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Immunohistochemical Demonstration of COX2 in Various Lesions of Oral Cavity

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ABSTRACT

Cyclooxygenase enzyme facilitates the conversion of arachidonic acid into pro-inflammatory compounds, resulting in formation of prostaglandins, which contributes substantially to the carcinogenic process. **Objective:** To analyze the immunohistochemical COX2 enzyme expression in various lesion of oral cavity. **Methods:** A total of 60 formalin fixed, paraffinized blocks (including 10 healthy oral mucosa cases, 10 cases of leukoplakia. 10 cases of oral sub mucosal fibrosis, 10 cases of dysplasia, 10 cases of well differentiated carcinoma, 10 cases of highly aggressive invasive squamous carcinoma) were randomly selected during the period of Jan, 2022 till Dec, 2023. Immunohistochemistry was done on each case for analyzing COX2 expression. Data was statistically analyzed by using chi square test. P value < 0.05 was taken as substantial. **Result:** It was found that the expression level was high in invasive carcinoma as compared with other oral lesion. **Conclusion:** Present study strongly supported the involvement of COX2 in the advancement of precancerous lesions of oral cavity to malignant one.

INTRODUCTION

Benign and malignant lesion of oral cavity are major health issue in developed and undeveloped countries. Because they are linked to a higher risk of oral cancer, especially Oral Squamous Cell Carcinoma (OSCC), Oral Potentially Malignant Disorders (OPMDs) pose a serious threat to global health. Globally, OPMD prevalence varies, with South Asian nations reporting greater rates. Given that OSCC's five-year survival rate is still less than 50%, early detection and intervention are essential [1]. In 2020, there were about 0.37 million new instances of oral cancer worldwide, with Asia accounting for a large portion of these cases [2]. High-risk lesions with a higher chance of malignant development, such as erythroplakia and non-homogeneous leukoplakia, are included in the classification of OPMDs [3].According to national cancer registry, Pakistan data Oral Cavity (OC) cancer is the leading No. 1 cancer in males of Pakistan and number three in females [4]. It has been observed that the percentage of progression from premalignant lesion of oral cavity to malignant is increasing despites marvelous advancement in diagnostic and surgical fields. Therefore, it is a dire need to search new molecular targets to prevent the progression of oral lesion toward malignancy [5]. In South East Asia especially in Pakistan the leading cause of oral lesions are mainly smoking, alcohol, betel nuts chewing and gutka eating. All these risk factors are somehow involved in

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causing chronic inflammation in buccal mucosa. In 1863, Virchow first hypothesized the connection between inflammatory process and carcinogenesis. An environment rich in inflammatory cells along with certain growth factors, enhance the potential for malignant transformation in proliferating cells [5]. Although inflammation has been regarded as a protective mechanism of body to various types of injurious stimuli, there has been growing evidence of its strong role in initiation or progression of various diseases especially cancer. It has been observed that inflammation accompanies many premalignant and malignant lesion of oral cavity. This results in elevation of COX 2 enzyme in local tissue, which converts into Prostaglandins (PGs) especially PGE2 (prostaglandin E2). It amplifies several key processes in tumor formation, such as angiogenesis, invasive capabilities, and cell proliferation [6]. COX2 mRNA has been found many folds high in oral cancer tissues compared with non-cancerous tissues. Likewise, buccal tissues of tobacco or gutka eaters expresses significant upregulation of COX2 mRNA levels than non-users. These patients exhibit increased COX-2 expression which has been linked to increased incidence of tumor progression from premalignant to malignant one resulting in lower 5-year survival rate [7].

This study aimed to evaluate the expression levels of COX2 in premalignant and malignant oral lesions.

METHODS

This was a retrospective observational study with immunohistochemical analysis, conducted at the Life Care Molecular and Polymerase Chain Reaction (PCR) Lab Services, Karachi, from January 2022 to December 2023, in collaboration with Fazaia Ruth Pfau Medical College, Karachi. Ethical approval was obtained from the Institutional Review Board (IRB) of Fazaia Ruth Pfau Medical College with reference number FRPMC/002/IRB/21. Permission for data collection was granted as per institutional guidelines. Since this was a retrospective study, informed consent was waived in compliance with institutional policies, ensuring strict confidentiality and anonymity of patient data. For sampling, a total of 60 Formalin-Fixed Paraffin-Embedded (FFPE) tissue blocks were retrieved from the Histopathology Department, Life Care Molecular Lab, covering the period from January 1, 2020, to December 31, 2023. These blocks contained tissues diagnosed as leukoplakia, oral submucosal fibrosis, dysplasia, carcinoma in situ, and invasive cancer, as well as ten blocks of large intestinal mucosa used as a control group. Since oral lesion biopsies are typically small and received for diagnostic purposes, normal mucosa from unaffected areas of the oral lesion could not be reliably used as a control. Instead, normal colonic mucosa was selected as a control group due to its larger tissue size and established expression of COX2, ensuring robust comparison of COX2 staining between normal and pathological samples. The colonic mucosa was obtained from biopsies submitted for unrelated diagnostic purposes, with care taken to sample areas confirmed as histologically normal. Tissue blocks from patients diagnosed with oral lesions, including leukoplakia, oral submucosal fibrosis, dysplasia, carcinoma in situ, and invasive cancer, large intestinal mucosa samples with histologically normal findings from diagnostic biopsies and cases with well-preserved histopathological features and complete clinical records were included for the study. Incomplete or degraded tissue samples and cases lacking essential clinical data or diagnostic confirmation were excluded. Furthermore, patients with a history of prior chemoradiotherapy or immunosuppressive therapy for oral lesions were also excluded. The sample size was calculated using data from a comparable study evaluating immunohistochemical expression of COX2 in similar oral lesions. The equation for sample size was derived using a single proportion formula: Using a single proportion formula:

