Original Article

# To analyse the mitotic and keratinisation correlation with *bcl-2* expression in varying grades of oral epithelial dysplasia and squamous cell carcinoma

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## Abstract

**Context:** The *bcl-2* proto-oncogene was discovered at the chromosomal breakpoint of t (14;18) found in follicular lymphoma. Histological changes in dysplasia are considered the earliest signs preceding the progression into squamous cell carcinoma. Serving as critical regulators of apoptotic pathways, *bcl-2* prohibits programmed cell death and subsequently assists in uncontrolled neoplastic growth.

**Settings and Design:** This study included 48 cases, eight each of epithelial dysplasia and squamous cell carcinoma. Immunohistochemical staining using *bcl-2* antibody was performed and different histological parameters were correlated with *bcl-2* positive cells in all the cases.

**Materials and Methods:** All 3 $\mu$m thick sections were stained with *bcl-2* antibody. After identifying four representative fields at 40x, their images were obtained for assessment of *bcl-2* labelled cells and their intensity along with different histological parameters in all the cases.

**Statistical Analysis:** The differences between different histological parameters were analysed using the Anova test, post hoc test and Bonferroni test. Pearson's Chi-square test was carried out to determine the level of correlation between the *bcl-2* positive cells in both epithelial dysplasia and oral squamous cell carcinoma cases.

**Conclusion:** Sequential increase in the bcl-2 expression was observed in increasing grades of epithelial dysplasia, whereas bcl-2 expression was significantly decreased in ascending stages of squamous cell carcinoma thus, suggesting a possible role of bcl-2 in disease progression from premalignancy to malignancy.

**Keywords:** Apoptosis, *bcl-2*, epithelial dysplasia, immunohistochemistry, oral squamous cell carcinoma

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## INTRODUCTION

Currently, oral cancer is the sixth most common type of cancer worldwide and is one of the most alarming health problems facing mankind.[1] Sometimes, due to genetic and epigenetic aberrations, certain progressive changes in cellular behaviour from slightly deregulated proliferation to full malignancy are observed.[2] Thus, cells with damaged genomes would not undergo apoptosis, allowing the defective genome to persist and replicate in the offspring cells.[3]

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The *bcl-2* gene family plays an important role in the regulation of apoptosis and modulation of cell cycle regulating proteins, illustrating the cross-play in mechanisms controlling cell death and proliferation.[4,5] The founding member of the *bcl-2* family of apoptosis-regulating proteins is B-cell lymphoma/leukemia-2 (*bcl-2*), which gets its name because of its involvement in B-cell lymphomas and leukemias.[6] The *bcl-2* proto-oncogene was discovered at the chromosomal breakpoint of t (14;18) found in human follicular lymphoma. The *bcl-2* oncoprotein is thought to regulate programmed cell death and facilitate cell survival and is associated with the mitochondrial membrane, nuclear envelope, and endoplasmic reticulum.[7]

The prognosis of oral cancer vastly depends upon the characterisation of the disease. Resultant genomic aberrations could help in the evaluation of high-risk premalignant lesions and their propensity for malignancy as molecular changes occur before histological manifestations.[8] The diagnosis of epithelial dysplasia is a subjective assessment of the discrepancy of epithelial maturation patterns and a variety of cellular changes implying an increased risk of malignant transformation that is relative to the grade of dysplasia.[9]

The present study is aimed to study the expression of *bcl-2* in epithelial dysplasia as well as in squamous cell carcinoma to search for the possible role of *bcl-2* in modulating the staging and the pathogenesis of squamous cell carcinomas. This study also aims at analysing the link between *bcl-2* and the malignant transformation of epithelial dysplasia into squamous cell carcinoma.

## MATERIALS AND METHODS

The study comprised of 48 cases of formalin-fixed, paraffin-embedded blocks, retrieved from the archives of the oral pathology department of I.T.S Centre for Dental Studies and Research, Muradnagar. The selected specimens consisted of 24 cases each of different grades of oral epithelial dysplasia and squamous cell carcinoma which were stained for *bcl-2* using the immunohistochemical method. Deparaffinization of 3-μm tissue sections was obtained by dipping in changes of xylene, then rehydrated in decreasing alcohol grades, and washed. The antigen retrieval method was conducted in two cycles further followed by *bcl-2* immunohistochemical staining. The lymph node was taken as a positive control for the expression of *bcl-2* and immuno-stained in the same manner as other study cases.

