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# Importance of bcl2 protein expression in histological negative margins after excision of oral squamous cell carcinoma

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## Abstract

***Background:*** B cell lymphoma-2 (bcl-2) is an intercellular membrane associated protein that functions as anti apoptotic protein via mitochondria mediated intrinsic pathway of caspase activation. We evaluated expression of bcl2 protein in patients with oral cancer and in adjacent normal tissue.

***Patients and methods*:** Bcl-2 protein expression was evaluated in patients with oral cancer undergoing surgical resection using immunohistochemistry (IHC) in the tumour tissue and the adjacent normal tissue.

***Results*:** Bcl2 protein expression was not seen in any tumour tissue however, in the normal margin 61 cases showed bcl2 expression. This expression was cytoplasmic in all the cases.

***Conclusions:*** This expression in margins correlated with lymphatic spread of the disease and suggest poor prognosis in this subset of the patients. The mRNA expression, polymorphism, mutations, SNP's were not looked at in the present study and hence, the exact significance of the results is not clear.

***Key words*:** apoptosis; oral cancer; tongue; buccal mucosa; alveolus; lymph node

## Introduction

B-cell lymphoma -2 (Bcl-2) is the second member of a range of proteins first discovered as the reciprocal gene translocation in chromosomes 14 and 18 locus in B-cell leukaemia. It is an intracellular membrane-associated protein that functions to block programmed cell death. Main role of Bcl-2 is in regulating a major apoptotic pathway [1]. This is the mitochondria-mediated or intrinsic pathway of caspase activation. The site of action for the Bcl-2 family is mostly on the outer mitochondrial membrane. Within the mitochondria are apoptogenic factors that if released activate the executioners of apoptosis, i.e. the caspases. Depending on their function, once activated, Bcl-2 proteins either promote the release of these factors, or keep them sequestered in the mitochondria [2].

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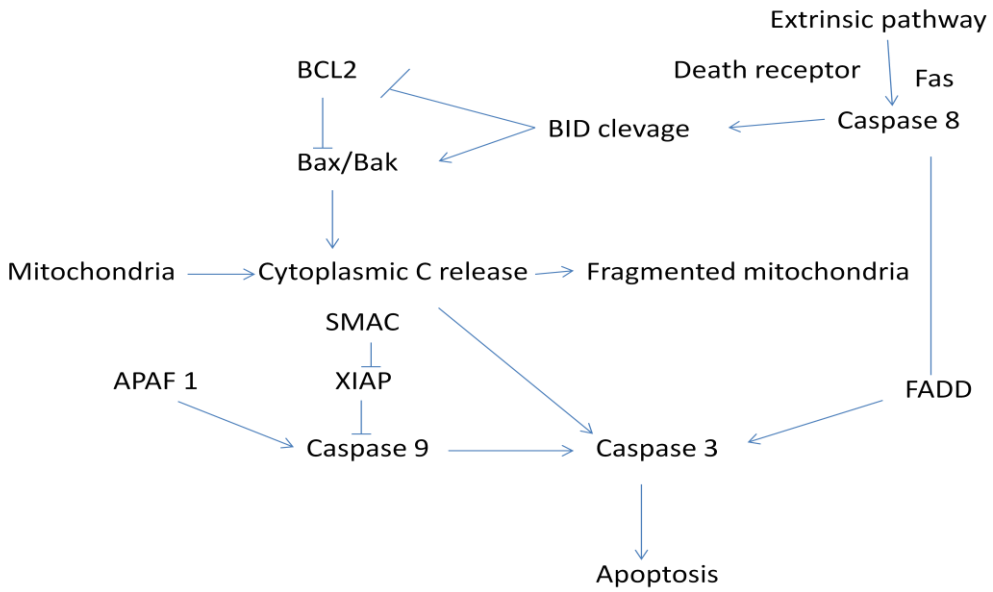


Figure 1: BCL2 apoptosis pathway and its regulation by intrinsic and extrinsic pathway

Bcl-2-regulate the pathway by controlling three major subgroups and the five pro-survival members, i.e. Bcl-2, Bcl-xL, Mcl-1, Bcl-w & A1. These are required for cell survival [3].

