Original Article

Bcl-2 expression in reactive oral lesions with atypical epithelium and in oral epithelial dysplasia associated with carcinogen exposure

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Abstract

Background: Reactive lesions of the oral cavity are nonneoplastic proliferations with very similar appearance to benign neoplastic lesions and are associated with chronic local irritation or trauma. Although these lesions are uncommonly associated with carcinogen exposure, at times, they present histopathologically with dysplastic epithelium, thus making it difficult to differentiate it from true potentially malignant disorders. Hence, the present study was conducted to evaluate the expression of Bcl-2 protein, an antiapoptotic marker, in reactive lesions with and without atypical epithelium and in true epithelial dysplasia, which clinically presents as premalignant disorders.

Materials and Methods: The samples included 15 cases each of normal oral mucosa (NOM), reactive lesions with and without dysplasia and oral epithelial dysplasia (OED) associated with carcinogen exposure. All the samples were subjected to immunohistochemical staining using Bcl-2 antibody. The total number of cells in the basal and parabasal layers in each field and total number of cells expressing Bcl-2 among them and the staining intensity were assessed.

Statistical Analysis: Kruskal–Wallis ANOVA test was used to compare the number of positive cells among the four groups. The comparison of average percentage of positive cells between the study groups was done using Mann–Whitney U-test.

Results: The immunohistochemical staining for Bcl-2 protein was identified in few cells in the basal layers of NOM, reactive lesions without atypical epithelium and in the basal and parabasal layers in reactive lesions with atypical epithelium and OED, as a granular cytoplasmic staining and as an accentuation around the nuclear membrane. There was a gradual increase in the expression and intensity of staining from Group I to IV.

Interpretation and Conclusion: The altered or increased expression of Bcl-2 oncoprotein in reactive lesions with atypical epithelium and in OED with carcinogen exposure may lead to prolonged cell survival and can be considered as an early molecular event in carcinogenesis, helping us in understanding the nature of dysplasia in reactive lesions, which was not considered during the histopathology reporting.

Keywords: Apoptosis, atypical epithelium, Bcl-2, oral epithelial dysplasia, reactive lesions

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INTRODUCTION

The oral cavity is constantly subjected to external and internal stimuli and therefore manifests a spectrum of diseases that range from developmental, reactive and inflammatory to neoplastic. The cells in the multicellular organisms are often inflicted with a variety of above challenges to which they react by regeneration, hyperplasia, dysplasia, hypertrophy, atrophy or metaplasia depending on the nature of the challenge.[1,2]

Normal tissue architecture is a result of balance in cell proliferation and cell death. Apoptosis, a mode of cell death which plays an imperative role in the development, regulation and maintenance of cell population in multicellular organisms, is implicated in both health and diseases. A dysfunctional apoptotic system can lead to either excessive removal or prolonged survival of cells.[1,3]

Apoptosis is brought about by interplay of numerous pro- and antiapoptotic proteins. The pro- and antiapoptotic markers help us to understand the nature of lesion and the process in progress.[3] The proteins include Bcl, TP53 and caspases and the various proteins of Bcl family include antiapoptotic (Bcl-2 and Bcl-xl) and proapoptotic (Bax, BAD, BOK and annexin).[4]

Apoptotic cell death plays an important role in the maintenance of the normal physiological state, and it may also be responsible for diseased state of the body. Bcl-2 is the first gene shown to be involved in apoptosis and is regarded as a proto-oncogene that suppresses the cell death rather than stimulating cell proliferation acting as an antiapoptotic marker. It regulates programmed cell death by allowing tumor cell to escape apoptosis, thereby promoting the survival of tumor cells and facilitating the acquisition of mutation.[5]

The human Bcl-2 protein is an intracellular, integral membrane protein with a molecular weight of about 26KD. They are the first member to be discovered and called as B-cell lymphoma-2 and located in chromosome 18. It is characteristic of follicular lymphoma, a human B cell malignancy (Tsujimoto *et al*, 1984). Several proteins of Bcl-2 family regulates the mitochondrial permeability events in apoptosis.[4]

The sensitivity of cells to apoptotic stimuli can depend on the balance of pro and anti-apoptotic bcl-2 proteins. When there is an excess of pro-apoptotic proteins the cells are more sensitive to apoptosis, when there is an excess of anti-apoptotic proteins the cells will tend to be more resistant.

