Original Article

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Cyclooxygenase-2 Expression in Oral Squamous Cell Carcinoma and Dysplasia of Oral Epithelium – A Comparative Study

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Abstract:

Objective: Cyclooxygenase-2 or COX-2 has been shown to play a vital role in pain and inflammation by mediating the conversion of arachidonic acid to prostaglandin, and the altered expression of cyclooxygenase-2 has been reported in carcinogenesis. The present study aimed to compare the expression of COX-2 in epithelial dysplasia (OED) and squamous cell carcinoma of the oral region (OSCC) via immunohistochemistry.

Material and Methods: The present study included 30 formalin-fixed paraffin-embedded samples, of which 10 were histopathologically diagnosed as oral epithelial dysplasia, 10 oral squamous cell carcinoma, and 10 normal oral mucosa. The samples were subjected to immunohistochemical staining with COX-2, following which the intensity and the area of staining were assessed and scored. The Kruskal-Wallis test was performed for statistical analysis with Statistical Package for the Social Sciences (SPSS) software version 23.0.

Results: A statistically significant increase in the expression of COX-2 was observed in OED and OSCC in comparison with the control.

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Conclusion: Thus, COX-2 expression could be further explored as a marker for early diagnosis and prevention of cancer progression.

Keywords: COX-2, oral epithelial dysplasia, oral squamous cell carcinoma

Introduction

Oral potentially malignant disorders (OPMDs) are a group of lesions or conditions that occur prior to the onset of squamous cell carcinoma of the oral region (OSCC)[1]. OPMDs include oral leukoplakia, erythroplakia, erythroleukoplakia, oral submucous fibrosis (OSMF), palatal lesions in reverse smokers, oral lichen planus, oral lichenoid reactions, proliferative verrucous leukoplakia, oral lupus erythematosus, and some hereditary conditions such as dyskeratosis congenita[2]. Most of these lesions may be asymptomatic in the early stages and could be detected during routine oral examination. However, these lesions could turn into a malignancy as they undergo dysplastic changes due to a lack of treatment and continuous exposure to carcinogens. The dysplastic changes are referred to as Oral Epithelial Dysplasia (OED).

OSCC is a common neoplasm of the head-and-neck region associated with a mortality rate of up to 50.0 % and is the third most common malignancy in South Central Asia. In India, 90 -95% of oral cancer is squamous cell carcinoma[3,4]. OSCC is an invasive neoplasm of epithelial origin with a variable amount of squamous differentiation, with or without a degree of keratinization[5]. The commonly affected sites include the anterior two-thirds of the tongue, buccal mucosa, hard palate, floor of the mouth, upper and lower alveolar ridges, retromolar trigone, and sublingual area[6]. The disease has multifactorial etiopathogenesis, including tobacco smoking, quid chewing, alcohol consumption, infections like human papilloma virus (HPV), and trauma due to a sharp tooth[7]. Gene mutations also play a major role in oral cancer. OSCC, if left untreated, progresses and invades adjacent tissues, including muscle, bone, neurovascular tissue, and lymph nodes[8]. A combination of surgery, chemotherapy, and radiotherapy enhances overall survival and remains the gold standard[9]. Early detection, assessment of severity, and prompt treatment are known to improve the five-year survival rate.

Hence, recent research has been focused on identifying diagnostic and prognostic markers for OSCC. Among the various tools to detect OSCC markers, the immunohistochemistry (IHC) procedure is the most cost-effective method.

The enzyme cyclooxygenase 2 (COX-2), an immediate-early response gene often induced by growth factors, oncogenes, and carcinogens, plays an important role in the conversion of arachidonic acid to prostaglandins, which in turn mediates inflammation and angiogenesis[10]. Elevated COX-2 levels have been found in potentially malignant lesions and malignant tumors, including breast, lung, pancreatic, gastric, oesophageal, liver, prostate, and stomach cancers[11].