Antiapoptotic BCL2 and BCL-x(L) are antiproliferative by facilitating G0 phase of the cell cycle while BAX is proapoptotic and accelerates S-phase progression [3]. When pro-apoptotic family members such as Bax and Bak are activated (Figure 1) then pores in the outer membrane of the mitochondria opens, allowing the release of proteins that initiate apoptosis [4, 5].

Bcl-2 is a tumorigenic protein that is expressed in cancers. It is elevated in many cancers, including breast, colon, prostate, small cell lung cancer, chronic lymphocytic leukaemia and low-grade lymphomas .

Bcl-2 expression in oral cancer has been reported in 50% to 75% of cases. In normal oral mucosa, Bcl-2 is not detectable or is expressed only occasionally in the basal cells [6]. Teni T *et al.*[7], found over expression of tumour specific cytoplasmic Bcl-2 in 56% and bax in 43% oral cancers. Expression of Bcl-2 was seen in 16% premalignant oral lesions comprising leukoplakia's and submucous fibrosis while expression of Bax was seen in 55% [7].

Unlike lymphoma, in the oral cancer, the reason for over expression is unknown because there is no genetic rearrangement. More so, over expression of Bcl-2 is not a universal feature of cancer as it is not seen in malignant melanomas. However, when expression of Bcl-2 is inhibited, some cancer cells lose their malignant behaviour. Bcl2 over expression has been studied in the head neck cancer and pre cancer [2, 6, 8-12].

It has been found to dysregulate cell-cycle, predict the response to chemotherapy and has also been found to be a useful prognostic marker. We carried out this study to look at the expression profile of bcl2 in oral cancers and correlate it with pathological markers.

## Patients and Methods

This study was carried out in the Department of Surgical Oncology and Department of Pathology, Institute of Medical Sciences Banaras Hindu Varanasi

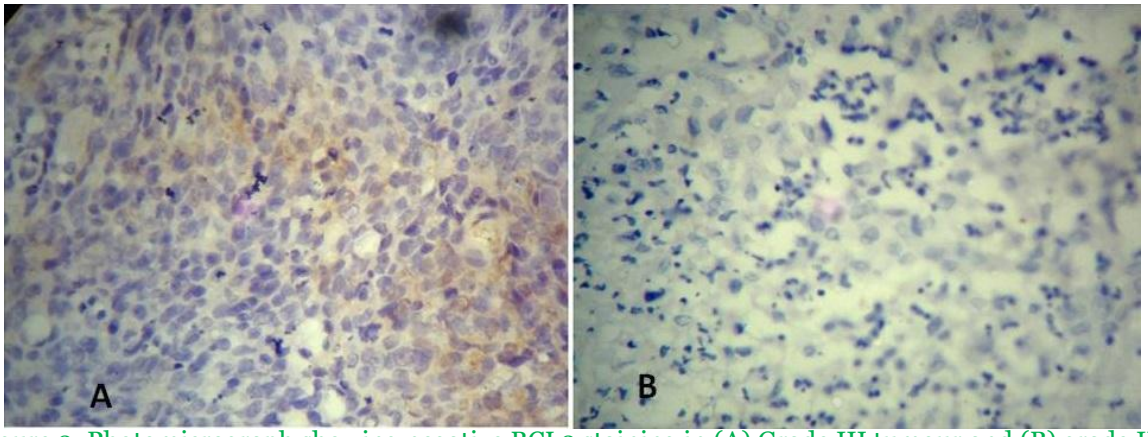

Figure 2: Photomicrograph showing negative BCL2 staining in (A) Grade III tumour and (B) grade II tumour.

between 2008 and 2012 with the approval of the Institute Ethics Committee. After obtaining written informed consent, tumour specimens from 100 patients oral Squamous cell carcinoma (OSCC) were collected along with the tissue from the margins at least 1 cm away from the primary tumours. Only patients diagnosed with primary OSCC without any prior treatment, where surgery as primary curative treatment was performed were included. Histological negative surgical margins were available from all 100 patients. Bcl-2 protein expression was determined by immunohistochemistry. The expression of Bcl-2 was also correlated with the grade of tumour, pathological lymph node metastasis and other findings in the specimens.

