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COMPARATIVE STUDYON THE EVALUATION OF CYCLO OXYGENASE 2 EXPRESSION ON DYSPLASTIC AND NON DYSPLASTIC ORAL LEISIONSBY IMMUNOHISTOCHEMISTRY.

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ABSTRACT

Tumors of oral cavity are characteristicallyprecededby Potentially Malignant Disorders which may elucidate various morphological and histopathological changes, known as Oral Epithelial Dysplasia. Histopathological evaluationof dysplasia has greater variabilityand may not always predict the malignant transformation rates.Identification of molecular markers which can augment the diagnosis before the malignant transformation can increase the survival rates.Hence to ascertain the prognostic significance of OED, we designed a study evaluating the expression of COX 2, a novel marker in histopathological confined OED.

Material and Methods: 20 cases of Oral epithelial dysplasia,20 cases of gingivitis, 10 cases of normal mucosa and positive control (colon cancer) were studied for COX 2 IHC expression.COX-2 staining was evaluated based on thepositive cells andstaining intensity.

Statistical Analysis: The statistical tests used in this study were t-test and Chi-square test using SPSS (version 22.0.0.0).

Interpretation and Result- In our study, COX 2 was significantly expressed on study group I, suggestive of its complementary roles during oral carcinogenesis.More over the expression of COX 2 was evident on all grades of dysplasia,even the mild dysplastic epithelium showed an increased staining intensity than a non-dysplastic lesion .Our findings indicate that COX 2expression can be a specific marker for PMDs and can reduce the risk of developing malignancy.Our study also suggests classification of epithelial dysplasia to be tailored along with molecular alterations so that it reduces the incidence of disease progression and mortality.

Key Words- Oral Epithelial Dysplasia, Chemo radiation,Cyclo oxygenase 2 ,Novel marker,Potentially Malignant Disorders.

Abrrevations PMD- Potentially malignant Disorder, OED- Oral Epithelial Dysplasia ,COX 2- Cyclo oxygenase 2,IHC- Immuno histo chemistry

INTRODUCTION

GlobalCancer Observatory (GLOBOCAN) data published in 2018, avowed that cancer is accountable for 9.6 million apparent deaths worldwide.Oral cancers are the most common malignancies worldwideand Oral Squamous cell carcinoma (OSCC) is the most common oral cancer in India.[1,2]OSCC usuallytrailed by a precursor lesion and the disease progression mainly involves numerous genetic events that alter the normal functions of oncogenic and tumor suppressor genes (TSG)[3,4,5]. This may result in increased production of growth factors , cell surface receptors, transcription factorsand intracellular messenger signaling[6].

Oral Cancers are recognized to produce more prostaglandins than the normal tissue.[3]Prostaglandin-endoperoxide synthase, generally called as cyclooxygenase (COX), is the key regulatory enzyme in tissue inflammation and is present chiefly as two isoforms COX-1 ,COX-2 and recently a split variant added called COX- 3.Cyclo- oxygenase(COX) is a rate limiting enzyme in the synthesis of prostanoids, with several important functions in physiology and disease. COX-2 is an inducible isoform which getsstimulated by cytokines, growth factors, oncogenes, inflammation and tumor promoting factors in tumeriogenesis[7]. COX 2 activates procarcinogens, endorse angiogenesis, augment production of free radicals, and inhibit apoptosis and immune surveillance[8-9].

Regardless of the recent advances in Oral Cancer Therapy, the mortality rate remains high. Hence further advancements in cancer therapy or chemoprevention is crucial to improve cancer survival rates, which is one of the prime goals of the World Cancer Declaration issued by Union of International Cancer Control.So in order to ascertain prognostic significance of COX 2 in the malignant transformation, we designed a study to evaluate significance of cyclooxygenase 2 by comparing its expression in different grades of epithelial dysplasia, gingivitis and normal mucosa.

METHODOLOGY

Twenty consecutive cases which were histological confirmed as epithelial dysplasia (n=20), gingivitis (20) and ten normal tissues from clinically apparent oral mucosa.(n=10) . The study was approved by the Institutional Review Board of Mar Baselios Dental College Kothamangalam Kerala (IEC/25/2012/MBDC)and informed consent was obtained from subjects prior to study.

