraacetate solution with pH 8 sections were covered with Mach 1 HP Polymer incubated with secondary antibody. Antigen-antibody binding was detected with Betazoid DAB Chromogen and sections were counterstained with CAT hematoxylin counterstain. Expression of the marker was evaluated using scoring methods [Tables 1 and 2].

Statistical analysis

The data obtained from the proteins expression were submitted for analysis of variance (ANOVA) to assess the statistical difference in percentage of expression and Q Score between the groups. Independent Student's *t*-test was applied to compare normal and submucous fibrosis groups. Chi-square test was applied to evaluate the degree of dysplasia in different grades and different expression.

RESULTS

COX-2 immunoexpression was done using standard immunohistochemical techniques. The study group comprised histologically confirmed specimens (n = 10 each) of EOSF, MOSF, AOSF and normal oral mucosa for comparison.

COX-2 was not expressed in morphologically normal mucosa [Figure 1]; out of 10 cases, 6 cases did not show any uptake, 3 cases showed 25% low-intensity expression and 1 case showed 50% low-intensity expression (mean: 17.5% of expression). The difference in percentage of expression in normal tissue and OSF was statistically highly significant ($P < 0.001$). Comparison of Q Score of normal and OSF tissue showed statistical difference in two groups (N mean = 25, standard deviation [SD] = 33.33; OSF mean = 138.33, SD = 90.195 $P < 0.001$). COX-2 protein found to be expressed in increasing intensity in OSF compared to normal mucosa [Graph 1].

Quantitative scoring methods

One-way ANOVA test was performed to evaluate COX-2 expression in different grades of OSF. Cytoplasmic staining assessed in terms of intensity, percentage of expression and Q Score did not show any statistical difference (percentage of expression F = 0.029, $P = 0.971$ [Table 3]; Q Score F = 0.154, $P = 0.858$). EOSF group showed 100% expression in 4 cases, MOSF in 3 cases and AOSF in 4 cases. Stronger intensity was found in EOSF [Graph 2]. However, strong immunostaining was observed in EOSF and MOSF [Figure 2] compared to AOSF even though the difference is statistically not

Table 1: Scoring of percentage of expression

Score	0	1+	2+	3+	4+
Positive cells (%)	<10	10–25	25–50	50–75	>75

Table 2: Scoring of intensity of expression

Score	1	2	3
Intensity of staining	Weak staining	Moderate staining	Strong staining

Table 3: Q score in different grades of dysplasia

Dysplasia	*n*	Mean	SD	Minimum	Maximum	*F*	*P*
Group							
Mild	14	101.79	60.815	25	225	3.571	0.028
Moderate	10	205.00	86.442	100	300		
Severe	1	150.00	.	150	150		
Nil	5	105.00	113.743	25	300		

SD: Standard deviation

significant (Q Score: EOSF and MOSF mean = 145 and AOSF mean = 125) [Graph 3].[11-13]

Increased uptake was observed with increased dysplasia, suggesting that increased COX-2 may contribute to malignant change in OSF cases ($P = 0.028$). Since only one case of severe dysplasia was seen, this aspect needs to be further evaluated with larger sample.

In samples showing 100% epithelial uptake, six cases showed moderate dysplasia and one case of severe dysplasia suggesting COX-2 is associated with dysplasia [Figure 3], whereas in cases <25% ($n = 4$) uptake showed no dysplasia in two cases mild dysplasia in two cases. The difference is not statistically significant when correlated. There is no statistical difference in degree of dysplasia between different grades of OSF.

Fifteen samples out of 30 samples have taken up COX-2 staining which accounts for 50%. No statistical difference was found between OSF grades with respect to connective tissue uptake of COX-2. However, with increase in percentage of expression in epithelium, increased connective tissue expression is observed [Graph 4].

Interestingly, 7 out of 30 cases showed intense uptake by minor salivary gland [Figure 4] and such expression was not seen in the normal mucosa. Ductal epithelium was taking more stain compared to acinic cells.