Studies have reported an upregulation of COX-2 at both mRNA and protein levels, which elevates prostaglandin synthesis, increases mitosis, reduces apoptosis, and promotes tumor angiogenesis and tumor invasion at early stages, even before the expression of apoptosis and angiogenesis markers[12,13]. However, there are very few studies that have compared COX-2 expression in epithelial dysplasia and oral squamous cell carcinoma. With this background, this study was undertaken to evaluate the immunohistochemical expression of COX-2 in OED and OSCC.

Material and Methods

Study design, sample size, and study groups

Institutional Ethical Committee approval was obtained before conducting the study: Ref 2019-MDS-BrV1- SHA-13/APDCH. The present case-control study was performed in 30 samples divided into 3 groups of 10 each. The sample size was determined using a convenience sampling method based on the availability of well-preserved, treatment-naïve formalin-fixed paraffin-embedded blocks meeting the inclusion/exclusion criteria and feasibility considerations for this exploratory, case–control investigation.

The samples were retrieved from archival samples based on the selection criteria as described below:

Group 1: 10 samples of histopathologically diagnosed cases of OED.

Group II: 10 samples of OSCC confirmed by histopathology.

Group III: 10 samples of control oral mucosa without abnormality or NOM that were obtained and archived following a third molar surgery, after obtaining informed consent.

Inclusion criteria

Group I: Histologically diagnosed cases of oral epithelial dysplasia of varying grades showing one or more architectural and cytological alterations.

Group II: Histologically confirmed cases of oral squamous cell carcinoma without prior treatment (chemotherapy/radiotherapy).

Group III: Normal oral mucosa from healthy individuals with no systemic disease, pregnancy, lactation, tobacco or alcohol related habits, or prolonged drug use.

Exclusion criteria

- Poorly preserved blocks.
- Archival samples that possess inadequate tissue.
- Samples with autolysis or artifacts that compromise histological interpretation.
- Patients who had received prior treatment (surgery, radiotherapy, or chemotherapy) or antioxidant therapy before tissue sampling.
- Presence of systemic diseases.
- Malignancies other than OSCC.
- Normal oral mucosa with inflammation.

Sample preparation

Serial sections of 3-micron thickness were made on 3-aminopropyl tri-ethoxysilane (APES) coated glass slides.

Immunohistochemistry

The expression of COX-2 was evaluated using a standard procedure using COX-2 (rabbit monoclonal antibody) [PathnSitu Biotechnologies Private Limited]. A histopathologically diagnosed formalin-fixed paraffin-embedded colon carcinoma sample retrieved from the archives was used as a positive control.

Preparation of sample

The mounting of serial sections (3 µm thick) taken from the archival formalin-fixed paraffin-embedded samples was done on 3-aminopropyl triethoxysilane (APES)-coated glass slides to ensure proper adhesion for IHC procedures.

Immunohistochemistry (IHC) staining procedure

The standard streptavidin-biotin-peroxidase method was used to assess the expression of COX-2.

Deparaffinization and Rehydration

Deparaffinization of the sections was performed in xylene (2 changes, 10 minutes each), and rehydration was achieved with graded alcohol and finally with distilled water.

Retrieval of antigen

Heat-induced antigen retrieval was performed with 10 mM citrate buffer (pH 6.0) in a pressure cooker for 10

to 15 minutes. Cooling was accomplished by washing the cooker with running water.

Endogenous peroxidase block

Treatment of sections with 3% hydrogen peroxide in methanol for 10 minutes to block endogenous peroxidase.

Primary antibody incubation

COX-2 primary antibody (rabbit monoclonal) was added to the slides, which were incubated for 60 minutes at room temperature, following the manufacturer's protocols.

Secondary antibody application

Slides were washed with phosphate-buffered saline (PBS), and sections were incubated with a secondary antibody conjugated with horseradish peroxidase (HRP) for 30 minutes.

Visualization

Chromogen diaminobenzidine (DAB) was added to visualize the antigen-antibody complex.

Counterstaining and mounting

Mayer's hematoxylin was added and dehydrated through graded alcohol, cleared in xylene, and mounted with DPX.