$$n = \frac{Z^2 \times P \times (1-P)}{d^2}$$

Where:

n= required sample size

Z = 1.96 (standard normal value corresponding to a 95% confidence level)

P=(Prevalence of Oral submucous fibrosis) 4.9% or 0.049 .

d=5% (margin of error)

Substituting the values into the formula

n=(1.96)2 x0.049x(1-0.049)(0.05)2

n=71.84

Since this could not get enough tissue samples and 60 samples for this study instead of the calculated 72. Although the ideal number is determined to be 72 by statistical power for necessary optimality, practical sample availability constraint forced us to use 60 as the sample size. In the same time, even when the sample size is 60, this number gives a quite meaningful insight and robust outcomes. Histopathological analysis was performed using fresh five-micron histopathological slides prepared from the retrieved tissue blocks, stained with hematoxylin and eosin (HandE), and examined independently by two consultant pathologists. HandE staining was performed as per the method described by Bancroft and Gamble (Theory and Practice of Histological Techniques, 8th edition) [9]. Large intestinal tissue from the colonic mucosa was used as a positive control for COX2 immunohistochemical staining. This tissue was chosen due to its reliable and established expression of COX2 in normal glandular epithelium and inflammatory contexts [10]. Sampling was

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limited to histologically confirm normal areas. Immunohistochemistry was performed from four-micron thick sections obtained from all tissue blocks. Sections were mounted on positively charged slides and underwent routine deparaffinization and antigen retrieval using an automated water bath (CytoTest, China). The primary antibody used was anti-COX2 monoclonal antibody (Clone CX-294, Dako, Denmark) at a dilution of 1:100. A secondary antibody conjugated with Horseradish Peroxidase (HRP) was used for signal amplification. A substrate (H_2O_2) and chromogen (3, 3'-diaminobenzidine; DAB) were added to form a brightly colored, insoluble product localized to antigenic areas. Positive expression was indicated by brown staining in tumor cells, visualized and photographed using a Leica 2500 optical microscope (Leica Microsystems, Germany). From each paraffin-embedded block, 3–5 slides were prepared to ensure adequate representation of tissue. For each slide, four fields were captured for analysis at a magnification of 40×. Images included a scale bar of 50 µm for reference. To ensure background or nonspecific staining did not interfere with results, negative controls were used, and antigen retrieval, antibody dilutions, and blocking steps were optimized. Endogenous enzyme activity was inhibited, and high-specificity antibodies were applied. Normal large intestinal tissue served as a control, and two pathologists independently reviewed the slides for consistency. Representative images were captured from three different paraffin-embedded tissue blocks, showcasing COX2 staining patterns in dysplasia, carcinoma in situ, and invasive cancer. Each image highlights the intensity and localization of staining at a magnification of 40× with a 50 µm scale bar. Immunohistochemical staining for COX2 expression was graded on a scale of 1–3 as 1+: Weak staining, 2+: Moderate staining, and 3+: Strong staining. The statistical analysis was conducted to evaluate the relationship between COX2 expression and the morphological grades of oral lesions. Descriptive Statistics was used to summarize COX2 expression across different lesions, calculating frequencies and proportions of staining intensities (+1, +2, +3, +4). Chi-Square Test was used to assess the association between lesion grade and COX2 expression intensity. A threshold of $p < 0.05$ was considered statistically significant, indicating the presence of a meaningful correlation.

