

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 immuno-histochemistry (IHC) in the tumor tissue and the adjacent normal tissue.

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

Conclusions:

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 leukemia. It is an intracellular membrane-associated protein that functions to block programmed cell death. Main role of Bcl-2 is 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, in 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].

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]. Anti-apoptotic BCL2 and BCL-x(L) are anti-proliferative by facilitating G0 phase of the cell cycle while BAX is pro-apoptotic and accelerates S-phase progression [3]. When pro-apoptotic family members such as Bax and Bak are activated then pores in the outer membrane of the mitochondria opens, allowing the release of proteins that initiate apoptosis [4, 5]. Bcl-2 is a tumori-genic protein that is expressed in cancers. It is elevated in many cancers, including

breast, colon, prostate, small cell lung cancer, chronic lymphocytic leukemia and low-grade lymphomas .

Bcl-2 expression in oral cancer has been reported at between 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 Tet al.[7], found over expression of tumor specific cyto-plasmic Bcl-2 in 56% and bax in 43% oral cancers. Expression of Bcl-2 was seen in in 16% premalignant oral lesions comprising leukoplakias and SMF and Bax 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] and has been found to dys-regulate cell cycle and predict the response to chemotherapy and has 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 between 2008 and 2012 after approval from the Institute Ethics Committee. After obtaining written informed consent, tumor 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 tumors. 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 immune-histochemistry. The expression of Bcl-2 was also correlated with the grade of tumor, 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 de-paraffinize 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 per-oxidase 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 were applied. Thereafter the slides were Incubated for over night at 40°C. 1-3 drops secondary antibody were 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 hematoxylin, 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).

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 tumor tissue 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 tumors 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 details 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 upto 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 tumor 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 tumors [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 Papilloma virus and have found poor prognosis in HPV +/BCL2- tumors [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 tumors 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

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None

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		.761589
Moderately differentiated	00				24		35		
Poorly differentiated	00				07		15		
Lymph Node Status									
Lymph node positive	00	80	P value = 1.0000 (It is not statistically significant)		61		19		<0.0001
Lymph node negative	00	20			00		20		
Sites									
Name of Site	Positive Cases	Negative Cases	Total cases	Positive Cases	Negative Cases	Total cases	.933174		
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			

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