The pro-apoptotic bcl-2 proteins are often found in the cytosol where they act as sensors of cellular damage or stress. Following cellular stress they relocate to the surface of the mitochondria where the anti-apoptotic proteins are located. This interaction between pro- and anti-apoptotic proteins disrupts the normal function of the anti-apoptotic bcl-2 proteins and can lead to the formation of pores in the mitochondria and the release of cytochrome C and other pro-apoptotic molecules from the intermembrane space. This in turn leads to the formation of the apoptosome and the activation of the caspase cascade.[6]

Bcl-2 family proteins play central roles in cell death regulation and are capable of regulating diverse cell death mechanisms that encompass apoptosis, necrosis and autophagy. Alterations in their expression and function contribute to the pathogenesis and progression of human cancers.[6]

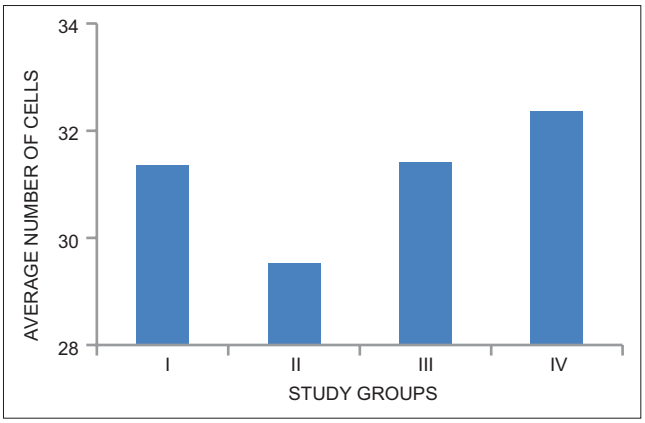
MATERIALS AND METHODS

The present cross-sectional retro-prospective study was conducted in the Department of Oral Pathology and Microbiology, College of Dental Sciences, Davangere. Sections from formalin-fixed paraffin-embedded tissue blocks of 15 cases of normal oral mucosa (NOM), oral epithelial dysplasia (OED) related to carcinogen exposure and reactive lesions of the oral cavity with and without atypical epithelium were retrieved from the archives, and immunohistochemical staining using monoclonal antibodies to Bcl-2 protein was carried out.

Table 1: Average number of cells in the basal and parabasal layers in the study groups

Groups	*n*	Average total cells	Mean rank	χ^2	*P*
I	15	31.35	28.13	2.21	0.529 (NS)
II	15	29.52	20.27		
III	15	31.40	27.60		
IV	15	32.36	21.13		

NS: Nonsignificant



Graph 1: Average number of cells assessed in the basal and parabasal layers in the study groups

Methodology

5 μm thick sections were cut from each block using the soft-tissue microtome and were mounted on precoated slides. They were heated at 60° on the slide warmer table. After deparaffinization and hydration, sections were incubated in 0.01 M tris ethylenediaminetetraacetic acid buffer for antigen retrieval which was followed by immunohistochemical staining for Bcl-2 antibody. The stained sections were observed under trinocular research microscope. Ten fields were selected randomly and the images were captured using 3-chip charge-coupled device camera attached to the microscope [Figure 1]. The Bcl-2 expression was assessed in the basal and parabasal layer in each section of different study groups. The total number of cells in the basal and parabasal layer in each field and the total number of cells expressing Bcl-2 among them and the staining intensity were assessed. The analyzed data were collected and subjected to statistical analysis. The statistical analysis was done using SPSS (Statistical Package for the Social Sciences) software version 20.0 (IBM Inc, Chicago, Illinois,USA).

