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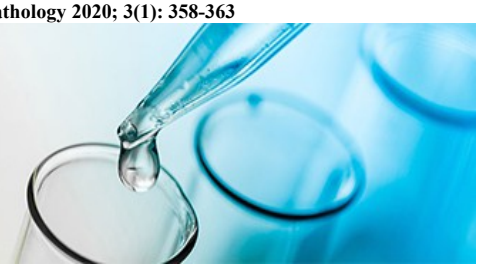

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Expression profile of apoptotic and proliferative markers in oral lesions and its clinicopathological significance

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Abstract
Introduction: Oral cavity is prone to a myriad of changes with advancing age as well as a result of the environmental and life style related factors. Genetic abnormalities are the primary cause of dysplastic, metaplastic, and neoplastic changes resulting in an imbalance between molecular mechanisms which regulate apoptosis and cell proliferation. Evaluating these parameters may not only help in identifying the individuals who are at a greater risk of developing carcinomas, but also carry significant prognostic value.
Aim: The present study was undertaken with the aim of evaluating the expression profile of Bcl2 and Ki-67 as biomarkers in assessing the apoptotic and proliferative activity of Oral lesions and compare the topographical aspect of these lesions.
Material and Methods: A cross sectional study of 31cases of oral lesions, immunohistochemically assessed for Bcl-2 and Ki67 for their expression pattern.
Results: Expression of Ki-67 showed a linear increase from normal oral epithelium through various histological grades of Oral squamous cell carcinoma (OSCC). Expression of Bcl-2 was variable, defined by cytoplasmic granular staining in the epithelial cells. A decrease in mean positivity was seen from well differentiated OSCC to Moderately differentiated OSCC. Weak intensity of Bcl2 staining was seen in all the cases except one case of poorly differentiated oral squamous cell carcinoma.
Conclusion: The pattern of expression of biomarkers, not only help in diagnosing oral lesion but also in identifying prognostic markers. Alterations in the expression of proteins related to cell proliferation and apoptosis are a strong indicator of the malignant transformation potential of certain lesions.

Keywords: Expression profile, apoptotic, proliferative markers, clinicopathological

Introduction
Oral cavity is prone to a myriad of changes with advancing age as well as a result of the environmental and life style related factors. They can occur as a result of infections, local trauma or irritation, systemic diseases and excessive consumption of tobacco, betel quid and alcohol [1]. These lesions may include leukoplakia, erythroplakia, oral submucos fibrosis, dysplasia and oral squamous cell carcinoma (OSCC) amongst others. The prevalence and incidence rates of oral mucosal lesions vary in Indian population due to the existence of cultural, ethnic and demographic differences [2].
Genetic abnormalities are the primary cause of dysplastic, metaplastic, and neoplastic changes. They result in an imbalance between molecular mechanisms which regulate apoptosis and cell proliferation. Apoptosis is a genetically programmed form of cell death which is indispensable for development and homeostasis of multicellular organism. Dysregulated apoptosis is implicated in pathogenesis of a variety of diseases including oral pathologies [3]. The anti-apoptotic mechanism is regulated by Bcl-2 (B-Cell Lymphoma 2) gene, while Ki-67 is expressed exclusively in nuclei of proliferating cells [4]. Ki-67 and MIB-1 (Molecular Immunology Borstel-1) monoclonal antibodies are directed against different epitopes of the same proliferation-related antigen [5]. Cell proliferation and a decreased rate of apoptosis are known to be involved in pathogenesis of carcinogenesis. Evaluating parameters such as cell proliferation and cell death may not only help in identifying the individuals who are at a greater risk of developing carcinomas, but they also carry a significant prognostic value and also represent a good model of tumor development [6, 7].

Material and Methods

The present study a cross-sectional study conducted in the Department of Pathology. Patients who had been exposed to risk factors, and had given consent were considered. A total of 31 patients from the various Out Patient Departments with suspicious oral lesions were included in the study which was carried out over a 3-month period the data was submitted under the Student Term Studentship program of Indian Council Medical Research (ICMR).

The harvested post-surgical specimens were processed in the histopathology section for routine paraffin embedding. Serial sections of 4micron uniform thickness were cut, stained with Hematoxylin and Eosin (H&E) stain and examined methodically to establish a diagnosis, histopathologically classify and assign a grade to the malignant cases.

