

**Expression of COX-2 in Normal Oral Mucosa, Oral Submucous Fibrosis and Oral Squamous Cell Carcinoma- An Immunohistochemical Study.**

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**Abstract**

**Background:** Oral Cancer is a major public health issue all around the world. Over 200,000 cases of head and neck cancer occur each year in India. Nearly 80,000 oral cancers are diagnosed every year in our country. Establishment of an early and reliable biomarker for oral carcinogenesis wherein the progress of the lesion can be monitored through non-invasive technique will enable early diagnosis of cancer.

In recent years, over-expression of Cyclooxygenase-2 (COX-2) has been reported in various cancers including oral squamous cell carcinoma. They play an important role in initiation and progression of Carcinomas of various organs.

Although many studies have shown over-expression of COX -2 in oral malignant lesions, only few researchers

have compared COX-2 in normal mucosa, pre-malignant conditions and oral squamous cell carcinoma.

**Aim & Objective:** To compare the expression of COX-2 in normal mucosa, Oral Submucous Fibrosis and Oral Squamous Cell Carcinoma through immunohistochemical staining technique.

**Material & Method:** The present study comprised of three groups: Group I: Twenty normal oral mucosa; Group II: Twenty established cases of Oral Submucous Fibrosis and Group III: Twenty established cases of Oral squamous cell carcinoma.

The COX-2 expression were studied on formalin fixed and paraffin embedded tissue section by the standard immunohistochemical techniques, using immunohistochemical detection system and as per the

protocols laid down by manufacturer. (Biogenex Life Sciences Private Limited, CA, USA).

**Statistical Analysis Used:** The expression of COX-2 was evaluated between the three groups and within the degree of differentiation of the tumors. Statistical analysis was done using Kruskal Wallis Test and correlation was assessed using Spearman's Rank correlation test.

**Result:** A statistical significant ( $p= 0.00$ ) and a positive correlation was found in the COX-2 expression between the three groups. COX-2 expression within the degree of differentiation of oral squamous cell carcinoma was found to be statistically non-significant and a negative correlation was found within the three grades of tumor.

**Conclusion:** These finding implicates that COX-2 plays a role in carcinogenesis and tumor transformation and progression. Further studies with larger sample size are needed for COX-2 to be a reliable diagnostic biomarker in cases of oral precancer and oral cancer.

**Keywords:** COX-2, Oral Squamous Cell Carcinoma, Oral Submucous Fibrosis, Biomarker.

## INTRODUCTION

Oral Cancers are ranked as second most common in males and third most common in females in India. Nearly 80,000 oral cancers are diagnosed every year in our country.<sup>i</sup>

The overall 5 year survival rate of oral cancer have remained low at approximately 50% for the past decade and have remained the worst of all cancer death rates.<sup>ii</sup> Establishment of an early and reliable biomarker for oral carcinogenesis whose expression can be monitored through non-invasive technique will enable early diagnoses of cancer and the best way for patient survival and improved quality of life.

Cyclooxygenases (COX's) are the rate limiting enzymes for high output production of prostanoids (Prostaglandins and Thromboxanes) from Arachidonic acid, which play an important role in various pathophysiological conditions.<sup>iii</sup>

Enhanced synthesis of Prostaglandins, which results from upregulation of COX-2, increases the proliferative activity of neoplastic cells, cancer invasiveness and metastasis. It also promotes angiogenesis and inhibits immune surveillance and apoptosis.<sup>iv, v</sup>

In recent years, over-expression of COX-2 has been reported in various cancers from colon,<sup>vi,vii</sup> stomach,<sup>viii</sup> breast,<sup>ix</sup> lung,<sup>x</sup> esophagus,<sup>xi</sup> pancreas,<sup>xii</sup> urinary bladder,<sup>xiii</sup> prostate,<sup>xiv</sup> and oral cancer<sup>xv</sup>. They play an important role in initiation and progression of carcinomas of various organs.

In the present study, we have compared expression profile of COX-2 in normal mucosa, Oral submucous fibrosis (OSMF) and Oral Squamous Cell Carcinoma (OSCC) through immunohistochemical staining technique.

## Materials and Method

### Sample Collection

In this study, 60 formalin- fixed paraffin embedded samples of which 20 cases were of normal oral mucosa, 20 cases of OSMF and 20 cases of OSCC were taken from the archives of Oral and Maxillofacial Pathology department, HKES's S.N. Institute of Dental Sciences & Research, Kalaburagi, Karnataka (India). The Ethical Committee of the Institution approved the design of this study. Only established cases of OSCC and OSMF and clinical and histopathological confirmed normal oral mucosal tissue were included in the study. Treated cases of OSCC, OSMF and inflammatory conditions of oral cavity were excluded from the study. All the cases of OSCC were later graded as well differentiated moderately differentiated and poorly differentiated according to Broder's classification.<sup>xvi</sup> Colon carcinoma was used as positive control. Negative control was established by omission of the primary antibody.