DISCUSSION

COX-2 is an inducible isoform of cyclooxygenase derived from arachidonic acid that plays an important role in various pathophysiologic conditions. COX-2 is normally not detectable in tissue but induced by trauma, pro-inflammatory or mitogenic stimuli.[14,15] COX-2 has been paid attention since it could play an important role in

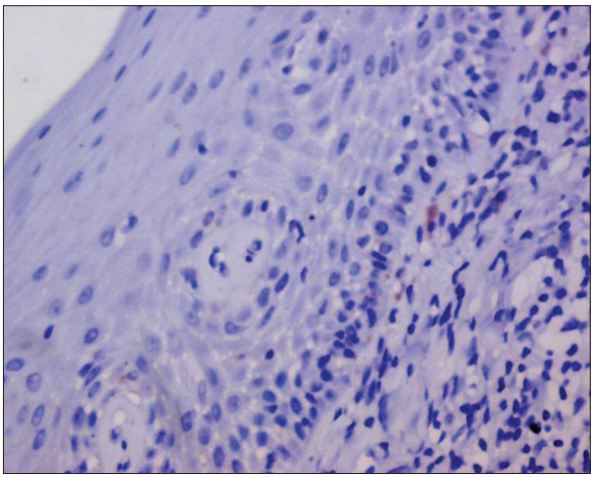


Figure 1: Complete absence of immunohistochemistry expression of cyclooxygenase-2 in normal mucosa

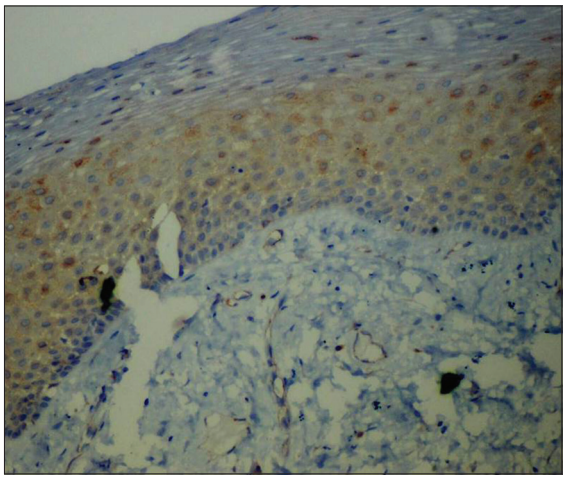


Figure 2: A diffuse dense cytoplasmic expression of cyclooxygenase-2 in basal parabasal and intermediate cells of epithelium associated with oral submucous fibrosis

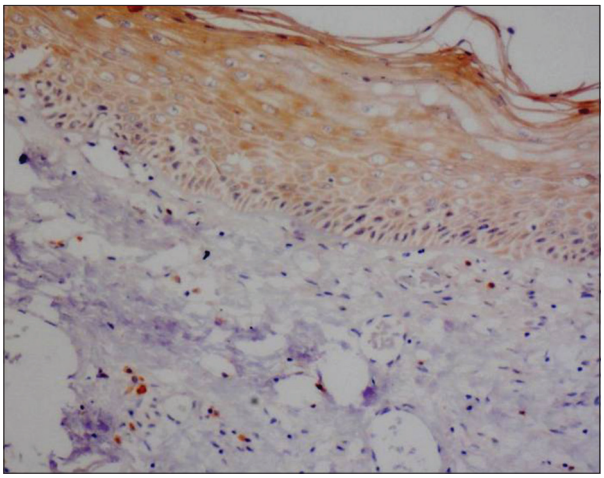


Figure 3: Dense diffuse cytoplasmic and membranous expression of cyclooxygenase-2 in dysplastic cells in entire thickness of the epithelium