Sections were also taken on poly-L-lysine coated slides and subjected to immunohistochemical (IHC) staining concurrently for Ki-67 and Bcl-2. Staining for Ki-67 was performed using pre-diluted ready to use mouse monoclonal antibody, standardized using Ultra View Universal DAB detection kit. Ki-67 positive nuclei were expressed as the percentage of total nuclei counted under 40X magnification. The labeling index for Ki-67 was calculated as following [8]

$$\text{Labeling Index} = \frac{\text{No.of cells showing positive staining}}{\text{Total No. of cells}} \times 100$$

IHC staining for Bcl2 oncoprotein was performed using Monoclonal mouse in 1:50 dilution. The immunohistochemical expression of Bcl-2 was defined by cytoplasmic staining in the epithelial cells. The percentage of positive cells were counted and classified as: More than 50% of cells positive (+++); 25-50%: Positive (++); 5-24% positive (+), and fewer than 5% positive or no staining (–). Lymphocytes were used as the internal positive controls. [9] Both markers were evaluated for intensity of staining also. Relevant statistical analysis was done on the data collected. The topographical aspect and degree of expression of Bcl2 and Ki-67 were also compared.

Observations and Results

The present study was conducted in the Department of Pathology on patients presenting in the various Out Patient Departments, with suspicious oral lesions. A total of 31 patients were evaluated of which, 30(97%) were males, and 1(3%) was a female. Age wise distribution of the patients showed maximum number of cases, 11(35%) in the age group 31-40 years, with a mean age of 44.45±11.23, followed by the age group 41-50 years, with 8(25%) patients. (Figure 1)

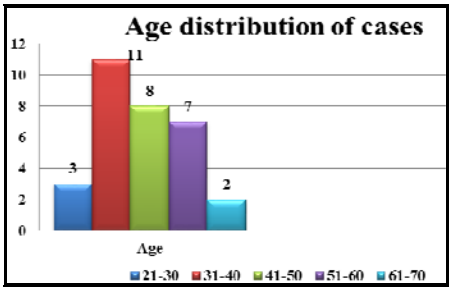

Fig 1: Distribution of cases according to age

On documenting the site of lesion, we found that the buccal mucosa was the most common site of involvement, with 12 cases (38.7%) followed by the lesions on the tongue. The distribution of cases on the basis of site of involvement is depicted in Table 1.

Table 1: Distribution of cases according to site of lesions

Site of Lesion	Number of Cases (31)	Percentage (%)
Buccal Mucosa	12	38.7
Left lateral border of tongue	6	19.3
Right lateral border of tongue	3	9.6
Base of tongue	3	9.6
Floor of mouth	1	3.2
Right gingivo-buccal sulcus	2	6.4
Left gingivo-buccal sulcus	1	3.2
Palate	3	9.6

Histopathological evaluation of the samples received in the laboratory was done; the oral lesions were diagnosed and categorized as depicted in Table II. The majority of the lesions were of Squamous cell carcinoma, 13cases (41.9%), out of which Moderately differentiated were 8 cases, Well differentiated 4 cases and poorly differentiated 1 case only. Dysplastic cases amounted to 6 while verrucous hyperplasia was seen in 3 cases. Non Dysplastic Oral leukoplakia was diagnosed in 5 cases. 12.9% of the cases were composed of normal oral epithelium on histopathology.

Table 2: Distribution of Cases According To Type of Lesion

Histopathological Diagnosis	No. of Cases	Percentage (%)
Normal Oral Epithelium	4	12.9
Leukoplakia	5	16.1
Verrucous Hyperplasia	3	9.6
Moderate Dysplasia	3	9.6
Severe Dysplasia	3	9.6
Well Differentiated OSCC	4	12.9
Moderately Differentiated OSCC	8	25.8
Poorly Differentiated OSCC	1	3.2

Immunohistochemical staining for Ki-67 was positive in all the cases, the LI for each was calculated. (Table 3) Intensity of staining for all the cases was strong.