IMMUNOHISTOCHEMISTRY TECHNIQUE

Immunohistochemical analysis was performed according to manufactures(Biogenex) guidelines and standardization of technique was done. Super sensitive one-step polymer-HRP technique (Biogenex Life Sciences, USA) was used for IHC. Paraffin-embedded tissue blocks were cut into 5 μm thick sections and transferredontoadhesive coated slides(2%, poly-L-Lysine -biogenex). Deparaffinized and rehydrated the sections through xylene and descending grades of alcohol. Antigen retrieval wasdone in domestic pressure cooker and the sections were placed in a container containing EDTA (pH-9.0) at 95°C .Slides were a cooled at room temperature for 15 minutes and were rinsed in phosphate-buffered saline (PBS). Later treated with peroxidase block with 3% H_2O_2 in water for 15 min to block the endogenous peroxidase activity.Power block was used to obstruct any nonspecific antigenic spots.The sections were then incubated for 2 h at room temperature with antibody(primary rabbit monoclonal antibody clone SP21 at 10–15 mg/ml). After PBS wash, the sections were then incubated with one-step polymer-HRP reagent for 30 min. DAB (diaminobenzidine tetrahydrochloride) was freshly prepared and applied. The slides were counterstained with Harris hematoxylin, later cleared and mounted with DPX.

Positive controls were run simultaneously.Assessment of COX-2 expression was determined based on the presence of brown-colored end product confined to the cytoplasm and perinuclear area. Positively stained cells were counted in randomly selected fields.

Statistical Analysis was carried out using statistical package, SPSS (version 22.0.0.0)

This study deals with analyzing whether there is any significant association among different grades of epithelial dysplasia, normal mucosa and staining intensity of epithelium, inflammatory cells and connective tissue. Chi-square test for association is used for the analysis. Bar graphs are also provided to visually understand the association. In all the analysis significance level is taken to be 0.05 (i.e., if the p-value is less than 0.05, reject the null hypothesis or it can be concluded that the null hypothesis is statistically significant) and the test are two-tailed.

RESULTS

The study sample contained two study groups and one control group. Study group I included subjects confirmed histologically as epithelial dysplasia which were graded according to WHO and study group II includedhistopathological confirmed as gingival inflammation. The control group which included is clinically normal appearing mucosa. The study evaluated the expression of COX 2 and compared among the study groups and with the control group.

In the present study, COX 2 was significantly expressed on study groups where as normal mucosa showed negative staining. In study group I all the subjects showed positivity (P-value<0.001) where as in study group II, 75% subjects shown positive staining but with lesser staining intensity compared to group I. We evaluated the expression of COX 2 in OED, gingivitis and normal mucosa of which OED showed higher positivity in all cases. Also group I basal cells of epithelium has shown more staining intensity than parabasal layer.

In between the two study groups (I &II) the epithelial component in dysplastic lesions are more intensely stained than non-dysplastic tissue lesions. In group I, we also evaluated the expression of COX-2 within different grades of epithelial dysplasia of which epithelial component does not show any statistical significance between different grades of dysplasia.Even the mild dysplastic epithelium showed an increased staining intensity than a non-dysplastic lesion

Connective tissue component and inflammatory cells between different grades of dysplasia have showed that in severe and moderate grades of dysplasia staining were predominantly seen when compared to mild dysplasia. COX 2 staining of inflammatory cells and connective tissue fibroblast in dysplastic lesions showed a greater predominance in dysplasia on comparing with non-dysplastic lesion .These finding indicate that COX 2 immunoexpression can be used as a specific marker for PMDs thereby reducing the risk of malignant progression.

Comparing the staining intensity of COX 2 among 3 study groups histopathologically confirmed epithelial dysplasia, gingivitis & normal mucosa and intensityof staining epithelium, inflammatory cells and connective tissue

Chi-square test for association is used to test the null hypothesis.