All the slides were viewed under a light microscope. The positively stained *bcl-2 cells* showed the uptake of brown colour. Evaluation of immunohistochemical staining intensity was conducted manually and was further compared in all epithelial dysplasia and squamous cell carcinoma cases. Staining intensity was categorized as a mild or intense expression using the intensity of the positive control as the benchmark.

### Selection of field for counting cells

From all positively stained areas, four representative fields at 40x were selected and counted that were not in continuum with each other to minimise any possible errors. The images of each of the four fields were obtained by binocular light microscope (40x) and then transferred to a grid for analysis. The immunoreactive score was then obtained by the assessment of *bcl-2* labelled cells and their intensity along with different histological parameters in all the cases.

### Statistical analysis

SPSS software was used for statistical analysis and the differences between different variables were analysed using the Anova test and post hoc test, followed by the Bonferroni test. Pearson's Chi-square test was carried out to determine the level of correlation between the study groups. The significance i.e., $P$ *value* $< 0.05$, was considered to be significant.

## RESULTS

As the epithelial dysplasia grade increased, a significant sequential increase in the *bcl-2* expression was observed which was lowest in mild dysplasia, followed by a gradual increase in moderate dysplasia and maximum in severe dysplasia ($p < 0.05$) [Figure 1]. An increase in differentiating grades of squamous cell carcinoma resulted in a decrease in *bcl-2* expression which was statistically significant except for two cases with an absence of *bcl-2* expression ($p < 0.05$) [Table 1].

The qualitative interpretation of the *bcl-2* expression in mild, moderate, and severe dysplasia was non-significant ($p > 0.05$) [Table 2A]. Similarly, the difference in *bcl-2* intensity expression in well-differentiated squamous cell carcinoma (WDSCC) moderately differentiated squamous cell carcinoma (MDSCC), and poorly differentiated squamous cell carcinoma (PDSCC) was non-significant ($p > 0.05$) [Table 2B].

When *bcl-2* expression was assessed with the histological parameter of mitosis in both epithelial dysplasia and squamous cell carcinoma (SCC) cases, it did not show any significant correlation. Out of eight cases of WDSCC, three showed the presence of mitosis while in five cases it was absent. Thus, the resultant $P$ value

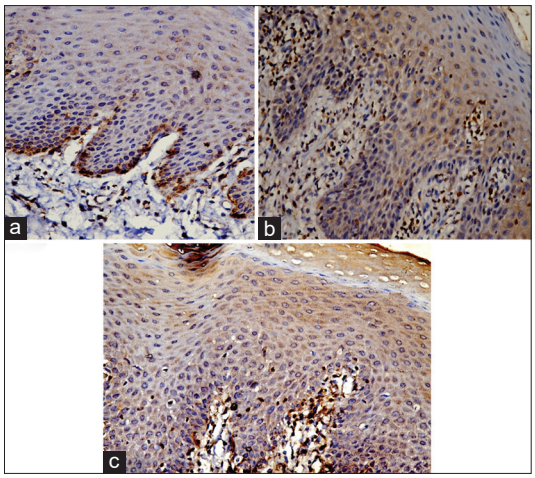


**Figure 1:** - (a) Mild epithelial dysplasia showing *bcl-2* nuclear and cytoplasmic staining in the lower one-third of the epithelium (×40). (b) Moderate epithelial dysplasia showing nuclear and cytoplasmic *bcl-2* staining until two-thirds of the epithelium (×40). (c) Severe epithelial dysplasia showing nuclear and cytoplasmic staining of *bcl-2* throughout the epithelium with less immunopositivity in the superficial layer (×40)