RESULTS

In our study, the immunohistochemical analysis for the expression of Bcl-2 antibody in the basal and parabasal layers in the above study groups was done which was in the form of granular cytoplasmic staining and as an accentuation around the nuclear membrane, and the expression of staining was assessed in the image analysis software.

Table 1 and Graph 1 show the average total number of cells assessed in the basal and parabasal layers in each group. The average number of cells in the basal and parabasal layers in Group I, II, III and IV was found to be 31, 29, 31 and 32 with a mean rank of 28.13, 20.27, 27.60 and 21.13, respectively. The data were analyzed using Kruskal–Wallis ANOVA and was found to be statistically nonsignificant with $P = 0.529$ and Chi-square value of 2.21.

Table 2 and Graph 2 show that the average percentage and average number of cells expressing Bcl-2 antibody in the

Table 2: Average positive cells in the basal and parabasal layers in the study group

Groups	*n*	Average number of positive cells	Average percentage of positive cells	Mean rank	χ^2	*P*
I	15	2.26	7.89	15.57	23.83	0.001
II	15	4.69	15.62	24.73		(HS)
III	15	8.38	26.34	38.03		
IV	15	9.66	30.98	43.57		

HS: Highly significant

Table 3: Intergroup comparison of average percentage of positive cells in the basal and parabasal layers

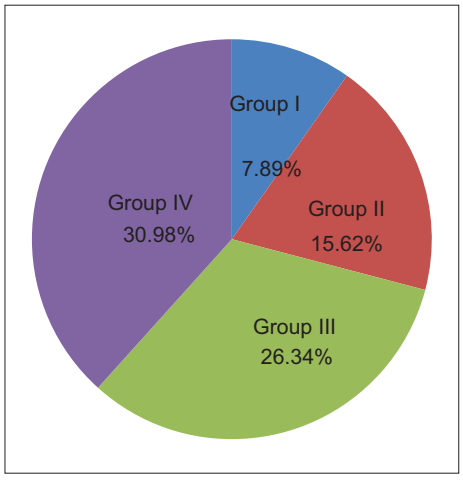
Groups	Mann-Whitney U-test value	*P*
I versus II	67.00	0.05 (S)
I versus III	27.00	0.0001 (HS)
I versus IV	21.00	0.0001 (HS)
II versus III	48 0.00	0.007 (S)
II versus IV	45 0.00	0.005 (S)
III versus IV	75.50	0.125 (NS)

NS: Nonsignificant, S: Significant, HS: Highly significant

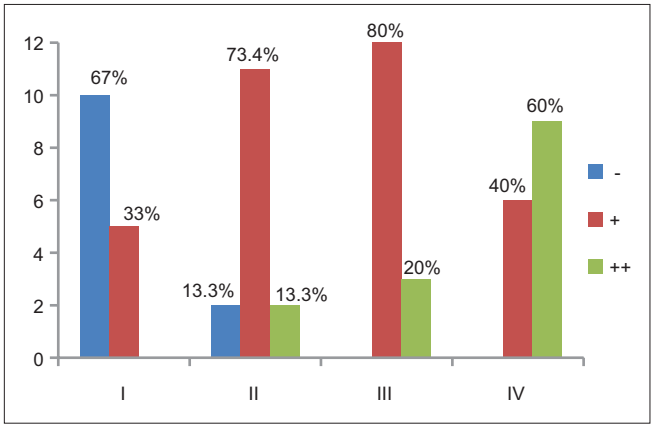
Table 4: Comparison of intensity of Bcl-2 staining among the study groups

Groups	*n*	–	+	++	χ^2	*P*
I	15	10 (67%)	5 (33%)	0 (0%)	28.468	0.001 (HS)
II	15	2 (13.3%)	11 (73.4)	2 (13.3%)		
III	15	0 (0%)	12 (80%)	3 (20%)		
IV	15	0 (0%)	6 (40%)	9 (60%)		
Total	60	12	34	14		

HS: Highly significant. –: Negative, +: Weakly positive, ++: Strongly positive



Graph 2: Average number of cells expressing Bcl-2 in the basal and parabasal layers in each group