### *Immunohistochemistry*

Clean glass slides with 95% ethanol were treated with 1% poly-L-Lysin solution and were air dried. 4 micron thick tissue sections were cut using microtome and slides were placed in incubator at 60°C for one hour. All slides were deparaffinize using 3x- Xylene for 5 minutes each , 2x- 100% ethanol for 5minutes each , 2x- 95% ethanol 5 minute respectively . Slides were washed and excess liquid was drained. Antigen retrieval was done in sodium citrate buffer (pH 6.0) solution and heated the slides at 95°C for 10 minute in microwave oven.

To quench endogenous peroxidase activity, slides were incubated for 5 minutes in 1-3 drops of peroxidase block. Rinsed with PBS and were transferred to a PBS wash for 2 minute on stir plate. Thereafter the slides were incubated for 20 minutes in 1-3 drops serum block, and Primary antibody in 1: 200µl dilution was applied. Thereafter the slides were incubated over night at 40°C. 1-3 drops secondary antibody was applied and slides were incubated for 30 minutes. This was followed by incubation for 30 minutes in 1-3 drops HRP- Streptavidin complex. After the washing 1-3 drops of HRP substrate was added to each slide.

Counterstaining of slides was done with haematoxylin, followed by dehydration with 2x 95% ethanol for 10 second each , and 2x 100% ethanol for 10 second each and 3x xylenes for 10 seconds each and were mounted.

### *Grading of the bcl2 staining:*

All slides observed by light microscope and staining was graded as follows:- (i) Positive (ii) Negative (iii) Equivocal.

Beside the following were also recorded for each slide; type of staining :- (i) Cytoplasmic (ii) Nuclear (iii) Membranous and staining was graded as: (i) 1+(<25%) (ii) 2+ (25-50%) (iii) 3+ (51-75%) and (iv) 4+ (>75% cells stained).

**Table 1: Showing expression profile by various pathological parameters**

| Variable | Bcl-2 Positive Tumor Samples | Bcl-2 Negative Tumor Samples | Bcl-2 Positive Adjacent Tissue Samples | Bcl-2 Negative Adjacent Tissue Samples | P value |
|---|---|---|---|---|---|
| Overall-100 | | | | | |
| | 00 | 100 | 61 | 39 | - |
| Staining | Cytoplasmic 00 | -- | Cytoplasmic 61 | 100 | |
| | Nuclear-00 | -- | Nuclear-00 | -- | |
| | Membranous-00 | -- | Membranous-00 | -- | |
| Scoring | + =00 | -- | + =15 | -- | |
| | 2+ =00 | -- | 2+ =20 | -- | |
| | 3+ =00 | -- | 3+ =18 | -- | |
| | 4+ =00 | -- | 4+ =08 | -- | |
| Degree of differentiation | | | | | |
| Well differentiated | 00 | 100 | 30 | 50 | .76158 |
| Moderately differentiated | 00 | | 24 | 35 | |
| Poorly differentiated | 00 | | 07 | 15 | |
| Lymph Node Status | | | | | |
| Lymph node positive | 00 | 80 | 61 | 19 | <0.001 |
| Lymph node negative | 00 | 20 | 00 | 20 | |
| Sites | | | | | |
| Name of Site | Positive Cases | Negative Cases | Total cases | Positive Cases | Negative Cases | Total cases | .93317 |
| Buccal Mucosa | 00 | 44 | 44 | 29 | 15 | 44 | |
| Tongue | 00 | 12 | 12 | 7 | 5 | 12 | |
| Alveolus | 00 | 19 | 19 | 11 | 8 | 19 | |
| Lip | 00 | 14 | 14 | 8 | 6 | 14 | |
| GB sulcus | 00 | 11 | 11 | 6 | 5 | 11 | |
| Total Sites | 00 | 100 | 100 | 61 | 39 | 100 | |

### *Statistical analysis*

The data is presented as categorical variables and the statistical analysis was done by Chi Square test.