Table 3: Expression of Ki-67 in various oral lesions

S. No	Category	No of Cases	Mean LI±SD Ki-67	Range (Min-Max)
1	Normal Oral Epithelium	4	10.4±2.08	6.4-11.2
2	Leukoplakia	5	16.6±14.37	8.6-46.2
3	Verrucous Hyperplasia	3	24.2±5.76	18.1-30.2
4	Moderate Dysplasia	3	29.4±2.64	27.4-32.4
5	Severe Dysplasia	3	32.4±2.2	30.2-34.4
6	WD OSCC	4	33.6±5.66	28.8-38.2
7	MD OSCC	8	60.4±13.23	39.8-82.2
8	PD OSCC	1	108	-

*WD: Well Differentiated, MD: moderately differentiated, PD: Poorly differentiated, OSCC: Oral Squamous Cell Carcinoma

Expression of Ki-67 showed a linear increase from normal oral epithelium through various histological grades of OSCC. However, mean LI of Ki-67 for oral epithelial

dyplasia was higher as compared to WDSCC and MDSCC but less than PDSCC. LI of verrucous hyperplasia (VH) was higher than non-dyplastic leukoplakia indicating proliferative activity.

Ki-67 expression in normal oral epithelium was predominantly seen along the parabasal region and sometimes the basal layer. Its expression in leukoplakia cases which were non dyplastic in our study was seen more in basal followed by parabasal and none in the suprabasal layer, similar expression was seen in VH cases however one case showed increased suprabasal expression. Ki-67 LI was seen in the basal and suprabasal layers of epithelial dysplasia and it was seen to increase according to the severity of dysplasia in the suprabasal layer. Increased expression of Ki-67 was seen in various grades of OSCC, the expression was prominent in the peripheral areas of tumor islands in two cases of well differentiated OSCC while it was diffuse in the remaining two cases. Moderately differentiated OSCC expression was diffuse in all cases including both tumor islands and adjacent areas while one case of poorly differentiated showed diffuse and intense staining of Ki-67. All cases showed strong immunostaining for Ki-67. Statistical tests of significance could not be applied to the current data due to small sample size.

The immuno histochemical expression of Bcl-2 was defined by cytoplasmic granular staining in the epithelial cells, immuno histochemical data and staining activity of Bcl2 is summarized in Table 4.

Table 4: Expression of Bcl2 in various oral lesions

S. No	Category	No. of cases	Positive cases	Percent Positive Staining Cells					Mean% cell positivity ±SD
				>50%	25-50%	5-24%	<5%	-ve	
1	Normal Oral Epithelium	4	3	0	0	0	3	1	2.7±0.75
2	Leukoplakia	5	2	0	1	1	0	3	20.2±8.48
3	Verrucous Hyperplasia	3	2	0	2	0	0	1	27.1±1.55
4	Moderate Dysplasia	3	3	0	2	1	0	0	29.3±2.96
5	Severe Dysplasia	3	3	0	3	0	0	0	30.6±4.00
6	WDOSCC	4	2	0	1	1	0	2	28.5±7.21
7	MDOCC	8	6	0	4	2	0	2	24.8±10.81
8	PDOSCC	1	1	1	0	0	0	0	58.2%

Expression of Bcl2 was variable; 9 cases out of 31 were negative. The use of the internal (lymphocyte) positive control suggests that the approach has been optimized for the samples under investigation. Immunostaining for Bcl2 was seen in 3 cases with <5% and very weak staining in the basal cells in normal oral epithelium, basal and parabasal in two (40%) cases of leukoplakia, the staining was seen to extend in to the suprabasal areas in VH. Dysplastic oral epithelium showed immunostaining for Bcl2 in basal and parabasal layers. 4/13 (30.7%) cases of OSCC showed complete negative expression of Bcl2. A decrease in mean positivity was seen from well differentiated OSCC to Moderately differentiated OSCC with staining prominent in peripherally placed tumor cells. A marked increase in expression was however seen in 1 case of poorly differentiated OSCC with a diffuse pattern of strong staining. Weak intensity of Bcl2 staining was seen in all the cases except one case of PDOSCC. No statistically significant differences between the expression Bcl-2 in oral epithelial dysplasia and OSCC and non-atypical and atypical lesions (dyplasia, OSCC) were observed ($P > 0.05$). Figures 2-5 depict the expression pattern of Ki-67 and Bcl2 in various lesions in the present study.