Graph 3: Comparison of intensity of staining among the study groups

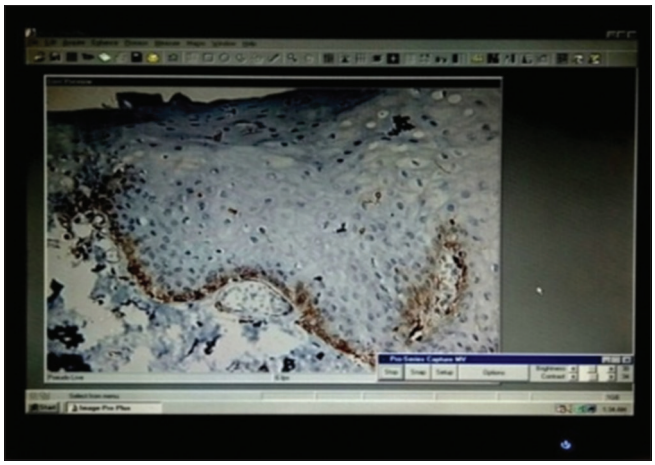


Figure 1: Image Pro Plus software used for capturing images

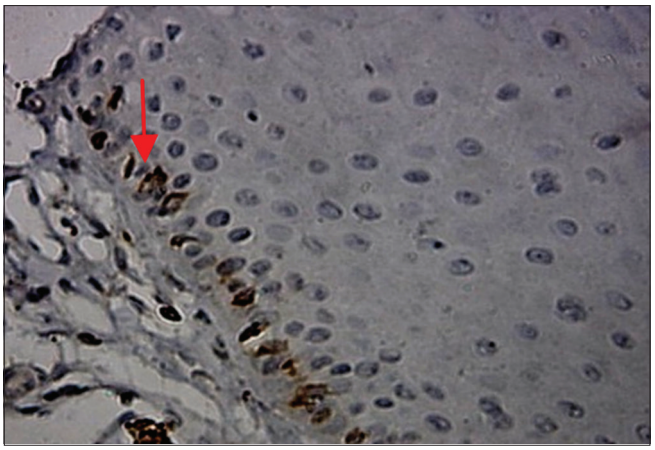


Figure 2: Bcl-2 expression in the basal layer of normal mucosa × 40

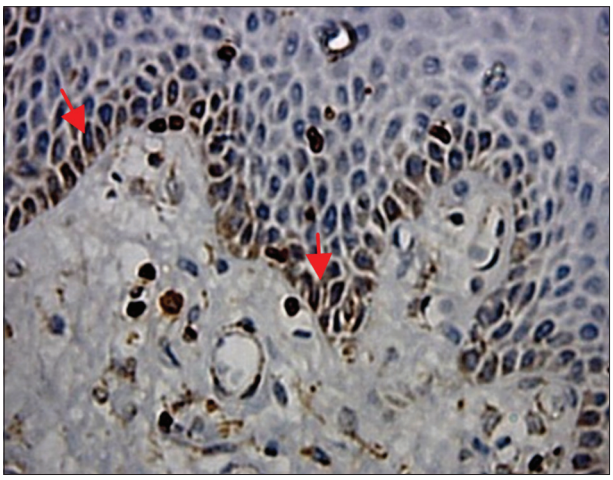


Figure 3: Bcl-2-stained section of reactive lesion without atypical epithelium (arrow showing positive cell in the basal layer) ×40