was non-significant ($p > 0.05$). The resultant *P* value was significant in MDSCC as out of eight cases, seven cases had the presence of mitosis while one was negative. Similarly, all eight cases of poorly differentiated squamous cell carcinoma showed the presence of mitosis and the *P* value for this grade was statistically significant ($p < 0.05$). Thus, mitotic activity was maximum in poorly differentiated type followed by moderately differentiated squamous cell carcinoma and least in well-differentiated carcinoma. Out of eight cases of mild dysplasia, four showed the presence of mitosis while in five cases it was absent, whereas in eight cases of moderate dysplasia, four cases had the presence of mitosis while the rest four were negative for it. Similarly, eight cases of severe dysplasia resulted in six cases being positive for mitosis, and two cases showed its absence. The resultant *P* value in all the three grades of dysplasia was not statistically significant (p > 0.05) [Table 3].

Out of eight cases, each of mild and moderate dysplasia, individual cell keratinisation, was not observed, whereas out of eight cases of severe dysplasia, only three cases displayed individual cell keratinisation. Thus, the resultant value was statistically non-significant ($p > 0.05$). Out of eight WDSCC cases, all cases showed the presence of keratin pearls, whereas in eight cases of MDSCC, only six cases had the presence of keratin pearls while eight cases of PDSCC did not show any evidence of keratin pearls. The resultant *P* value was highly significant ($p < 0.05$) [Table 4]. The evaluation of the *bcl-2* expression in different locations in epithelial dysplasia showed a significant sequential increase in *bcl-2* expression from the basal layer to the superficial layer [Table 5A], whereas, in squamous cell

**Table 1: Comparative quantitative assessment of *bcl-2* expression in varying grades of oral epithelial dysplasia and squamous cell carcinoma**

| *bcl-2* expression | Mean | Standard deviation | *P* |
|---|---|---|---|
| Mild dysplasia (*n*=8) | 35.67 | ±19.07 | 0.000(S) |
| Moderate dysplasia (*n*=8) | 53.13 | ±8.14 | Significant |
| Severe dysplasia (*n*=8) | 85.19 | ±2.61 | |
| WDSCC (*n*=8) | 66.37 | ±13.69 | 0.006(S) |
| MDSCC (*n*=8) | 75.23 | ±9.24 | Significant |
| PDSCC (*n*=8) | 84.95 | ±5.04 | |

**Table 2A: Qualitative assessment of *bcl-2* expression in varying grades of oral epithelial dysplasia**

| Staining intensity | GROUP | | | Total | *P* |
|---|---|---|---|---|---|
| | Mild | Moderate | Severe | | |
| Mild | | | | | 0.42 (NS) Non Significant |
| Count | 4 | 1 | 3 | 8 | |
| % within GROUP | 50.0% | 12.5% | 37.5% | 33.3% | |
| Intense | | | | | |
| Count | 4 | 7 | 5 | 16 | |
| % within GROUP | 50.0% | 87.5% | 62.5% | 66.77 | |
| Total | | | | | |
| Count | 8 | 8 | 8 | 24 | |
| % within GROUP | 100.0% | 100.0% | 100.0% | 100.0% | |

**Table 2B: Qualitative assessment of *bcl-2* expression in varying grades of squamous cell carcinoma**

| Staining intensity | GROUP | | | Total | *P* |
|---|---|---|---|---|---|
| | WDSCC | MDSCC | PDSCC | | |
| No Stain | | | | | 0.866 (NS) Non Significant |
| Count | 2 | 0 | 0 | 2 | |
| % within GROUP | 25.0% | 0.0% | 0.0% | 8.3 | |
| Mild | | | | | |
| Count | 2 | 3 | 3 | 8 | |
| % within GROUP | 25.0% | 37.5% | 37.5% | 33.3% | |
| Intense | | | | | |
| Count | 4 | 5 | 5 | 14 | |
| % within GROUP | 50.0 | 62.5 | 62.5 | 58.4 | |
| Total | | | | | |
| Count | 8 | 8 | 8 | 24 | |
| % within GROUP | 100.0% | 100.0% | 100.0% | 100.0% | |

carcinoma, entire islands and peripheral island cells having central keratinisation core showed *bcl-2* expression in well and moderately differentiated squamous cell carcinoma [Figure 2a, 2b] and PDSCC showed *bcl-2* expression throughout the sheet pattern [Figure 2c]. Two cases of WDSCC showed negative *bcl-2* expression which was non-significant [Table 5B]. In the present study, the mean *bcl-2* count in epithelial dysplasia was comparatively higher (mean-736.42) when compared with OSCC (mean-703.2) [Table 6].