## Results

Of the 100 patients, 44 were of cancer of the buccal mucosa, 12 of tongue, 19 lower alveolus, 14 lip, and 11 gingivo-buccal complex. Bcl2 protein expression was not

seen in any tumour tissue (Figure 2) however, in the normal margin 61 cases showed bcl2 expression. This expression was cytoplasmic in all the cases. Of these, 29 were of buccal mucosa, 7 tongue, 11 alveolus, 6 GB sulcus and 8 cases were of lip. In 30 of these the tumours were well differentiated, 24 moderate differentiation and 7 were poorly differentiated. The bcl2 staining was seen only in cases with lymph node metastasis (61/80), the 20 patients without lymphatic spread did not show any positivity for bcl2 staining. This difference was statistically significant. The detail of the expression profile is given in table 1. Of the 61 cases, the 15 cases showed 1+, 20 showed 2+, 18 showed 3+, and 8 showed 4+ cytoplasmic positivity.

## Discussion

The Bcl2 family proteins comprise the sentinel network that regulate the mitochondrial or intrinsic apoptotic response [13]. The bcl2 expression in the cancers of the head neck including oral cavity has been studied [2, 8-10, 14-21]. The expression is seen in up to 40% of the cases and higher expressions have been reported in premalignant lesions like leukoplakia and submucous fibrosis. In the present study however, none of the tumour showed positivity for bcl2, while 61% of the normal margin tissue showed bcl2 expression. The expression was significantly found to correlate with aggressive disease i.e. patients who were cervical lymph nodes positive compared to those who were negative suggesting that bcl2 expression in the margins can be used as a marker of aggressive disease. Earlier studies too has found bcl2/bax ratio as predictor of aggressive disease [14].

Due to the sharing of the same microenvironment, adjacent normal tissue also may show molecular similarity to tumour tissues [22, 23], it has been shown that the adjacent tissue may produce growth factors and may also help modify the matrix thereby modifying the disease progression. Again in these situations expression of bcl2 and alteration of bcl2/bax ratio may play a very significant role [23].

The expression has also been correlated with the response to chemotherapy, radiotherapy and presence of human papilloma virus [8, 18, 19, 22, 24, 25]. Deng *et al*., showed lowering of expression in malignant tissues compared to that in normal tissues [21], similar to ours where malignant tissue showed no expression at all. Studies of Teni *et al*., [7] has suggested that bcl2 expression is an early even in oral carcinogenesis and hence, it is possible that in our cases expression was not seen as most cases were advanced. Crowe *et al*., showed that the response of p53 is mediated by bcl2 in oral cancer cells [26]. Similarly, expression of bcl2 has been found to increase after vitamin A therapy suggesting its potential role in carcinogenesis [20]. Studies have also looked at the bcl2 pleomorphism and BCL2 ala43ala genotype is found to be at increased risk for developing tumours [27], however BCL2 (-938 C>A) failed to show any association with carcinogenesis [28], but on the other had was found to effect overall and relapse free survival [12]. A few studies have also looked at bcl2 expression and human palilloma virus and have found poor prognosis in HPV +/BCL2- tumours [18]. A meta analysis on biological markers in oral cancer showed some improvement in survival in patients expressing bcl2, however, due to smaller studies and in absence of enough power no significance can be attached to these results [29].

## Conclusions

Results of the present study show that the tumours did not express bcl2 while 61% of the healthy margins expressed bcl2. This expression in margins correlated with lymphatic spread of the disease and suggest poor prognosis in this subset of the patients. The mRNA expression, polymorphism, mutations, SNP's were not looked at in the

present study and hence, the exact significance of the results is not clear. Further studies incorporating all aspects and up and downstream signalling are needed for definite conclusions.

## Authors' contribution

DKR- literature search, experiment and data collection

MK- Data collection, design and interpretation of results

MS- Interpretation of results and preparation of manuscript

MP- design of the study, conceiving of the idea and preparation of manuscript.

## Ethical considerations

The study was approved by the Institute ethics committee

## Funding

None

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