		Intense	Moderate	Mild	Faint	Negative	Total
Epithelium	Dysplasia	2	9	6	3	0	20
	Gingivitis	0	1	12	3	4	20
	Normal	0	0	0	0	10	10
	Total	2	10	18	6	14	50
Connective tissue	Dysplasia	1	6	5	6	2	20
	Gingivitis	0	5	4	6	5	20
	Normal	0	0	0	0	10	10
	Total	1	11	9	12	17	50
Inflammatory cells	Dysplasia	0	6	7	6	1	20
	Gingivitis	2	3	6	6	3	20
	Normal	0	0	0	0	10	10
	Total	2	9	13	12	14	50

Table 1: Frequency distribution

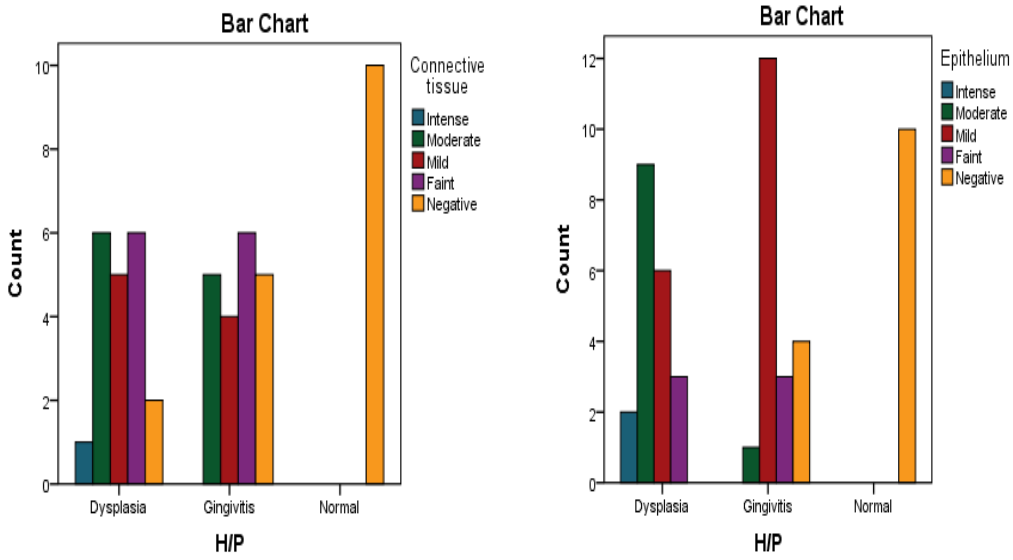
From the above table it can be observed that all the patients in normal group have negative staining intensity of epithelium, inflammatory cells and connective tissue. The results of the chi-square test are given below.

	Chi-square value	Df	Asymp. Sig. (2-sided)
Epithelium	46.571	8	.001
Connective tissue	26.429	8	.001
Inflammatory cells	36.346	8	.001

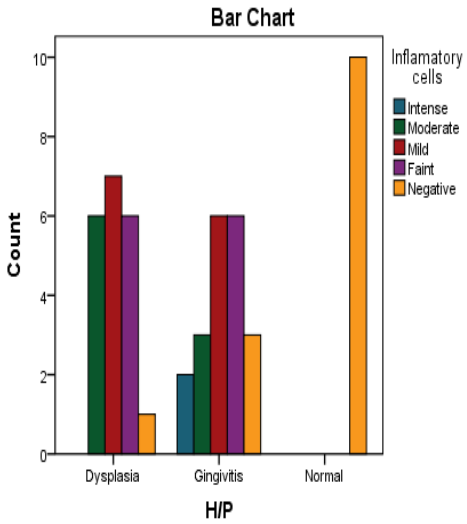
Table 2: Chi-square test

The above results suggest that there is significant association among H/P diagnosed study groups of dysplastic &non-dysplastic lesions and staining intensity of epithelium (chi-square (8) = 46.571, p-value < 0.001), inflammatory cells (chi-square (8) = 36.346, p-value < 0.001) and connective tissue cells (chi-square (8) = 26.429, p-value = 0.001).

The bar charts are given below.



Graph 1: Cox 2 Staining Intensity For EpitheliumGraph 2: Cox 2 Staining Intensity For Connective Tissue



Graph 3: Cox 2 Staining Intensity For Inflamatory Cells

Association among different grades of dysplasia & staining intensity of epithelium, inflammatory cells and connective tissue.Chi-square test for association is used to test the null hypothesis that there is no association among Dysplasia and staining intensity of epithelium, inflammatory cells and connective tissue. The frequency distribution is given below.