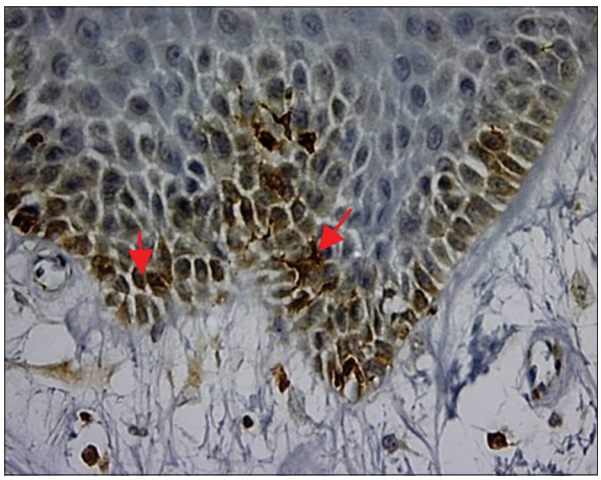


Figure 4: Bcl-2-stained section of reactive lesion with atypical epithelium (arrow showing positive cell in the basal and parabasal layers) ×40

basal and parabasal layers in Group I, II, III and IV were found to be 7.8% (2.26), 15.62% (4.69), 26.34% (8.38) and 30.98% (9.66), respectively. Kruskal–Wallis ANOVA test was used to compare the number of positive cells among the four groups, and statistically significant difference was found with $P = 0.001$ and Chi-square value of 23.830. This indicates that there was an increase in the expression of Bcl-2 in the basal and parabasal layers from Group I to Group IV.

Table 3 shows the comparison of average percentage of positive cells between the study groups. When Mann–Whitney U-test was applied for comparison, between Group I and II, a statistically significant difference was found ($P = 0.05$). Between Groups I and III and Groups I and IV, there was a statistically highly significant difference ($P = 0.0001$). Again, when Mann–Whitney U-test was applied between Groups II and III and II and IV, statistically significant difference was found ($P = 0.007$ and 0.005). There was no statistically significant difference between Groups III and IV.

Table 4 and Graph 3 show the comparison of intensity of staining among the study groups, which was graded as negative (−), weakly positive (+) and strongly positive (++).[7]

In Group I, among 15 samples, 10 samples (67%) were negative and 5 (33%) were weakly positive.

In Group II, of 15 samples, 2 samples (13.3%) were negative, 11 (73.4%) were weakly positive and 2 (13.3%) were strongly positive.

In Group III, of 15 samples, 12 (80%) were weakly positive and 3 (20%) were strongly positive.

In Group IV, of 15 samples, 6 (40%) were weakly positive and 9 (60%) were strongly positive. There was a gradual increase in the intensity of staining from Group I to

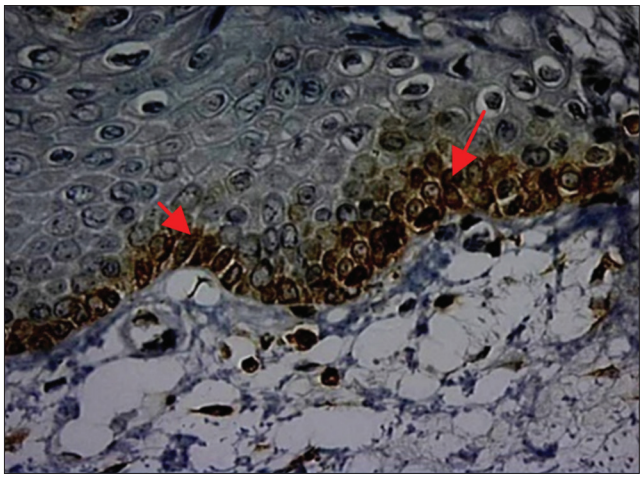


Figure 5: Bcl-2-stained section of epithelial dysplasia (arrow showing positive cell in the basal and parabasal layers) ×40

Group IV. On comparing these data using Pearson Chi-square test, the difference in intensity was found to be statistically highly significant ($P = 0.001$) with $\chi^2 = 28.5$.

DISCUSSION

There is an increased awareness of the importance of apoptosis in carcinogenesis. In normal proliferating epithelium, Bcl-2 is expressed in cell zones such as basal layers, where it acts to prevent the cell death in the regenerative compartment. Overexpression of Bcl-2 results in an alteration of programmed cell death with the persistence of cells that fail to die.[1]

Bcl-2 protein overexpression has been found in the early phase of epithelial carcinogenesis and also in precancerous lesion of the oral cavity. Overexpression of Bcl-2 protein has been demonstrated in the carcinomas of nasopharynx, lung, colorectum, esophagus and stomach, suggesting its association with early oncogenesis in these organs.