## DISCUSSION

In our study, it was observed that as the grade of epithelial dysplasia increased, there was a gradual increase in *bcl-2* expression which was significant ($p < 0.05$-Table 1). These findings were also corroborated by Pallavi *et al.*, Singh *et al.*,

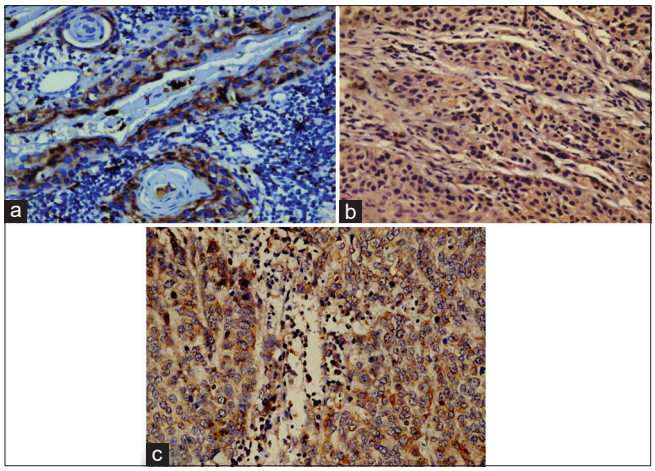


**Figure 2:** - (a) Tumour islands of WDSCC showing *bcl-2* expression at the periphery and diminished expression at the center (×40). b) Tumour islands of MDSCC showing cytoplasmic and nuclear expression of *bcl-2* (×40). (c) PDSCC showing sheets of tumour cells having *bcl-2* positivity in both cytoplasm and nucleus (×40)Table 3: Quantitative assessment of the presence of mitosis in different grades of epithelial dysplasia and squamous cell carcinoma

and Juneja *et al.* in their respective studies.[9-11] Similarly, sequential grades of SCC demonstrated an increased expression of *bcl-2* which was again significant [Table 1] and was consistent with the findings of Singh *et al.*, *Chen et al.,* and Suri *et al.*[10,12,13] Hence, this concept could be put forth that the *bcl-2* oncoprotein expression is inversely proportional to the degree of keratinisation/differentiation as suggested by Jordan *et al.*[14] and Nitya K *et al.*[15] that this up-regulation allows a selective growth advantage to the dysplastic cells by prolonging cell survival, thus leading to malignancy. This result is contradictory to the findings by Loro *et al.*[16] who observed that *bcl-2* expression was suppressed in PDSCC and stated that this difference might be topographical or because of different laboratory protocols and antibodies used in various studies.

The qualitative *bcl-2* expression evaluation in epithelial dysplasia showed that 16 cases were intensely stained while eight cases stained mildly [Table 2A]. It was evident that an increase in epithelial dysplasia grades resulted in an escalation of *bcl-2* staining intensity, thus indicating that this oncoprotein may play a key role in relative early events in the development and progression of oral neoplasia.[10]

Qualitative assessment of all squamous cell carcinoma cases revealed that as the degree of differentiation decreased, there was an increase in the staining intensity except for two cases of well-differentiated squamous cell carcinoma, which did not take up the stain [Table 2B]. The *bcl-2* expression in the majority of moderately and poorly differentiated squamous cell carcinoma cases was intense which was

**Table 3: Quantitative assessment of the presence of mitosis in different grades of epithelial dysplasia and squamous cell carcinoma**

| Group | Count | % of cells | Count | % of cells | Count | % of cells | *P* |
|---|---|---|---|---|---|---|---|
| Mild Dysplasia | 3 | 37.5% | 5 | 62.5% | 8 | 100% | 0.024(S) |
| Moderate Dysplasia | 4 | 50.0% | 4 | 50.0% | 8 | 100% | Significant |
| Severe Dysplasia | 6 | 75.0% | 2 | 25% | 8 | 100% | |
| WDSCC | 3 | 37.5% | 5 | 62.5% | 8 | 100% | 0.001(S) |
| MDSCC | 7 | 87.5% | 1 | 12.5% | 8 | 100% | Significant |
| PDSCC | 8 | 100% | 0 | 0.0% | 8 | 100% | |