Discussion

Lesions in the oral cavity can be grouped into different categories according to the pathological process, like inherent (congenital or hereditary), inflammatory, infectious, iatrogenic and idiopathic (oral premalignant lesion or neoplasm).[10] In developing countries, where oral-health-care resources are meagre, oral cancer (OC) is a major health problem representing the leading cause of death.[9] The aetiology is multifactorial and the most important risk factors include tobacco and alcohol consumption.[11,12,13] A total of 31 cases were included in our study with oral lesions varying from unremarkable oral epithelium through leukoplakia, VH, epithelial dyplasia to OSCC. A predominance of male population comprising of 30(97%) of the cases and 1(3%) female were part of the study, attributed to traditionally males more likely to display oral habits such as tobacco smoking and betel quid chewing, known risk factors for oral lesions especially oral cancers. The site of involvement of lesions varies but buccal mucosa followed by the tongue, are the most common sites of involvement [14, 15, 16] a similar trend was seen in our study however involvement of mucosa and tongue were in equal proportions. Age is an important factor in oral lesions, triggering dysplastic changes and carcinogenesis. The mean age in the present study was 44.4 years, in concordance to other authors [17] while it was lower than 63.7 years observed by some [18].

Stratified squamous epithelium is the lining epithelium of the oral mucosa and results from cell proliferation and sequential differentiation. Proliferation, apoptosis and differentiation are the fundamental aspects of tumor biology [19]. Ki-67 is a peri-chromosomally located, cell cycle-related antigen. Its expression is strictly restricted to dividing cells in all active phases of the cell division cycle. Therefore, it is regarded as one of the most potent proliferation marker in the field of molecular pathology. Our study was undertaken to demonstrate expression patterns of Ki-67 in different oral lesions. The mean value of LI was found to increase as the nature of the lesion changed especially from dysplasia to SCC. In the present study, normal oral epithelium expressed the lowest Ki-67 LI while the proliferative activity of non dyplastic leukoplakia was higher, similar to Mondal *et al.* [20] however this was contrary to, Dwivedi *et al.* [21] and Kumar *et al.* [22] who noticed very little disagreement between the mean LIs of normal oral epithelium (13.65%) and nondysplastic OL (12.78%). In our study, the discrepant and relatively higher mean LIs within leukoplakia lacking any dysplasia are

possibly a consequence of smaller sample sizes. Ki-67 expression in normal oral epithelium was predominantly seen along the parabasal region. Its expression in leukoplakia cases which were non dyplastic in our study was seen more in basal followed by parabasal and none in the suprabasal layer in concordance with other authors [21, 22, 23]. Oral VH resembles verrucous carcinoma both clinically and histologically, however the exophytic growth pattern of VH, and endophytic invasive growth pattern associated with verrucous carcinoma differentiates the two lesions. Ki-67 LI index of VH in the study was in concordance with others [18]. A basal pattern of expression has been described in literature in VH [18, 24] a similar finding was observed by us however one case showed suprabasal staining, this could be indicative of transition from hyperplastic to dyplastic change.

Increase in Ki-67 LI has been seen in the basal, parabasal and spinous layers of epithelial dyplasia as proliferative activity increases due to cellular alteration. Ki-67 in the basal and suprabasal layers of epithelial dysplasia increased according to the severity of dyplasia in the suprabasal layer [8, 21]. A similar trend was seen in the present study. Epithelial dysplasia presents as an alteration of the cellular maturation in the epithelium and as increase in the proliferative activity in suprabasal layers. Studies have revealed that Ki-67 positivity increased according to the proliferative activity and degree of epithelial dysplasia [8], thus implicating it as a marker of proliferation and exhibiting the degree of severity of dyplasia. The expression of Ki-67 was seen to linearly increase from normal mucosa through various grades of OSCC. Our results were in accordance with other authors [21, 25]. The expression correlated with the grading of OSCC as it depicts the growth of the tumor and its proliferative status. It increased according to the aggressiveness of the tumor.

Ki-67 positive cells in WDOSCC were located in the periphery of the tumor nests where frequent mitoses were observed than the central areas where cells are more differentiated. In MDOSCC however, Ki-67 expression was observed in both peripheral and central layer as cells. More so, in PDSCC, Ki-67 expression was diffuse as the cells were less differentiated than lower grades. More number of cells were in proliferative phase and hence showed an increase Ki-67 LI than WDSCC and MDSCC. These results in the present study were similar to others reported in literature [8, 21, 25, 26].