Although reactive lesions of the oral cavity are uncommonly associated with carcinogen exposure, at times, they present histopathologically with dysplastic epithelium, thus making it difficult to differentiate it from true potentially malignant disorders. Little is known about the presence of Bcl-2 protein in normal and reactive lesions of the oral cavity with and without atypical epithelium.

Hence, the purpose of our study was to evaluate the expression of Bcl-2 protein, an antiapoptotic marker, in reactive lesions with and without atypical epithelium and in true epithelial dysplasia, which clinically presents as potentially malignant disorders.

The study consisted of a total of 60 tissue blocks which were divided into four groups. The sections obtained from the blocks were subjected to immunohistochemical analysis using Bcl-2 monoclonal antibody. The expression of Bcl-2 was assessed in the basal and parabasal layers which presented as granular cytoplasmic staining and as an accentuation around the nuclear membrane.[6]

The expression of Bcl-2 protein in the study groups showed a gradual increase from Group I to Group IV, which was expressed in percentage as 7.8%, 15.62%, 26.34% and 30.98%, respectively, which was found to be statistically highly significant. This indicates the decrease in apoptosis of cells from Group I to IV.

In our study, NOM expressed Bcl-2 (7.8%) in the basal layer [Figure 2], which was similar to the study done by Suri,[6] Singh *et al*.,[7] Piattelli *et al*.[8] and Kummoona *et al*. (5%–24%, 2007).[9] Various studies done on uterine cervix, epidermis and other organs have also expressed Bcl-2 in the basal layers of normal epithelium. Studies with no basal cell staining in NOM were also reported by Thomas and Sethupathy.[10] The expression of Bcl-2 in the proliferative layer of the epithelium may protect it from apoptosis, thereby ensuring continuous supply of cells for differentiation.

In reactive lesions without atypical epithelium, the expression of Bcl-2 was mostly confined to the basal layers of epithelium (15.6%) [Figure 3], which was similar to the study by Núñez *et al*[11] on inflammatory fibroepithelial hyperplasia and Nakamura[12] on pyogenic granuloma, who suggested that antiapoptotic activity was higher when compared to proliferative activity as compared to NOM.

In our study, the reactive lesions with atypical epithelium showed increased expression of Bcl-2 (26.3%), seen both in basal and in few parabasal layers [Figure 4]. This expression of cells, both in the basal and parabasal layers, could be due to the increase in the number of basal cells as a result of injury or inflammation. Although these reactive lesions in the oral cavity occur in the mucosa as a result of injury or any local factors, at times, they may be associated with atypia which can be attributed to the progression of the lesion.

And also, on retrieving the history, majority of the cases were found to be associated with tobacco habits without any clinical potentially malignant disorders. This increase in the expression of Bcl-2 protein in these lesions could be attributed to the field injury, due to tobacco leading to chronic irritation and inflammation and causing changes at

the cellular level. To the best of our knowledge, literature research did not reveal any studies related to Bcl-2 expression in reactive oral lesions with atypical epithelium.

Increased expression was also observed in the studies done by Laban *et al*.[13] and Arjunan *et al*.[14] on endometrial hyperplasia. A gradual increase in the expression of Bcl-2 from normal endometrium, simple endometrial hyperplasia to complex hyperplasia and complex hyperplasia with atypia was observed. However, authors suggested that these findings could be due to the influence of hormonal stimulation which may have more antiapoptotic activity and also suggested that the patients with complex hyperplasia and atypical endometrial hyperplasia were more likely to develop endometrial carcinoma than those lesions without atypia.