RESULTS

The immunohistochemical analysis of COX2 expression in various oral lesions revealed significant variations across different morphological types. A higher intensity of COX2 expression was observed in more advanced lesions such as poorly differentiated Oral Squamous Cell Carcinoma (OSCC), as compared to less aggressive lesions like leukoplakia and normal buccal mucosa. In normal buccal mucosa, 90% of cases exhibited weak (+1) COX2 expression, with only 10% showing moderate (+2) expression. Leukoplakia showed a notable increase in COX2 expression, with 60% of cases displaying moderate (+2) staining. Oral submucosal fibrosis showed a mix of moderate (+2) and strong (+3) expressions in 50% of cases. Dysplastic lesions had a balanced distribution across mild, moderate, and strong expressions (+1 to +3). Well-differentiated OSCC and poorly differentiated OSCC demonstrated the highest levels of COX2 expression, with the majority of cases showing strong (+3) and very strong (+4) staining (Figure 1). Table 1 presents the distribution of COX2 cytoplasmic expression in different oral lesions, categorized by proportional value. A significant increase in COX2 expression is observed from normal mucosa to invasive carcinoma.

Table 1: COX-2 Cytoplasmic Expression in Different Oral Lesions

Morphological Type of Specimen	Total Cases Analyzed	Proportional Value				Probability Value
		+1	+2	+3	+4	
Normal Mucosa	10	09	01	-	-	0.001
Leukoplakia	10	04	06	-	-	
Oral Submucosal Fibrosis	10	02	03	05	-	
Dysplasia	10	01	02	02	05	
Well Differentiated OSCC	10	-	01	03	06	
Poorly Differentiated OSCC	10	-	-	03	07	

Proportional value was designated as +1: 5-10% of cells positive, +2: 11-40% of cells positive, +3: 41-70% of cells positive, and +4: 71-100% of cells positive.

Table 2 categorized COX2 expression by morphological grade. A statistically significant correlation was found between the grade of the oral lesions and the intensity of COX-2 expression ($p < 0.05$). Higher grades of lesions showed an increase in the intensity of COX2 staining.

Table 2: Graded Analysis of COX-2 Cytoplasmic Expression in Oral Lesion

Morphological Type of Specimen	Total Cases Analyzed	Proportional Value				Probability Value
		0	+1	+2	+3	
Normal Mucosa	10	10	-	-	-	0.001
Leukoplakia	10	00	10	-	-	
Oral Submucosal Fibrosis	10	00	04	06	-	
Dysplasia	10	00	02	03	05	
Well Differentiated OSCC	10	00	00	04	06	
Poorly Differentiated OSCC	10	00	00	02	08	

Grading was designated a proportional values as Grade 0: Negative COX-2 expression, Grade +1: Mild COX-2 expression, Grade +2: Moderate COX-2 expression, and Grade +3: Strong COX-2 expression. A and B represented negative COX-2 staining in normal mucosa of grade 0 indicating no significant expression in normal Mucosa. C and D represent mild positive COX-2 immunostaining expression in an oral fibrotic lesion (oral submucosal

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fibrosis) showing mild oral fibrosis of grade 1. E and F represent moderate immunostaining in a dysplastic lesion, showing an increased number of positive cells of grade 2. G and H represent strong expression in a well-differentiated OSCC,severe positive immunostaining in poorly differentiated OSCC of grade 3 with significant tumor cell show intense staining.