## Immunohistochemistry

Immunohistochemical staining was performed as per the protocol laid by Biogenex Life Sciences Private Limited, CA, USA. Two to three sections of 3-micron size were taken. These sections were deparaffinized in three changes of xylene and rehydrated in graded alcohol. For antigen retrieval, the deparaffinized slides in citrate buffer with pH = 6 were put in the pressure cooker for 2 whistles. Incubating the sections with peroxidase block for 20 minutes blocked endogenous peroxidase activity followed by gentle wash with Tris buffer Saline solution (pH = 7.4). Non-specific antibody binding was blocked by pretreatment with protein block solution for 10 minutes. Then the sections were incubated with the primary antibody, which is ready to use monoclonal rabbit anti-human COX 2 antibody (Clone Number: SP 21; Biogenex Life Sciences Private Limited, CA, USA) for one hour. After primary antibody application, these sections were again washed with Tris- Buffered Saline. In next step super enhancer reagent was applied on the sections for 20 minutes in order to enhance the penetration of the subsequent polymer reagent. The sections were then incubated with the secondary antibody (Poly Horse radish Peroxidase reagent) for 30 min. Diaminobenzidine (DAB) was used as chromogen. The sections were counterstained with Harris hematoxylin. Finally slides were dehydrated in graded alcohol and then cleaned with xylene and covered with a coverslip.

## Evaluation of cox- 2 immunostaining

Two independent pathologists in order to eliminate inter-observer bias observed prepared slides independently with an optical microscope (OLYMPUS, BX-41). Presence of red/ brown colored end product at the site of target antigen was indicative of positive reactivity. The negative control tissue demonstrated absence of specific staining.

The evaluation of staining for the expression of COX 2

was assessed on the basis of the intensity and pattern of staining and the location of staining. Intensity of staining was assessed using a semi-quantitative method.

COX- 2 positive cells, which showed staining for IHC, were then subjected to manual counting. Percentage of positive cells in individual cases was counted in five high-power fields in hot spots on slide. The stained sections were observed for positivity of COX-2 expression. The positivity was expressed in terms of percentage of tumor cells positive for COX-2 expression. Absent (0) - Negative staining; For Positive staining: Mild: <5%; Moderate: 5–30% and Intense: >30

Negative and <5% COX-2 positivity were taken as negative expression and  $\geq 5\%$  COX-2 positivity as positive expression group. The >30% COX-2 positivity was taken as over-expressed group and  $\leq 30\%$  COX-2 positivity was taken as under-expressed group

## Statistical Analysis

Using SPSS version 17 IBM Software, the statistical analysis of observation data was done. The relationship with histological grade and expression of COX-2 (Ordinal data) were analysed using Kruskal Wallis Test. Using Kruskal Wallis and Mann Whitney-U Test, the comparison between ordinals of the two groups was performed. The relationship between COX-2 expression among the cases of normal oral mucosa, oral submucous fibrosis and oral squamous cell carcinoma were also studied using same test. The correlation was done using Bi-variate analysis and Spearman Rank Order Correlation test. Significance value was considered when p value was less than 0.05 ( $p > 0.05$ )

## Observations

A total of sixty histopathologically confirmed cases were included in the present study of which twenty cases were of normal oral mucosa (Mean age of  $36.05 \pm 11.062$  years); twenty cases of oral submucous fibrosis (Mean age

of  $26.65 \pm 6.976$  years) and twenty cases of oral squamous cell carcinoma (Mean age of  $42.65 \pm 9.582$  years). [Table-1].

Among the 20 cases of oral squamous cell carcinoma, there were 8 cases of well-differentiated squamous cell carcinoma, 7 cases of moderately differentiated squamous cell carcinoma and 5 cases of poorly differentiated squamous cell carcinoma. [Table-3]

There was statistically significant difference in the expression of COX-2 among three groups. The result was even statistically significant when comparison was done between normal mucosa with OSCC, normal mucosa with OSMF (p value 0.00; Table-3) and OSMF with OSCC and was found to be statistically significant (p value 0.00; Table-3)

COX-2 expression were noted in all three groups and analyzed statistically. There was statistically significant difference in the expression of COX-2 among three groups. The result was even statistically significant when comparison was done between normal mucosa with OSCC and normal mucosa with OSMF (p value 0.00; Table 3). The correlation between OSMF and OSCC was also found to be statistically significant (p value 0.00; Table 3). The result of statistical tests was confirmed with Mann-Whitney U Test, which revealed similar results.

Correlation of expression of COX-2 with normal mucosa, OSMF and OSCC were established using Spearman correlation test. The analysis revealed statistically significant correlation between these two variables. (p value 0.001, Table-4 ). Thus it can be concluded that the expression of intensity varies with the group.