Components		Intense	Moderate	Mild	Faint	Negative	Total
Epithelium	Severe	2	3	0	0	0	5
	Moderate	0	5	3	2	0	10
	Mild	0	1	3	1	0	5
	Total	2	9	6	3	0	20
Connective tissue	Severe	1	3	1	0	0	5
	Moderate	0	3	4	3	0	10
	Mild	0	0	0	3	2	5
	Total	1	6	5	6	2	20
Inflammatory cells	Severe	2	2	0	1	0	5
	Moderate	0	3	4	3	0	10
	Mild	0	1	3	0	1	5
	Total	0	6	7	6	1	20

Table 1: Frequency distribution

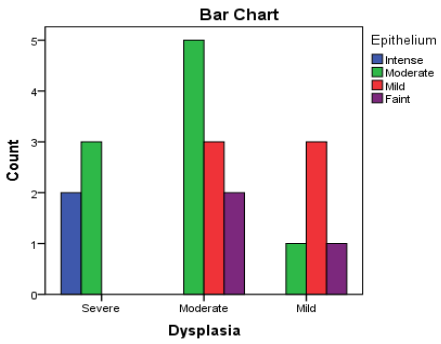
The results of the chi-square test are given below.

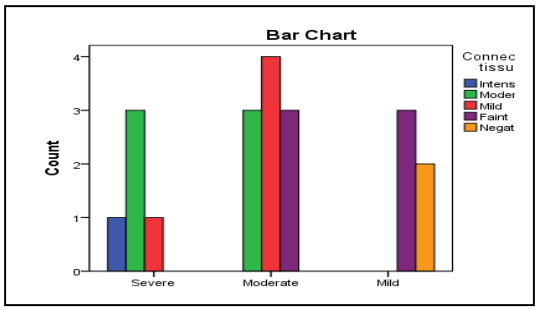
	Chi-square value	df	Asymp. Sig. (2-sided)
Epithelium	11.000	6	0.088
Connective tissue	17.200	6	0.028
Inflammatory cells	9.048	6	0.171

Table 2: Chi-square test

The above results suggest that there is no significant association among Dysplasia and staining intensity of epithelium (chi-square (6) = 11.000, p-value = 0.088), inflammatory cells (chi-square (6) = 9.048, p-value = 0.171).But there is significant association among Dysplasia and staining intensity of connective tissue cells (chi-square (6) = 17.200, p-value = 0.028).

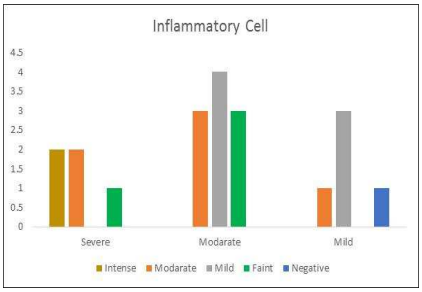
The bar charts are given below.





GRAPH 4- COX-2 Staining Intensity Among Different Gradeof Dysplasia

Graph 5: Cox-2 Staining Intensity Of Connective Tissue Among Different Grades Of Dysplasia



GRAPH 6: COX-2 Staining Intensity Of Inflamatory Cells Among Different Grades Of Dysplasia

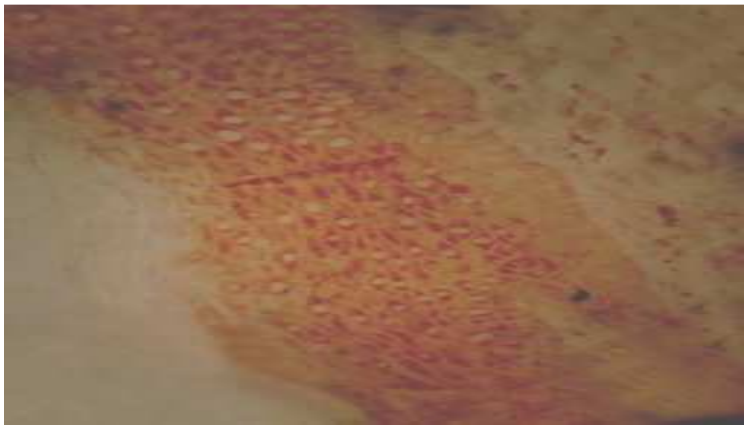


FIGURE 1: COX 2 showing cytoplasmic staining in Severe Dysplasia (40X)