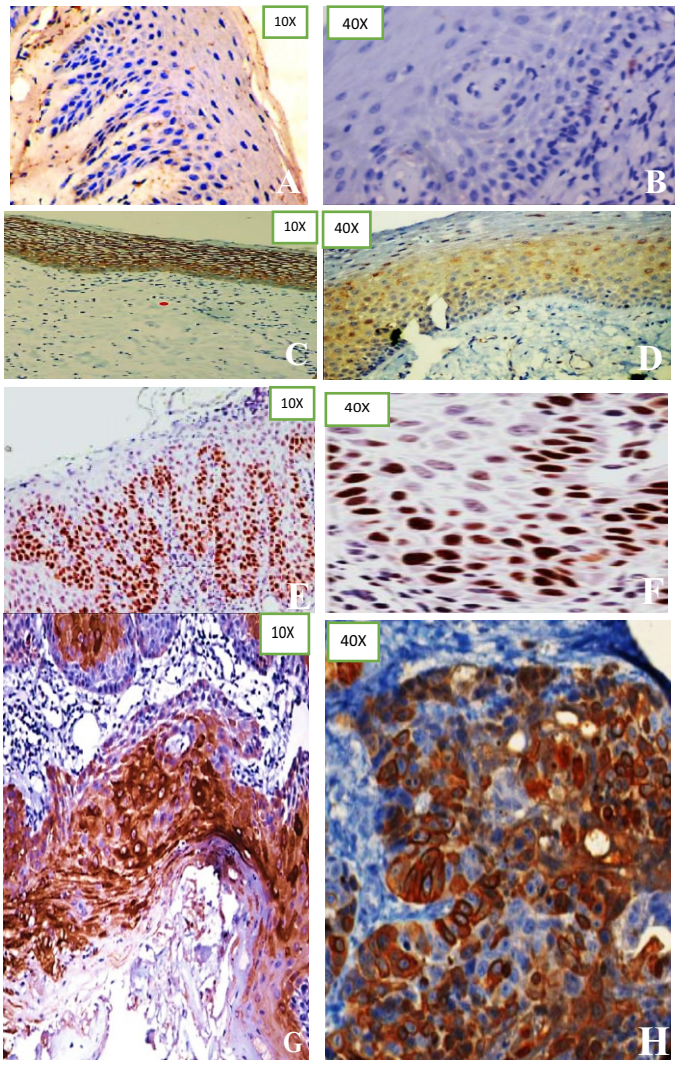

Figure 1: Immunohistochemistry of COX-2 Expression in Oral Lesions

DISCUSSION

The current study's findings revealed a progressive upregulation of COX2 expression in oral cavity lesions, transitioning from premalignant to malignant stages, as well as in normal buccal mucosa, thereby substantiating the involvement of COX2 in the carcinogenic process (Figure 1). Cyclooxygenase (COX) is a member of myeloperoxidase family which is involve in the synthesis of prostaglandins from arachidonic acid [11]. The Cyclooxygenase (COX) enzyme exists in three isoforms, namely COX1, COX2, and COX3, each with distinct properties and functions. COX 1 and COX3 are encoded by the same gene. Difference in both COX enzyme is that COX3 is a posttranscriptional modification of COX1. The expression of COX1 is predominantly localized to the Central Nervous System(CNS)and the aortic wall. While the full significance of COX3 remains to be fully understood, it is known to possess important pathophysiological properties that contribute to various biological processes [12]. COX1 is responsible for the maintenance of normal bodily homeostasis. Somehow, it is also being found involved in the pathogenesis of atheromatous plaques and inflammatory foci in rheumatoid arthritis [13]. In humans, COX 2 is present in very low concentration and is rarely detected in healthy individuals. Nevertheless, it is involved in various physiological functions in GIT, renal, CVS, CNS, Eye and the reproductive system. Upregulation of COX-2 has been implicated in the development and progression of various cancers, including esophageal cancer, urinary bladder cancer, and notably, head & neck cancers, as well as oral cancers [14]. Over expression of COX2 genetically and phenotypically change premalignant cells to a malignant one. It also disturbs cell growth cycle, apoptosis and the immune response enabling cancer cells to proliferate, survive, enhancing neovascularization, and promotes cancer cell invasion [15]. This study revealed the presence of COX2 expression in normal buccal mucosa as well as various benign and malignant lesions of oral cavity. Or results showed negative expression in healthy oral mucosa while COX 2 expression was found to be raised as disease progress from benign to malignant conditions. Table 1 and 2 results are in accordance with the study of which stated that simultaneous upregulation of COX2 expression was detected as the disease progress from dysplasia to invasive oral squamous cell cancer. This suggests a crucial role for this enzyme in the progression of premalignant lesions to malignancy [16]. Similar results were also stated by their research also revealed that COX-2 expression is significantly higher in oral OSCC (Oral Squamous Cell Carcinoma) compared to premalignant lesions [17]. These results are in accordance with the study of which also showed COX2 expression is significantly higher in dysplasia and oral squamous cell carcinoma as compared to normal mucosa [18]. Same results have been reported by for head and neck SCC and by and about OSCC [19]. These results are in accordance with the study of that a significant variation in COX2 expression was also noted among OSCC (Oral Squamous Cell Carcinoma), oral leukoplakia, and oral fibrous lesions. Higher expression was found in oral squamous carcinoma than leukoplakia and fibrosis[20].

CONCLUSIONS

This study investigates the expression of COX2 in various oral lesions, including normal oral mucosa, to provide a comprehensive understanding of its role in oral pathology. The results obtained clearly demonstrate the increasing

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level of expression of COX2 among benign to cancerous oral cavity lesions. This clearly point toward the positive role of COX2 in progression of premalignant to malignant oral cavity lesions. Since COX 2 enzyme has a rate limiting function in inflammation, anti COX2 medicines could be used to prevent the transformation of pre malignant lesions to malignant one. Additionally, these drugs may potentiate the effects of chemotherapy, allowing for reduced treatment durations and minimizing the risks associated with prolonged exposure to chemotherapeutic agents.

Authors Contribution

Conceptualization: SZ
Methodology: NQ, HK, MS, SS, FM
Formal analysis: NQ, HK, MS, SS, FM
Writing, review and editing: NQ, HK, MS, SS, FM

All authors have read and agreed to the published version of the manuscript

Conflicts of Interest

All the authors declare no conflict of interest.

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