Positive control: Histopathologically confirmed colon carcinoma tissue was used as a positive control.

Negative control: Sections were processed without the primary antibody[14].

Analysis and interpretation

Intensity and area of staining expression of COX-2 were assessed in 5 random fields at 40X magnification under a light microscope. Two blinded examiners independently assessed the staining intensity and area. The intensity of staining (extent of color uptake) was assessed to determine the extent of stain uptake. The area of staining (percentage of positive cells) was analyzed to determine the protein expression levels and their pattern. Quadratic-weighted κ score for interobserver variability for the intensity of staining was 0.908, and the area of staining was 0.895. The scoring criteria are described in Supplementary Table 1[15].

Statistical analysis

The data obtained were subjected to statistical analysis using the Statistical Package for the Social Sciences. {(SPSS) version 23, IBM}. Kruskal-Wallis test (non-parametric test) was performed to compare the intensity and the area of staining among the 3 groups owing to the low sample size.

Results

Tables 1 and 2 depict the statistically significant variations in staining patterns. Normal oral mucosa had scores of 1 and 2, followed by 1, 2, 3, and 4 in OED, with high scores of 3 and 4 in OSCC. Mild staining was exhibited by NOM, followed by moderate staining in OED and intense staining in OSCC.

Table 1 Determination of the staining intensity and the area of COX-2 among study groups

Variable	Total cases (n)	Test statistics	df	p-value
Intensity of staining (IOS)	30	19.569	2	<0.001
Area of staining (AOS)	30	19.447	2	<0.001

Staining pattern in OED

Of the cases, 20.0% showed intense staining, 40.0% showed a moderate intensity of staining, 20.0% expressed mild staining intensity, and the remaining 20.0% did not show any staining (Figure 1a, b). Considering the staining area, 40.0 % of the cases showed staining of 26.0% to 50.0%, whereas only 20.0% of cases expressed staining of more than 75.0%. (Figures 2a, b).

Staining pattern in OSCC

Of the cases, 70.0% showed intense staining and 30.0% of the cases showed moderate staining (Figures 3, 4, 5). Considering the staining area, 40.0% of the cases showed more than 75%, and 60% of the cases showed staining of 51.0 % to 75.0%.

Staining pattern in NOM

Of the cases, 80.0% did not show any staining, and 20.0% of the cases showed a mild staining intensity.

Inference

Results depict a statistically significant enhanced expression of COX-2 in OSCC, followed by epithelial dysplasia, and the lowest expression was seen in normal oral mucosa.

Table 2 Pairwise comparison of the staining intensity between study groups

Comparison	Test statistic	Std. error	Std. test statistic	p-value
NOM vs OED	9.100	3.780	2.407	0.016
NOM vs OSCC	16.700	3.780	4.417	<0.001
OED vs OSCC	7.600	3.780	2.010	0.044

NOM=Normal oral mucosa, OED=oral epithelial dysplasia, OSCC=oral squamous cell carcinoma