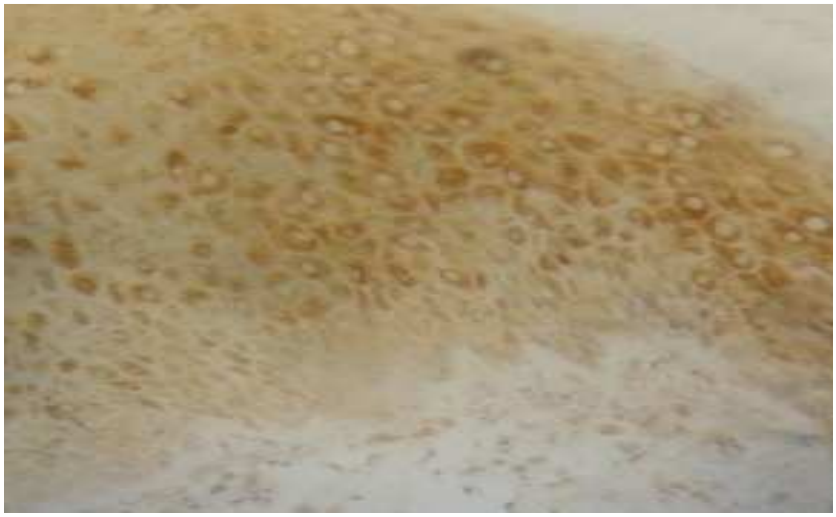


FIGURE (2): COX 2 showing cytoplasmic staining in Moderate Dysplasia (40X)

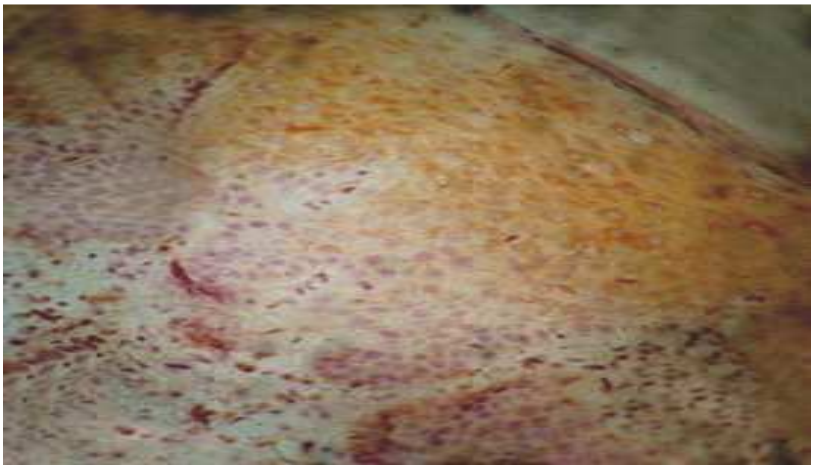


FIGURE (3): COX 2 showing cytoplasmic staining in Mild Dysplasia (40X)