The balance between cell proliferation and apoptosis determines growth in both normal tissue and in cancer. Bcl-2 family members are apoptosis regulatory proteins. Bcl2 is an anti apoptotic protein that plays a role in determining the cell fate. Bcl-2 expression appears to be altered to varying degrees in oral mucosal lesions. In normal proliferating epithelium, while Bcl-2 is expressed in stem cell zones such as the basal layers, where it acts to prevent the death of cells in the regenerative compartment, its expression is very infrequent. Very low expression of Bcl2 was seen in the normal epithelium in our study, similar to reports in literature [27, 28]/. Reports of complete absence of Bcl-2 expression in normal epithelium are seen in literature [27, 28] In the present study, 2 positive (40%) cases of leukoplakia showed basal and parabasal expression. Bcl2 expression in leukoplakia has been reported predominantly in basal with few cases in suprabasal areas also. Increase Bcl2 expression in suprabasal region may also be correlated to the basal cell degeneration along with its risk of carcinoma development.[28] Limited expression of Bcl2 in the verrucous lesions reported by Thennavan *et al*. has been explained on the basis of a high Ki-67 LI. However in contrast to this we observed Bcl2 expression in all the three cases which also expressed high Ki -67 LI. An increase in anti-apoptotic marker, Bcl-2 resulting in the down regulation of proliferation marker, Ki-67 has been reported by Piattelli *et al*.[29] The increased expression of Bcl-2 is not only essential to oral carcinogenesis, it also influences progression of the disease by increasing the survival rate of neoplastic cells, allowing new genetic mutation to occur.[9] Overexpression of Bcl-2 has been reported in oral dysplastic lesions in various studies conducted on oral tissues and has been suggested to play an important role in oral tumorigenesis [30, 31, 32]. A proportional increase in Bcl-2 oncoprotein in all categories of dysplasia was seen in the present study, in agreement with Ravi *et al*. and Singh *et al*. [30, 32] On the contrary, other studies have reported a sporadic Bcl-2 expression or lack of expression in oral dysplasias [33, 27]. These reports suggest that dysregulation of the Bcl-2 gene may be one of many genetic aberrations in the progression of epithelial tumors [9].

Variable results in Bcl2 expression have been reported in OSCC, 25% [32] 36.1% [28] 60% [31] 83.3% [34], 69.2% in the present study. The differences in Bcl-2 expression from various studies may reflect subtle inherent differences in upstream genetic events between the different population groups, as also the environmental differences.[35] The differences in immunolocalization of Bcl-2 in various epithelia and neoplastic tissues may be topographical in nature or as a result of different laboratory protocols and antibodies used in the different studies [9] The pattern of staining of Bcl2 showed peripheral staining of tumor cells in well differentiated OSCC than central cells, similar results were observed by Juneja *et al*.[9] and Piatelli *et al*.[29] These observations might be attributed to down-regulation of Bcl-2 expression concomitant with terminal cell differentiation [32]. A decrease in mean positivity was seen from Well differentiated OSCC to Moderately differentiated OSCC with staining prominent in peripherally placed tumor cells, in agreement with Jessica *et al*.[4] Stronger expression of Bcl-2 oncoprotein was seen in poorly differentiated OSCC which is consistent with the findings of Juneja *et al*. [9] Sudha *et al*. [28] The increased Bcl-2 expression in poorly differentiated carcinomas may reflect the loss of ability of malignant keratinocytes to differentiate terminally.

Conclusions

The pattern of expression of biomarkers, not only help in diagnosing oral lesion but also in identifying prognostic markers. Alterations in the expression of proteins related to cell proliferation and apoptosis are a strong indicator of the malignant transformation potential of certain lesions. Although our study was limited by low sample size and unequal distribution of samples, we conclude that Ki-67 is a reliable proliferative marker which can be used for the diagnosis of lesions. Ki-67 increased with increasing grades of dysplasia and also linearly through various histological grades of oral squamous cell carcinoma indicating that they may be used as predictive markers in oral cancer development. Expression and distribution of Bcl2 showed

that positive expression was associated with a more aggressive histopathologic behavior, indicating an unfavorable clinical outcome may provide a better understanding of the biological behavior of oral lesions especially dysplastic and cancerous lesions which, in the future, may have important therapeutic and prognostic implications. We emphasize that difference in topographical expression, and degree of expression of Bcl-2; can be better appreciated with investigation of more number of cases. The identification of molecular markers may provide a useful insight into the potential behavior or aggressiveness of tumors which is an essential step for the improvement of cancer treatment and prognosis.

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