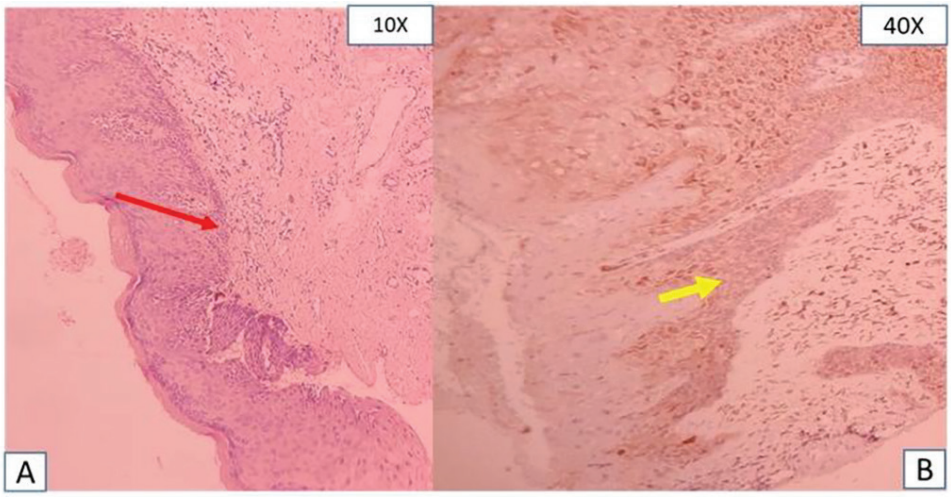

Figure 1 (A) Photomicrograph of H&E-stained section depicting epithelial dysplasia, indicated by the red arrow at 10x magnification (B) Photomicrograph of IHC-stained section depicting moderate expression of COX-2 in moderate epithelial dysplasia, indicated by the yellow arrow, lower power magnification (10x)

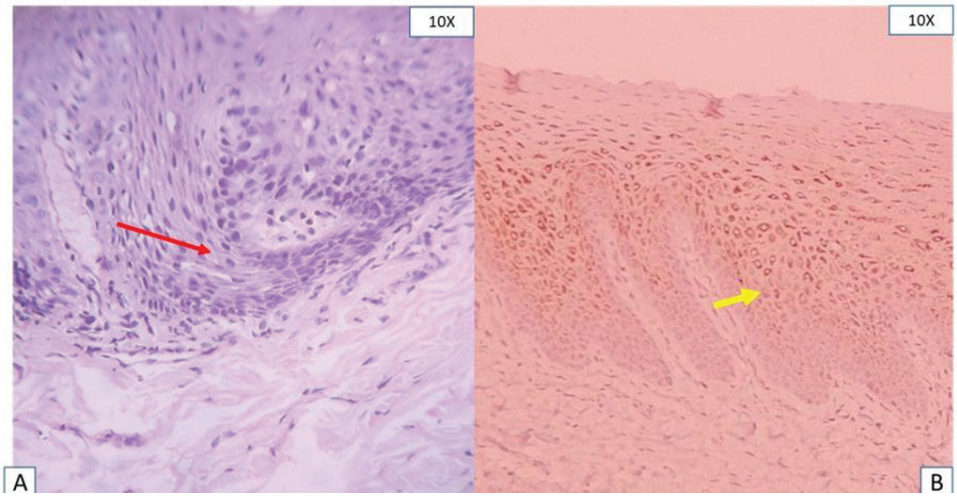

Figure 2 (A) Photomicrograph of a low-power view of an H&E-stained section showing severe epithelial dysplasia, indicated by the red arrow (B) Photomicrograph of IHC-stained section showing intense expression of COX-2 in severe epithelial dysplasia, indicated by the yellow arrow at lower power magnification (10x)