The expression of Bcl-2 protein in OED with carcinogen exposure of our study was higher (30.9%) than any other group and was observed in the basal and parabasal layers [Figure 5]. This aberrant expression was also found in the studies done by Singh *et al*,[7] Nunez *et al*,[13] Juneja *et al*,[15] Bhattacharya *et al*[16] in the oral cavity. A similar increase in the expression was also observed by Walker *et al*.[17] on dysplastic bronchial epithelium, Kamaraddi *et al*.[18] on the cervix and Li *et al*.[19] on urothelial dysplasia. This abnormal expression could be because of the genetically modified tumor cells, contributing to the expansion of the damaged cell clone by preventing the cell turnover due to programmed cell death, leading to cellular immortalizations. It also suggests that overexpression of Bcl-2 in dysplastic lesions may have an important role in the progression of dysplasia to malignancy. However, studies showing decreased expression of Bcl-2 in moderate and severe dysplasia compared to normal and hyperplastic epithelium were also observed, and the authors suggested that the dysregulation of Bcl-2 gene may be one of the many genetic aberrations in the progression of epithelial tumors.[3]

On multiple comparisons between our study groups, a statistically significant difference was observed when Group I (7.8%) was compared with Group II (15.6%). This result was also coinciding with the study by Nunez *et al*[11] on NOM and inflammatory fibroepithelial hyperplasia also, Laban *et al*[13] and Arjunan *et al*[14] on the studies done comparing the normal endometrium, simple endometrial hyperplasia and complex endometrial hyperplasia without atypia.

Furthermore, when Group I (7.8%) was compared with Group III (26.3%) and Group IV (30.9%), a statistically highly significant difference was found, indicating a significant increase in the expression of Bcl-2 in reactive lesions with atypical epithelium and OED when compared with NOM indicating high antiapoptotic activity in these groups.

Similar results were observed in studies by Singh *et al*.,[7] Juneja *et al*.[15] and Sudha and Hemavathy,[20] where they found an increase in the expression of Bcl-2 in epithelial dysplasia as compared to NOM. This result was also in accordance with the studies on the bronchial epithelium, endometrium and urinary bladder by Walker *et al*.,[17] Arjunan *et al*.[14] and Kamaraddi *et al*.,[18] respectively.

Statistically significant difference was observed in the expression of Bcl-2 when Group II was compared with Group III and Group IV. These results were comparable with Núñez *et al*., as there was a significant increase in the expression of Bcl-2 in OED as compared to inflammatory fibroepithelial hyperplasia. The difference in the expression of Bcl-2 in our study could be due to chronic irritation, inflammation and tobacco exposure, causing changes at the cellular level in Groups III and IV.

Although there was a difference in the expression of Bcl-2 in reactive lesion with atypical epithelium (26.3%) and in OED with carcinogen exposure (30.9%), it was found to be statistically nonsignificant.

In our study, the staining intensity was evaluated and categorized as negative (−), weak positive (+) and strong positive (++), similar to that of Laban *et al*. on the endometrium.[9]

When the staining intensity of Bcl-2 protein was evaluated among the study groups, most of the cases of NOM (67%) showed negative staining (−) and 33% showed weak positivity (+). However, a study done by Thomas and Sethupathy[10] reported negative staining in all NOM samples.

In our study, reactive lesions without and with atypical epithelium, 73.4% and 80% showed weak positivity and 13% and 20% were strongly positive, respectively; also, 13.3% of reactive lesions without atypical epithelium showed negative staining.

In cases of OED associated with carcinogen exposure, 40% were weakly positive and 60% were strongly positive. Interestingly, both of the dysplastic groups (Groups III and IV) did not show any negative staining.

The increased number of cases showing the dark intensity of expression in reactive lesions with atypical epithelium and in OED with carcinogen exposure indicates the

increased activity of the Bcl-2 protein, reducing the apoptosis in dysplastic epithelium associated with habits when compared with other two study groups.

CONCLUSION

The increased expression of Bcl-2 (antiapoptotic protein), in reactive lesions with atypical epithelium, similar to that of OED associated with carcinogen exposure of our study may impose us to report the dysplasia observed in the reactive lesions and addresses the need to follow-up, similar to the OED lesions.

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Conflicts of interest
There are no conflicts of interest.

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