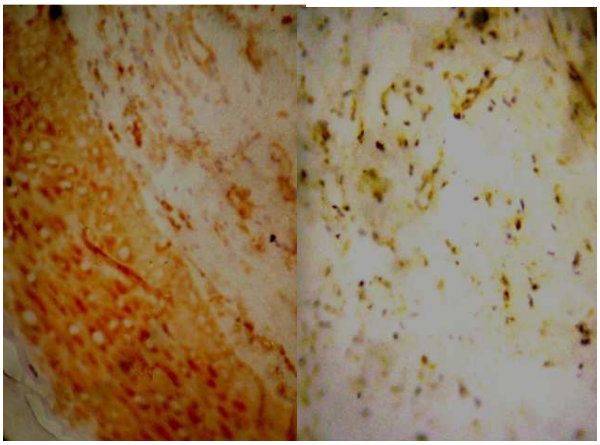


FIGURE (4): COX 2 Positivity in Fibroblast and inflammatory cells in Dysplasia

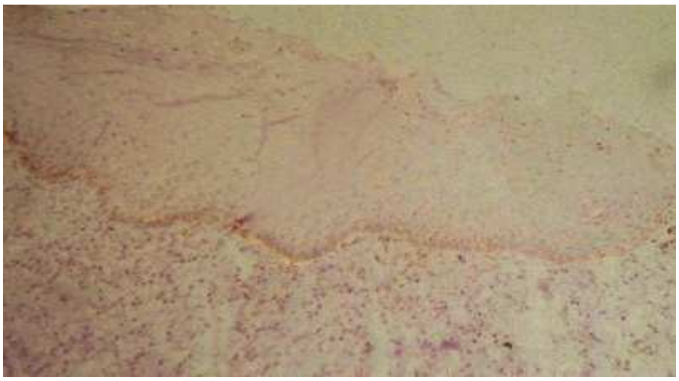


FIGURE (5): COX 2 showing mild positivity in Gingivitis (10X

DISCUSSION

Lumerman[10]*et al* defined epithelial dysplasia as 'A diagnostic term used to describe histopathological changes seen in chronic progressive and premalignant disorders of oral mucosa.

The histopathological evaluation of epithelial dysplasia can be relatively slanted and shows intra and inter-observer variability[11,12]. Somolecular surrogate markers can aid for the true prediction of precancerous stage of epithelium. .

Virchow in 1863 hypothesized that malignancy arises in sites of inflammation[13].Numerous studies suggested the effect of inflammation on the multistage development of oral cancers. Inflammatory mediators up regulated in chronic inflammation can increase the mutation rates,

produces genomic instability and enhance the proliferations of mutated cellsduring initial phases of carcinogenesis.[14,15,16]

Francesco Collotta *et al* surmise that Cancer Related Inflammation as the seventh hall mark of cancer.[17]. Upregulation of COX-2 prolongs the survival of abnormal cells thereby favors the accumulation of sequential genetic changes which increases the risk of tumorigenesis[17,18]. Furthermore, the expression of COX-2 valor light over the pathophysiology and clinical activities of oral cancers.

In the present study COX-2 expression shown a wide distinction in staining intensity depending on the degree of differentiationand was analogous to the study by Chan *et al*.[18].In our study group I, basal cells of epithelium has shown more staining intensity than para basal layers which is in accordance with the study of Rahul Pal*et al*[19]whichsuggested early neoplastic features in OED are first evident at basal epithelium positioned at epithelial-connective tissue interference (ECTI), separating the basal epithelium from underlying lamina propria. In between the two study groups , epithelial component in dysplastic lesions are more intensely stained (p-value <0.001) and even the mild dysplastic epithelium showed an increased staining intensity than a non-dysplastic lesion .This substantiate the role of COX 2 in invasion and metastasis of cancer.Shibata*et al*[20]suggested the expression of COX 2 is correlated with early tumeriogenesis which was comparable to the present study that showed positivity irrespective of WHO grading of epithelial dysplasia (p-value $\leq$0.001) .

Banerjee AG*et al*[21]confirmed that the early COX 2 overexpression in precancerous is an early casual event for tumor initiation through conversion of procarcinogens to carcinogens.

In our study in group I we also evaluated the expression of COX-2 in epithelial component within different grades of epithelial dysplasia(p value=0.088) of which does not show any statistical significance. This finding may be because the COX-2 liberated in different grades of OED, may be the same despite of the grading. This also emphasize more on the inter and intra observer variability in grading of dysplasia. The development of cancer as a continuum from mild dysplasia has also well established[22] .In an agreement with our study Goulalart *et al*and*I*toh*et al*found no significant difference in COX 2 expression between low grade and high grade OSCC[23-4]. Maryam [25]*et al* suggested no significant correlation between the histological grading of dysplasia and COX 2 expression which also appendto our findings. The consistency

of the grading of OED and scoring system will indeed to be assessed and refined for better clinical management of precancerouslesion.

In our study connective tissue component and inflammatory cells between stainingwere more predominantly seen in severe and moderate dysplasia when compared to mild dysplasia(p-value =0.028) .COX 2 staining of inflammatory cells and connective tissue fibroblast in dysplastic lesions showed a greater prevalence on comparing with non-dysplastic lesion. In support to our study Muellar*et al*[30]suggested stromal micro environment is known to play a major role in tumour invasion and progression .In the present study positive staining was observed mainly in epithelial cells, fibroblast and inflammatory cells dispersed throughout lamina propria .Recently many studies suggested that the tumour micro-environment and their stroma, influence tumour growth which add on to our study and showed an increased expression of COX 2 in connective fibroblasts of epithelial dysplasia

Though COX 2 localization in tumorsiscontroversial, in our study there is an increased expression of COX 2 protein in both epithelial component and connective tissue stroma. COX 2 is said to be increased in stromal component of tumors where as another school of thought suggested an increased COX 2 in epithelial components.