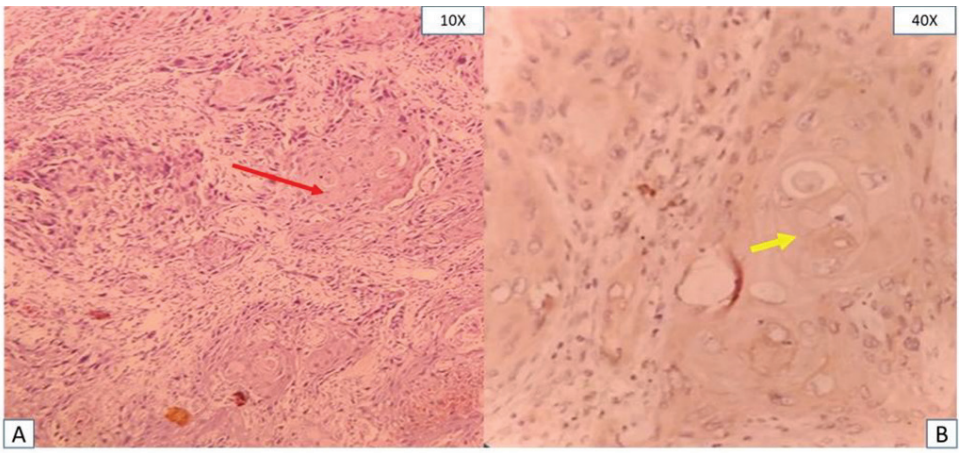

Figure 3 (A) Photomicrograph of H $ E H&E-stained section of well-differentiated squamous cell carcinoma, indicated by the red arrow at 10x magnification (B) Photomicrograph of IHC-stained section showing mild expression of COX-2 in well-differentiated squamous cell carcinoma at low power magnification (10x), indicated by the yellow arrow

Hence, COX-2 could be considered as an early marker of carcinogenesis.

Discussion

Among the well-known cyclooxygenase enzymes, COX-1 and COX-2, COX-1 plays a role in regulating the physiological functions of the gut and kidney. COX-2 enzyme is involved in inflammation and repair. COX-1 is an important housekeeping enzyme responsible for maintaining basal prostaglandin levels, which in turn are vital for tissue homeostasis. COX-1 is found to be expressed in the kidneys, lungs, and stomach, whereas COX-2 expression is absent in normal tissue. COX-2 is an inducible enzyme that can be detected during inflammation and tumorigenic settings[16].

Human malignancies, including OSCC, are a multistep process involving several somatic mutations and alterations. Several studies have indicated a relationship between chronic inflammation and a variety of cancer types, including OSCC. Also, expression of inflammatory markers in OSCC patients has been linked to poor prognosis. Inflammatory responses play a vital role in all stages of tumor development, including initiation, promotion, growth, invasion, and metastasis. The inflammatory response is considered a defense mechanism during tissue injury through the action of a variety of inflammatory mediators, including cytokines, prostaglandins, cyclooxygenase (COX) enzymes, and matrix metalloproteinases (MMPs). These inflammatory mediators cause genetic and epigenetic changes, thereby suppressing the tumor suppressor genes. This, in turn, can lead to cancer development and progression[6].

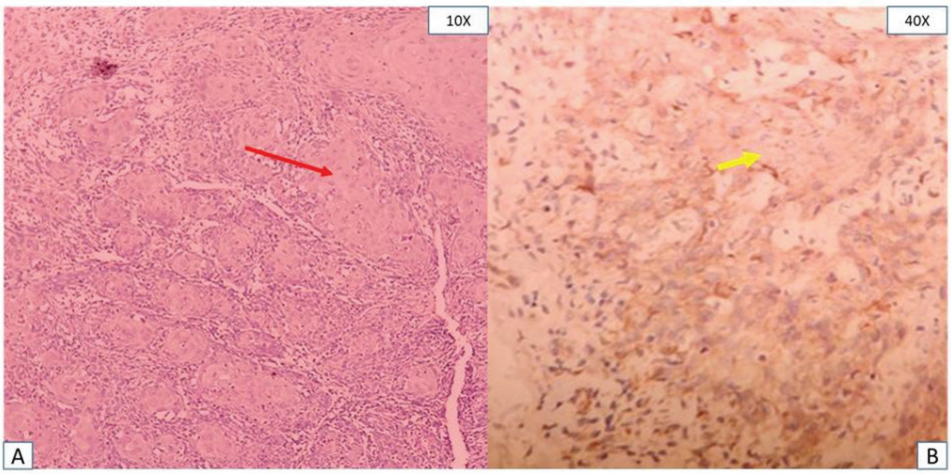

Figure 4 (A) Photomicrograph of an H&E-stained section depicting a moderately differentiated oral squamous cell carcinoma, indicated by the red arrow at 10x magnification (B) Photomicrograph of an IHC-stained section showing moderate expression of COX-2 in moderately differentiated squamous cell carcinoma at low power magnification, indicated by the yellow arrow