Analysis of degree of differentiation and expression of COX-2 among three subtype of oral squamous cell carcinoma was done using Kruskal Wallis test. The ordinals were converted into rank value. The test revealed that there is no significant difference in the expression of

COX-2 when compared among well, moderately and poorly differentiated variants of OSCC (p= 0.367, Table-5). The correlation between degree of differentiation and expression of COX-2 was negative when analyzed using Spearman correlation (p = 0.214, Table-6). The correlation coefficient was 0.291 that shows no correlation among degree of differentiation and expression of COX-2.

### Discussion

Cyclooxygenase (COX) is the key-enzyme catalyzing the first step in the biosynthesis of prostaglandins, molecules that play a pivotal role in the initiation and progression of many cancers.<sup>xvii</sup> In particular COX-2 seems to act synergistically with cytosolic phospholipase A2, the other key enzyme of prostaglandin synthesis, to contribute to the process of oral carcinogenesis and both enzymes are overexpressed in oral dysplasia and carcinoma. Cell culture lines studies have shown that COX-2 contributes to the neoplastic potential of epithelial cells by increasing adhesion to extracellular matrix and making them resistant to apoptosis.<sup>xviii</sup>

The contribution of COX to carcinogenesis is due to its involvement in several key-mechanisms: 1) the conversion of pro-carcinogens to carcinogens as a consequence of arachidonic acid metabolism, 2) stimulation of cell growth, 3) inhibition of apoptosis, 4) stimulation of Vascular Endothelial Growth Factor (VEGF) and angiogenesis, 5) promotion of invasion and metastasis via matrix metalloproteinases 6) immunosuppression by Interleukin-10 (IL-10) induction.<sup>xix</sup>

In the present study, we evaluated COX-2 expression in twenty normal oral mucosa, twenty OSMF cases and twenty OSCC cases. We correlated the expression of COX-2 in normal mucosa, OSMF and OSCC and to degree of differentiation in tumors.

In our study we found faint COX-2 expression in 7 (35%) out of 20 normal mucosa samples. These focal COX 2

expressions were located in the cytoplasm of basal cells, often being concentrated around peri-nuclear area forming a discrete “halo”. Few COX-2 expressions were positive in endothelial cells, smooth muscle cells and lymphocytes of the stroma. Studies have shown that these served as internal positive control.<sup>xx</sup> **Shiotani et al**<sup>xxi</sup> found weak basal staining of COX-2 in normal tongue epithelium of rats. **Shibata et al**<sup>xxii</sup> obtained rare expression ( $3.8 \pm 1.2$ ) in 9 normal oral mucosa samples. **Tsai**<sup>xxiii</sup> obtained very faint normal expression, which is limited to lamina propria. We believe that faint COX-2 positivity could be due to antibody sensitivity.<sup>20, 24</sup>

In our study 13 (65%) out of 20 normal mucosa samples were totally negative in COX-2 expressions. **Minter et al**<sup>20</sup> found total negative COX-2 expressions in 6 out of 23 normal samples. **Renoken et al**<sup>xxiv</sup> found 25% of 38 samples of normal tongue were totally negative result. **Syedmijadi et al**<sup>xxv</sup> found absence of normal mucosa staining in 19 out of 20 samples. This is attributed to the fact that COX-2 is absent under normal conditions and expressed mainly in response to inflammatory and mitogenic stimuli.<sup>xxvi</sup>

In our study, COX-2 immunoreactivity was seen in all the cases of Oral Submucous Fibrosis. These expressions were located in cytoplasm of basal cells of epithelium. COX-2 expressions were seen in few fibroblasts, lymphocytes, endothelial cells and smooth muscle cells. **Patil et al**<sup>xxvii</sup> too found COX-2 expressions in cytoplasm and restricted to basal cells in OSMF cases. They obtained weak COX-2 expression in early OSMF cases but strong expression in advanced OSMF cases. In vitro studies indicate that buccal mucosal fibroblasts do not constitutively express COX-2. However, when fibroblasts are challenged with agents causing OSMF, upregulation of COX-2 has been observed as early as 30 minutes later.<sup>23</sup> Our findings regarding COX-2 expression suggest that as

OSMF progresses the population of epithelial cells immunoreactive for COX-2 also increases. **Tsai et al** found COX-2 expression in sub-epithelial and deeper layer in early to moderate OSMF cases while in advanced OSMF cases weaker and less frequency is seen.<sup>23</sup>

In our study, positive COX-2 staining was seen in 95% cases of Oral Squamous Cell Carcinoma. Our finding correlated with various studies. In a study conducted by **Timo Atula et al**<sup>19</sup> found expression of COX-2 in 88% of Squamous cell carcinoma. **Minter et al**<sup>20</sup> found all tumor samples stain positively for COX-2 expressions. Few studies exhibit 44-96% expression of COX-2 in squamous cell carcinoma cases.<sup>xxviii, xxix</sup>