In our study on inflammatory cell component, we found an increased COX 2 expression in epithelial dysplasia.An inflammatory component is present in the tumor micro environments which are not epidemiologically related to normal inflammation. Tumor inflammation is recognized as stimulators of angiogenesis and they release COX 2, VEGF, IL-8 etc. COX 2 liberated in tumour stroma affects immune surveillance. These immune cells that infiltrate tumors engage in an extensive and dynamic cross talk with cancer cells.

Inflammation can also be abeneficial response activated to restore tissue injury. Acute inflammation is a response to an alteration induced by a pathogen or a physical or chemical insult which functions to eliminate the source of damage and restore homeostatic to the affected tissue. In our study group II, (non dysplastic/gingivitis without periodontal destruction) were considered as the study subjects hence COX 2 expression was less compared to group I but higher than normal mucosa. Numerous literatures suggested that the expression of COX 2 in gingivitis when compared with dysplasia is very mild which is consistent with our study[27-8]. The

mild expression of COX 2 in gingival inflammation is an adaptive immune response where as those expressed in epithelial dysplasia may be associated with tumour associated inflammatory cells.

In a nut shell our study emphasize the role of COX2 in tumeriogenesis and can also be a potent molecular marker .Our study on COX 2 also throws light on the use of COX 2 inhibitors as an alternative therapeutic modality for oral epithelial dysplasia to be evaluated on future researches on a wider angle . The expression of COX2 in both epithelium and stroma of mild dysplasia is suggestive that molecular aberrations are occurring which are not markedly evident in the cellular level under light microscopy. Therefore grading epithelial dysplasia into mild, moderate and severe in cellular level needs further investigation and reclassification.

CONCLUSION

We coined our study on COX 2 as it is considered not only as a prognostic marker but also a chemo target. Only few studies have evaluated the significance of COX 2 in different grades of oral epithelial dysplasia.In our study we found WHO grading of OED is subjective and predictability doesn't account for malignant transformation between the grades of dysplasia as COX 2 expression was found in all grades of OED which is suggestive of molecular mutations and risk of development of malinancy in early dysplastic lesion. Further studies with larger sample size are required to emphasize the realiabity of our study. Evaluation of markers like COX 2 can render early treatment of pre-cancerous lesions that are at higher risk thereby preventing the progression of oral epithelial dysplasia to a malignant tumor.

Conflict of Interest Statement –Authors declare no conflict of interest.

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BIBILOGRAPHY

1. Ravi Mehrotra ,Shruti Pandya,Ajay Kr Chaudary et l Prevalance Of Oral Premalignantand Malignant Leisions at a Tertiary Level Hospital Allahabad ,Indian Asian Pacific Journal of Cancer Prevention 2008: 263-65