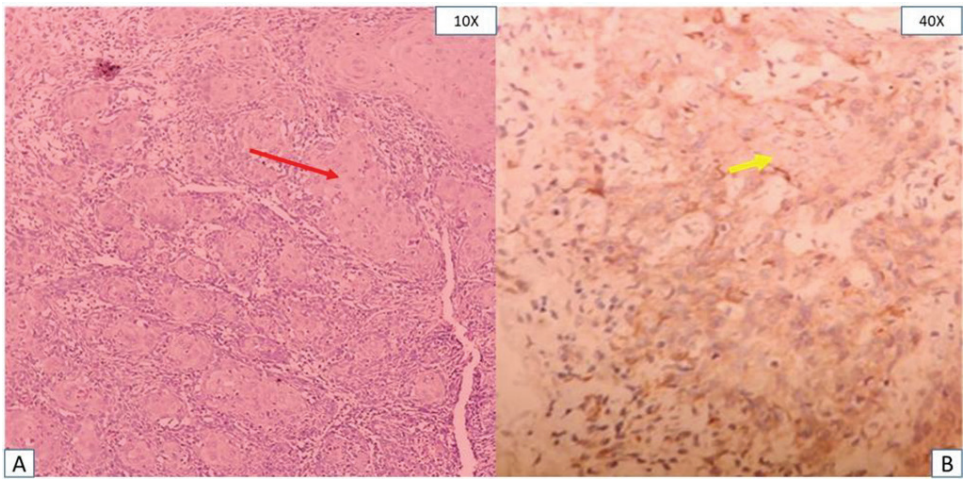

Figure 5 (A) Photomicrograph of H&E-stained section depicting poorly differentiated oral squamous cell carcinoma, indicated by the red arrow at 10x magnification (B) Photomicrograph of IHC-stained section showing intense expression of COX-2 in poorly differentiated squamous cell carcinoma at low power magnification (10x), indicated by the yellow arrow

COX-1 and COX-2 convert arachidonic acid into biologically active prostaglandins (PGD 2, PGE 2, PGF 2, and PGI 2) and thromboxane (TXA 2). COX-2 and PGE2 have been reported to play a role in carcinogenesis by immune evasion, and studies have reported increased expression in various cancers[17-19].

However, there is limited information on the comparative expression of COX-2 in oral epithelial dysplasia and oral squamous cell carcinoma.

With this background, the present study was conducted to compare the expression of COX-2 in oral epithelial dysplasia, OSCC, and normal tissue; 20.0% of the cases of oral epithelial dysplasia (OED) showed intense staining, 40.0% showed moderate intensity of staining, 20.0% expressed mild staining intensity, and the remaining 20.0% did not show any staining (Figure 1). Considering the area of staining in OED, 40.0% of the cases showed about 26.0% to 50.0% of staining, whereas only 20.0% of cases expressed more than 75% of staining (Figure 2). These findings are concurrent with those of Sawhney et al. (2007)[20], Sun et al. (2005)[21], Yamaguchi S et al. (2014)[22], Sankar et al. (2017)[23], and Shibata et al. (2021)[24]. Their results revealed a gradual increase from normal mucosa to dysplasia. This proves that COX-2 expression may be involved in cell proliferation and cell regulation from normal mucosa to moderate and severe dysplasia.

Of the cases, 70% of oral squamous cell carcinoma (OSCC) showed intense staining, and 30.0% of the cases showed moderate staining (Figures 3 and 4). Considering the area of staining, 40% of the cases showed more than 75.0% of the area of staining, and 60.0 % of the cases showed about 51.0% to 75.0% of staining; 70% of the OSCC cases in the present study showed intense staining, and these results are similar to those of Thomas et al. (2019)[25]. In their study, most of the OSCC cases showed intense staining (Figure 5).

Similarly, in a study reported by Pandey et al[26]. and Zhang et al.[27], an increased expression of COX-2 was

observed in OSCC in comparison with OED and NOM. Their findings are also consistent with our study. Our study results are also similar to those of Chan et al. (2009), who reported a significant expression of COX-2 in OSCC[28]. In the present study, expression of COX-2 was not only seen in tumor cells but also in inflammatory cells, such as lymphocytes, which is similar to the study published by Chan et al. (2009)[28].