**Table 4: Quantitative assessment of individual cell keratinization in all grades of epithelial dysplasia and presence of keratin pearls in all grades of squamous cell carcinoma**

| Group | Count | % of cells | Count | % of cells | Count | % of cells | *P* |
|---|---|---|---|---|---|---|---|
| Mild Dysplasia | 0 | 0.0% | 8 | 100% | 8 | 100% | 0.083(NS) |
| Moderate Dysplasia | 0 | 0.0% | 8 | 100% | 8 | 100% | Non Significant |
| Severe Dysplasia | 3 | 37.5% | 5 | 62.5% | 8 | 100% | |
| WDSCC | 8 | 100% | 0 | 0.0% | 8 | 100% | 0.000(S) |
| MDSCC | 6 | 75% | 2 | 25% | 8 | 100% | Significant |
| PDSCC | 0 | 0.0% | 8 | 100% | 8 | 100% | |

**Table 5A: Quantitative assessment of *bcl-2* expression in different locations in all grades of epithelial dysplasia**

| *bcl-2* staining | GROUP | | | Total cases | *P* |
|---|---|---|---|---|---|
| | Mild | Moderate | Severe | | |
| Only Basal | 7 | 6 | 0 | 13 | 0.000(S) |
| Count | 87.5% | 75.0% | 0.0% | 54.2% | Significant |
| % within GROUP | | | | | |
| Suprabasal | | | | | |
| Count | 1 | 1 | 1 | 3 | |
| % within GROUP | 12.5% | 12.5% | 12.5% | 12.5% | |
| Superficial | | | | | |
| Count | 0 | 1 | 7 | 8 | |
| % within GROUP | 0.0% | 12.5% | 87.5% | 33.3% | |
| Total | | | | | |
| Count | 8 | 8 | 8 | 24 | |
| % within GROUP | 100.0% | 100.0% | 100.0% | 100.0% | |

confirmed by Nair *et al.*[17] that this upregulation of *bcl-2* may reflect the lost ability of malignant keratinocytes for terminal differentiation and suggested that those cells overexpressing *bcl-2* have a stem cell phenotype. The finding of negative *bcl-2* staining has also been substantiated by Kannan *et al.*[18] who demonstrated an inverse correlation between p53 and *bcl-2* expression in oral squamous cell carcinoma, suggesting that one of these proteins could substitute the other during carcinogenesis and that the p53 gene can downregulate the *bcl-2* gene during the apoptotic process.

We also observed that when epithelial dysplasia was correlated with the presence of mitosis in both basal and suprabasal layers, a greater number of mitotic figures were seen as the grade of dysplasia increased from mild

**Table 5B: Quantitative assessment of *bcl-2* expression in different locations in all grades of squamous cell carcinoma**

| *bcl-2* staining | GROUP | | | Total cases | *P* |
|---|---|---|---|---|---|
| | WDSCC | MDSCC | PDSCC | | |
| Entire island | | | | | 0.304 (NS) |
| Count | 5 | 7 | 1 | 13 | Non Significant |
| % within GROUP | 62.5% | 87.5% | 12.5% | 54.17% | |
| Periphery | | | | | |
| Count | 1 | 1 | 0 | 2 | |
| % within GROUP | 0.0% | 0.0% | 87.5% | 29.17% | |
| No stain | | | | | |
| Count | 2 | 0 | 0 | 2 | |
| % within GROUP | 25.0% | 0.0% | 0.0% | 8.33% | |
| Total | | | | | |
| Count | 8 | 8 | 8 | 24 | |
| % within GROUP | 100.0% | 100.0% | 100.0% | 100.0% | |