2. G Sreedharan Epidemology ,control and prevention of Tabacco induced oral mucosal lesions in India Indian J Cancer 2014: 80-5
3. F A Olshan , Epidemology Pathogenesis of Oral Squamous Cell Carcinoma J Oral Clin Patho: Molecular Pathol 2000;53:165-72
4. P C Caldeira , Aberue MH, Carmo MA, Binary System of Grading Oral Epithelial Dysplasia: Evidence of a bearing to the scores of an immuno histochemical study: J Oral PatholMed 2012; 41:452-53
5. J J Pindborg, Reichart P Smith CJ, Van der Waal I World Health organization: histological typing of cancer and precancer of the oral mucoasa Berlin:Springer –Verlag 1997
6. Gayani Pitying, Tilakaratne WM, Tavassoli M , Warnakulasurya S: Molecular Markers in Oral epithelial Dysplasia - A Review; J Oral PatholMed 2009; 38:737-52
7. Claria; Cyclooxygenase to Biology; CURRPHARMDES 2003; 2177- 90
8. Cherie –Ann O Nathan ,Igor L Leskov, Meihong Lin, Fluerette W Abreo ,Runhua Shi eta l COX 2 Expresssion in Dysplasia of the Head and Neck Correlation with Eif4E 2000:1888-1985
9. Lumerman H Freedman P, Kerpal S Oral epithelial dysplasia and development of invasive squamous cell carcinoma, Oral Surg Oral MedOral Pathol Oral Radiol Endod 1995 321-9
10. Warnakulsuriya,J Reibel ,J Bouquot,E Dabelsteen Oral epithelial dysplasia classification systems :Predictive value,utilityweakness,scope for improvement J Oral Pathol Med ,2008 127-133
11. Geetha M Leeky ,TV Narayan ,S Sadhana ,J Saleha;Grading of dysplasia :Points to ponder Journal of Oral and Maxillofaxial pathology ,2015198-204
12. Jerry E Bouquet Paul M SPEIGHT Paula M Farthing Epithelial Dysplasia of oral mucosa-Diagnostic problems and prognostic featutres;Current Diagnostic Pathology 2006,11-21
13. Fran Balkwill,Alberto Mantovani Inflamation and cancer back to Virchow –A review Lancet 2001:357:539-45
14. Naipier SS CG Cowan T A Gregg Potentially malignant oral leisions in Northern Ireland Oral Dis 2003 129-37

15. Robert D Schrebier Role of Immunology in human cancer research Science 331 2011
16. Michael M KIM, Joseph A Califano Molecular pathology of Head & neck Cancer: International journal of cancer 2004, 545-53
17. Francescco Colotta,Paola Allavena,Antonoi Sica Ceeillia Garlanda Alberto Mantovani:Cancer Related Inflammation,the seventh hallmark of cancer :links to genetic instability Carcinogrnesis 2009 1073-1081
18. Chan G, Boyle JO, Yang EK, Zhang F, Sacks PG, Shah JP, et al. Cyclooxygenase-2 expression is up-regulated in squamous cell carcinoma of the head and neck. Cancer Res 1999;59:991-4.
19. Rahul Pal, Tuyu Schilagard Jiniping Yang ,Paula Villarreal :Remodelling of epithelial dysplasia connective tissue interface(ECTI) as visualized by non –invasive 3D imaging Cancer research 2016:10-11
20. Shibata K Kodani I,Osaki M,Araki K ,Adachi H ,Ryoke K et al Cyclooxygenase 1 and 2 expression in human oral mucosa,dysplasia and squamous cell carcinoma and their pathological significance Oral Oncol 2005:41 304-12
21. Banerjeee AG,Gopalakrishnan VK,BhattacharyaI, Vishwanatha Desregulated cyclooxygenase -2 expression in oral premalignant tissues J K Mol Cancer Ther 2002 :14 1265-71
22. S Fedele Diagostic aids in the screnning of oral cancer Head & neck Oncology 2009: 5-11
23. Goulart Filho J A,Nonaka C F,da Costa Miguel M C ,de Almeida Freitas R,Galvao H C .Immuno expression of cyclooxygenase 2 and p53 in oral squamous cell carcinoma.Am J Otolargyngol 2009:30:89-94
24. Itoh S,Matsui K,FurutaI,Takano Y.Immunohisochemical study on over expression of cyclo oxygenase 2 in squamous cell carcinoma of oral cavity:Its importance as a prognostic predictor.Oral Oncol 2003;39:829-35
25. Maryam Amirchaghmaghi,Nooshin Mohtasham ,Pegah Mosannen Mozaffari:Comparision of COX 2 Expression in between Oral squamous cell carcinoma ,Leukoplakia and Normal mucosa :Journal of contemporary Dental Practice 2012:13(2):205-09

26. Mueller MM ,Fuseing N E :Tumor –stroma interactions directing phenotype and progression of epithelial skin tumor cells 2002;70;486-97
27. Morton R S, Dongari-Bagtzoglou A, Cyclogenase-2 is up regulated in inflamed gingival tissues ; J Perdontol; 2001, 72; 461-9
28. Yucel-Lindberg T, Hall storm T Kats A, Mustafa M, Modeer T-Induction of microsomal prostaglandin E synthase-1 in human gingival Fibroblasts; Inflammation 2004 Apr 28(2): 89-95