In the current study, in normal oral mucosa (NOM), 80.0% of the cases did not show staining, and 20.0% of the cases showed mild staining intensity. The findings are in contrast with the study conducted by Thomas et al.[25] and Nathan et al.[29] where all the cases of normal oral mucosa showed negative staining. This could be attributed to the presence of inflammation in histologically normal tissue as reported by Sawhney et al. (2007)[30] and Singla et al. (2015)[31]. There were statistically significant variations in the intensity of staining and the area of staining among all 3 study groups (OED, OSCC, and NOM) (p-value of 0.000).

An intragroup comparison was also performed for both the intensity of the staining and the area of staining for COX-2 expression. A p-value of 0.133 (p-value<0.05) for staining intensity and a p-value of 0.49 (p-value<0.05) for the area of staining were obtained on comparison between OED and OSCC. These results are similar to the results obtained by Singla et al. (2015)[31], who also reported a higher expression of COX-2 in the OSCC group than in dysplasia, and low positive staining in NOM. Elevated levels of COX-2 expression have also been reported in head-and-neck SCC by Gregor et al. (1999)[32] and esophageal SCC by Zhi et al. (2006)[33], which is similar to the higher expression in OSCC. Similarly, Yazdandoust et al.[34] reported significantly higher mRNA and protein expression of COX-2 in OSCC, followed by OLP, with the lowest in NOM (1.08±0.79; P <0.01). Another study by Aparnadevi et al.[35] also showed a statistically significant increase in the expression of COX-2 in OSCC and OSMF in comparison with normal oral mucosa, which is concurrent with our findings, suggesting the role of COX-2 in carcinogenesis.

The present study is different from previous research comparing the expression of COX-2 in OSCC, OED, and normal mucosa; whereas most of the studies have assessed COX-2 expression individually in OSCC or OED and the lack of comparison between the two, our findings have shown that COX-2 expression was found to increase from NOM to OED to OSCC, which highlights the role of COX-2 in early tumorigenesis and progression.

Elevated COX-2 expressions in OED and OSCC should be viewed not only from a diagnostic point of view but also from a therapeutic standpoint. The enzyme COX-2 not only plays a role in carcinogenesis but also modulates dendritic cells, T cells, and natural killer T cells, thereby suppressing immunity against tumors and playing a pivotal role in tumor immune evasion[36,37]. In other words, COX-2 plays the role of an oncogene by suppressing tumor immunity. Hence, researchers have demonstrated that specific inhibitors of COX-2 have aided in tumor reduction and the prevention of carcinogenesis in several cancers[37,38]. Hence, with the observed elevated expression of COX-2 in OED and OSCC in the present study, specific pharmacological inhibitors of COX-2 could be researched as a preventive malignant transformation of OED or an adjunctive therapeutic strategy for improving the prognosis of OSCC.

The limitations of the study include a small sample size. The present study also did not compare the staining intensity and areas in different histopathologic grades of dysplasia and OSCC. Also, the expression of COX-2 in the different stages of OSCC was not determined. However, COX-2 could be explored further as a diagnostic and prognostic marker for OED and OSCC with future studies involving a larger sample size.

Conclusion

With the limitations of the present study clear, including its sample size and lack of comparison of staining expression with different histopathological gradings of OED and OSCC, we conclude that significant differences in immunohistochemical expression of COX-2 were observed between normal oral mucosa, OED, and OSCC. Marked expression of this enzyme in OED and OSCC supports its role in carcinogenesis. Hence, COX-2 inhibitors could be used to prevent the transformation of oral premalignant lesions to oral squamous cell carcinoma.

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Conflict of interest

None

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Supplementary Table 1 Scoring criteria for COX-2 staining

Score	Intensity of the	Area of staining (%)
0	No staining	0
1	Mild staining	0–25
2	Moderate staining	26–50
3	Intense staining	51–75
4	Not Applicable	76–100