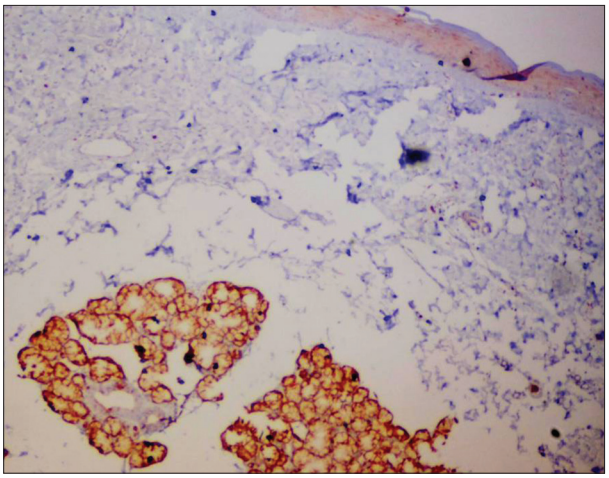
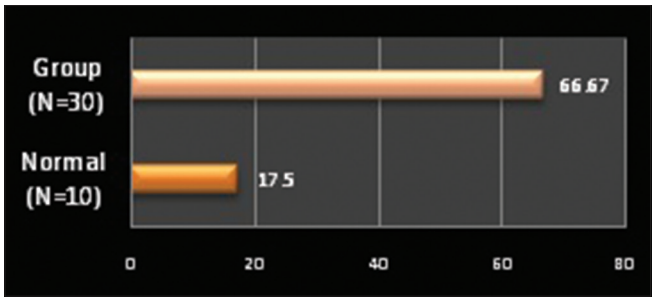
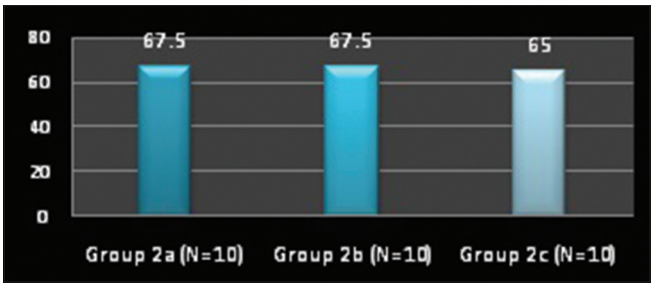


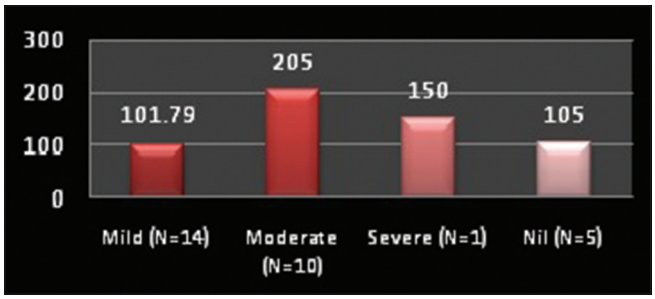
Figure 4: Immunohistochemistry expression of cyclooxygenase-2 in complete thickness of epithelium and minor salivary gland



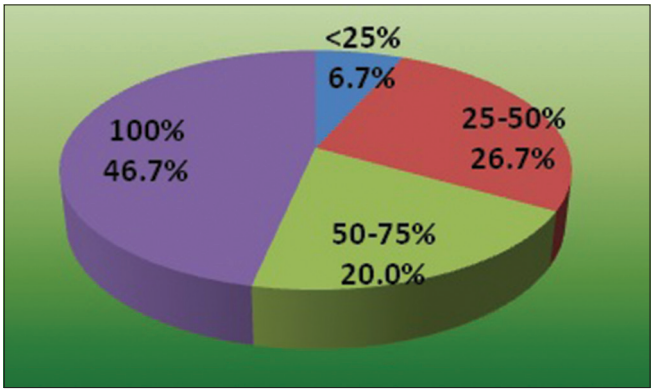
Graph 1: Comparison of mean percentage of expression of cyclooxygenase-2 in normal and submucous fibrosis group



Graph 2: Comparison of mean percentage of expression of cyclooxygenase-2 in different grades of submucous fibrosis



Graph 3: Comparison of mean Q Score to degree of dysplasia



Graph 4: Distribution of percentage of expression to connective tissue uptake (*n*=13)

the initiation and progression of carcinomas of the various organs.[6,16-18] Upregulation of COX-2 is associated with increased angiogenesis, proliferation of cancer stem cells and inhibition of apoptosis.[19]