In our study COX-2 staining was located in cytoplasm of basal layer of epithelium in well and moderately differentiated oral squamous cell carcinoma. This is conjunct with the finding of **Minter et al**<sup>20</sup>, who found COX-2 expressions in the basal layer of oral squamous cell carcinoma. Our findings also correlated with **Renoken et al**<sup>24</sup> and **Nathan et al**,<sup>xxx</sup> which implicates that COX-2 expressions correlates with cell proliferation, hence more commonly seen at basal layer. In some areas invading malignant epithelium surrounded by mononuclear leukocytes also stained positive with stromal fusiform fibroblasts, contributing overall up-regulation.

COX-2 positivity with variable staining intensity and heterogeneous in distribution is found in cytoplasm of neoplastic cells. This finding is supported by various studies.

Few stromal cells such as macrophages, some neutrophils, fibroblasts and vascular endothelial cells showed COX-2 positivity. This finding suggests that immunoreactivity for COX-2 may be modulated by interaction of stromal cells with the neoplastic cells in the process of destructive invasion. **Minter et al**<sup>19</sup> found highest expressions with perinuclear localization at leading edge of tumor. **Jaeckal**

et al<sup>xxxix</sup> found COX-2 highly expressed in keratin whorls of well-differentiated tumors. Haginomori et al<sup>xxxix</sup> found COX-2 expression in mitosis of oral cancer cells and diffuse faint COX-2 expression in carcinoma cells without mitosis.

A statistically significant difference and a positive correlation were found in the COX-2 expression within normal mucosa, oral submucous fibrosis and oral squamous cell carcinoma. Minter et al<sup>19</sup> found COX-2 was overexpressed in tumors compared with separate samples of normal epithelium. Wang et al<sup>xxxiii</sup> in their meta-analysis found COX-2 expression in cancer tissue was significantly higher than normal and benign tissues.

Our finding is consistent with Shamma et al<sup>xxxiv</sup>, Shibata et al<sup>22</sup> and Pannone G et al<sup>xxxv</sup> who reported a higher COX-2 expression in premalignant lesion than in malignancies. On contrary to our finding, Segawa et al<sup>xxxvi</sup>, Nystrom et al<sup>xxxvii</sup>, Sawhney et al<sup>xxxviii</sup> and Sakurai K et al<sup>xxxix</sup> reported a progressive increase of COX-2 expression from normal oral mucosa towards invasive carcinoma. Our data are in agreement with all the studies that report a higher expression of COX-2 in precancerous lesions than in malignancies and that hypothesize a correlation between COX-2 activation and the early stages of carcinogenesis.

When comparing COX-2 expression with the histological grade of squamous cell carcinoma, we found it to be statistically non-significant. ( $p = 0.367 < 0.05$ ) and a negative correlation ( $p = 0.214$ ) was found within the degree of differentiation. This finding was consistent with few of the studies while it is contradicting with few other studies.

Kyzas et al<sup>28</sup> found strong, heterogenous pattern of Cox-2 staining which was strongly correlated with Lymph node metastasis and with higher clinical staging but found no correlation with COX-2 expression and histological grade

of differentiation. Shigeto Itoh<sup>xl</sup> found no correlation between COX-2 expression and histological grade of tumor. Amirchmaghi et al<sup>xli</sup> found that COX-2 expression in spinous layer of normal tissues was significantly lower than Squamous cell carcinoma total. They obtained no significant difference between three grades of Squamous cell carcinoma. In agreement with our study, Goulart Filho et al<sup>xlii</sup> also found no significant difference in COX-2 expression between low grade and high grade OSCC.

Shibata et al<sup>22</sup> found inverse correlation with histological differentiation of Squamous cell Carcinoma, value being lowest for poorly differentiated Squamous cell carcinoma with a significant difference ( $p < 0.05$ ).

Renoken et al<sup>24</sup> found COX-2 expression is closely related with histological grade of Squamous cell carcinoma of tongue. Cao et al<sup>xliii</sup> found high expression of COX-2 in oral squamous cell carcinoma and are correlated with tumor size, lymph node metastasis and histological grade.

### Conclusion

Our study indicates that COX-2 activity could be involved in the process of tumor transformation. We hypothesize that COX-2 expression, which is absent in normal cells, is required during initial stages of carcinogenesis. They are fully activated during pre-malignancy (With all the samples clearly expressing COX-2). Later on, in the more advanced stages of tumor it is well established, that the involvement could be less critical and for this reason COX-2 starts to be downregulated. This finding implicates that COX-2 may play a role in tumor growth, inflammation being a mediator in carcinogenesis.

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