**Table 6: Quantitative assessment of total *bcl-2* expression in epithelial dysplasia and squamous cell carcinoma**

| Lesion | Mean | Std deviation | *P* |
|---|---|---|---|
| Epithelial dysplasia | 736.42 | ±360.6 | 0.775 (NS) |
| Squamous cell carcinoma | 703.2 | ±421.01 | Non Significant |

to severe, which was significant ($p < 0.05$-Table 3). It was suggested that loss of *bcl-2* in oral keratinocytes may be involved in dysplastic and malignant progression of oral epithelium by making oral keratinocytes more responsive to mitotic stimuli.[19] In squamous cell carcinoma cases, fewer abnormal mitoses were evident as the grade of differentiation increased [Table 3]. Another observation in our study was, as the number of mitoses increased due to the progression of the disease, that there was a simultaneous increase in the intensity of *bcl-2* expression. The significant increase in numbers of mitoses from normal to dysplasia to carcinomas possibly represents increased stem cell turnover, thus leading to early invasive tumour development.[4,20]

Out of the many dysplastic features, one essential component for malignant transformation is the presence of individual cell keratinisation.[9,21] As the disease progressed from mild to moderate dysplasia, individual cell keratinisation was absent except in three cases of individual cell keratinisation evident in severe dysplasia [Table 4], which suggested that as the dysplastic grade increases, it leads to increased *bcl-2* expression, thus enhancing its further malignant transformation potential. It was also evident that as the degree of differentiation in squamous cell carcinoma increased, there was a simultaneous increase in the number of keratin pearls which was significant [Table 4].

Our findings suggest that as the grade of dysplasia increased, it resulted in a sequential increase in the level of *bcl-2* expression from the lowermost basal layer up till the superficial layer which was significant [Table 5A]. The expression of *bcl-2* in superficial layers proposed a modulation of its expression due to the binding of *bcl-2* to other cellular proteins as the *bcl-2* expression is only confined to the basal cell layer.[13] The present study noted that the entire islands devoid of central keratinised core showed *bcl-2* positivity, whereas the tumour islands having central keratinisation stained only in the peripheral cells with very less or no immunopositivity in the centre. Cases of poorly differentiated squamous cell carcinoma showed immunopositivity throughout in the pattern of sheets [Table 5B]. Similar results were found by Suri *et al.*[13] and Sudha *et al.*[22] that this overexpression possibly reflected the resistance of these tumour cells to apoptosis and the loss of ability of malignant keratinocytes to differentiate terminally. In contrast to our study, Teni *et al.*[23] reported that in well and moderately differentiated cases, *bcl-2* staining was restricted to the cells within the centre of tumour islands.

In our study, it was reflected that the mean *bcl-2* expression was higher in epithelial dysplasia than in squamous cell carcinoma [Table 6]. Pallavi *et al.*[9] showed similar findings that *bcl-2* immunoreactivity was more in epithelial dysplasia and had diminished activity in squamous cell carcinoma cases. Positive *bcl-2* expression was also perceived in the endothelial cells lining the blood vessels that might be due to increased production of interleukin-8 (IL-8) resulting in enhanced trans-membrane endothelial cell permeability, thus promoting tumour angiogenesis.[24] Our study revealed that the expression of *bcl-2* was directly proportional to the degree of dysplasia, whereas an inverse correlation was found between *bcl-2* positivity and degree of differentiation in cases of squamous cell carcinoma, thus probably contemplating that *bcl-2* may play an important role in early events of tumour progression. In previous studies, only quantitative and qualitative analysis of *bcl-2* has been done in epithelial dysplasia and squamous cell carcinoma cases, but this is the first study that has attempted to correlate the histopathological parameters along with quantitative and qualitative analysis of *bcl-2* in varying grades of both the lesions.

## CONCLUSION

Additional follow-up studies are the need of the hour to extract the intricate molecular mechanisms substantiated with other molecular markers in predicting the sequential progression from premalignancy to malignancy which might be instrumental in advocating the use of *bcl-2* judiciously and could further pinpoint and emphasize the

role of *bcl-2* in the pathogenesis and progression model of oral squamous cell carcinoma.

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## Conflicts of interest

There are no conflicts of interest.

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