Tissue inflammation is believed to play an important role in occurrence of tissue fibrosis. OSF and lichen planus are potentially malignant disorders where immunoinflammatory processes are implicated in pathogenesis and malignant transformation. Very few studies have been done to evaluate the COX-2 expression in OSF. Tsai *et al*.[20] demonstrated that COX-2 was significantly higher in OSF specimens and expressed mainly in epithelial cells, endothelial cells and fibroblasts. They also observed that COX-2 expression in cells treated with arecoline was upregulated as early as half an hour, suggesting that COX-2 is an early cellular response. Studies have shown 1.4–3.4-fold increase of PGE2 production and 1.1–1.7-fold increase of PGE 1 when gingival keratinocytes exposed to areca nut extracts.[21,22]

The present study was done to record the immunohistochemical expression of COX-2 in normal oral mucosa and different grades of submucous fibrosis. Increased uptake of COX was seen in OSF specimens compared to normal mucosa. Our result is similar to those obtained in the previous studies that found an increased COX-2 expression from normal to oral potentially malignant disorders to OSCC, but it is especially in accordance to the results obtained by Shibata *et al*.,[7] who evaluated the expression of COX-1 and COX-2 in oral carcinogenesis and found that COX-2 expression was higher in oral dysplasia than in OSCC. COX (COX-2) expression analysis by Singh *et al*.[23] Immunocytochemistry and Western blot found synchronization in both the assays which support the finding that COX-2 expression is upregulated in OSF specimens compared to normal oral submucosal cells. Strong immunostaining for COX-2 was detected in arecoline exposed normal oral mucosal cells and in OSF samples.

Immunoreactivity for COX-2 was mainly found in the cytoplasmic compartment. COX-2 uptake was limited to suprabasal layers in EOSF and connective tissue uptake was seen in advanced cases with dysplastic changes although the difference could not be statistically proved. COX-2 was cytoplasmic in cancer cells, and it was also observed in the stromal components, especially in inflammatory cells, suggesting that the immunoreactivity for COX-2 may be modulated by interaction of the stromal cells with cancer cells in the process of destructive invasion. Cytoplasmic staining was also assessed in terms of the intensity of the immunopositive reaction. No statistical difference was found among the groups. Similar immunoreactivity has been reported by Itoh in oral squamous cell carcinoma.[24]

Statistically, we could not find a correlation between the COX-2 overexpression and histological grades of

submucous fibrosis. Strong immunostaining was observed in EOSF and MOSF compared to AOSF comparable to a study by Gallo *et al*.[25] where biopsies from buccal mucosa of OSF cases and controls were stained for COX-2 by IHC and revealed that there was increased expression of the enzyme in moderate fibrosis, and this disappeared in advanced fibrosis. This finding is compatible with the histology of the disease, as there is a lack of inflammation in the advanced disease.

OSF is characterized by the formation of thick bands of collagen fibers and hyalinization extending deep into the submucosal tissues and decreased vascularity. Inflammation and fibrosis of minor salivary glands and muscle degeneration will occur in advanced stages of OSF. On histological examination, varying degree of fibrosis of minor salivary gland has been observed with degenerative change in mucous acini.[26,27] We observed increased uptake of immunostaining by the minor salivary glands in OSF group, suggesting alteration in salivary secretions may be part of pathogenesis COX-2 immunoreactivity might be modulated by the interaction of stromal cells and cancer cells during progression to advanced disease or invasion. *In vivo* autofluorescence from the buccal mucosa seems to be an interesting noninvasive tool to differentiate normal mucosa from OSF and early carcinoma.[28]

Nevertheless, due to the small number of samples included in this study, general statements regarding correlation between the degree of severity of the OSF pathology and the quantitative expression of these potential markers cannot be made. Prospective studies with larger samples may be of greater clinical importance and reliable prognostic indicator.

CONCLUSIONS

Our findings regarding COX-2 expression suggest that as OSF progresses the population of epithelial cells immunoreactive for COX-2 also increases. This indicates that COX-2 may be an important marker of disease progression. Current failure in treatment of submucous fibrosis is due to our inability to target molecular mechanisms. COX-2 can serve as predictor toward disease progression and malignant transformation. Further studies on this can help in early intervention with COX inhibitors or immune modulators for the benefit for the humankind.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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