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To Evaluate the Immunohistochemical Expression of Bcl2 and Bax in Oral Squamous Cell Carcinoma

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Abstract: The present study was carried out in the Department of Oral and Maxillofacial Pathology and Microbiology, Modern Dental College and Research Centre, Indore to evaluate the Immunohistochemical Expression of Bcl2 and Bax in Oral Squamous Cell Carcinoma. The mean number of cells expressing Bcl2 and Bax in different nodal status was found to be non-significant in Bax and significant for Bcl2. Increase mean number of cells in cases with nodal involvement demonstrates increase expression with more aggressive phenotype.
Keywords: Immunohistochemical, Bcl2, Bax & Oral Squamous Cell Carcinoma.

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the most prevalent pathologic type and accounts for over eighty percent of head and neck malignancies [1]. Carcinogenesis of OSCC is a multistage multi factorial process involving the activation of oncogenes and inactivation of tumor suppressor genes with imbalance of cell death and growth.

Apoptotic processes are of widespread biological significance, being involved in development, differentiation, proliferation/homoeostasis, regulation and function of the immune system and in the removal of defect and therefore harmful cells. Thus, dysfunction or deregulation of the apoptotic program is implicated in a variety of pathological conditions. Defects in apoptosis can result in cancer, autoimmune diseases and spreading of viral infections, while neurodegenerative disorders, AIDS and ischaemic diseases are caused or enhanced by excessive apoptosis [2].

Considerable interest has recently been focused on the identification of regulator of apoptosis that may be a potential and key influence on the balance of cell death and cell growth in cancer. Bax and Bcl-2 are two most commonly investigated effectors during apoptosis and previous studies have indicated the potential correlations between the expression of Bax and Bcl-2 and the prognosis in the patients with OSCC [3].

MATERIAL & METHOD

The present study was carried out in the Department of Oral and Maxillofacial Pathology and Microbiology, Modern Dental College and Research Centre, Indore after approval from institutional ethical committee.

The study material was obtained from the archivals of Oral Pathology Department, Modern Dental College and Research Centre, Indore as well as from Central Lab, Indore (courtesy of Dr Vinita Kothari, Managing Director Central Lab). The study sample comprised of ten normal oral mucosa cases and thirty known cases of oral squamous cell carcinoma. Study sample is described as follows.

1 Ten normal oral mucosa samples were obtained with informed consent from the individuals in the age group 18 to 45 years without any clinically observable lesion, that were undergoing minor oral surgical procedures like surgical removal of impacted third molar or alveoloplasty. Exclusion criterias followed were that the patient should not have any abusive habit or suffering from any other major illness.

2 Thirty known cases of oral squamous cell carcinoma, ten in each group as per the classification into well, moderate and poorly differentiated were selected. Detailed information about age, type and duration of habit, size of lesion, histopathologic grading, TNM staging and treatment with follow up as far as available was obtained.

All the biopsy specimens were routinely fixed in 10% neutral buffered formalin solution and embedded in paraffin. Three 4µm thick sections were taken, one on albumin coated slide for hematoxyline & eosin examination and two sections on silane coated for BAX and Bcl2 immunohistochemical examination.

Reagents

- Distilled Water
- Citrate Buffer (pH 6)
- Tris Buffer Saline (pH 6.4 to 7.6)
- Antigen retrieval Solution
- Isopropyl Alcohol
- Xylene
- Hematoxylin (Ehlrich's)
- DPX Mountant

Polymer Kit

- Peroxidase Block
- Polymer HRP reagent
- Substrate DAB Buffer
- Liquid DAB Chromogen

Antibody

Mouse Monoclonal antihuman antibody (Bcl2). Mouse polyclonal antihuman antibody (Bax)

Hematoxylin & Eosin Staining

Procedure

- The sections were dewaxed and hydrated through descending grades of alcohol to water.
- They were stained with Ehlrich's hematoxylin for 10 minutes.
- Then washed under running water for 5 minutes
- They were then differentiated with 1% acid alcohol by giving 2-3 dips.
- Again washed under running tap water until the sections were blue i.e. approximate1y 5minutes.
- They were stained with water soluble 1% eosin for 1 minute.
- After this, sections were washed well under running tap water for 1-5 minutes.
- This was followed by dehydration through ascending grades of alcohol.
- Next step was clearing in xylene and mounting with DPX.

RESULTS

Nuclei	:	Blue/ black
Cytoplasm	:	Varying shades of pink
Collagen	:	Pale pink to red
RBCs	:	Orange to red

The histopathological features of the stained slides were assessed under light microscope.

IMMUNOHISTOCHEMICAL METHOD

Principle

An enzyme labeled antibody is used to link a cellular antigen specifically to a chromogen that can be more readily visualized under light microscope.

Polymeric method

This technique permits binding of large number of enzyme molecules to a secondary antibody via the dextran backbone. It has benefits like increased sensitivity, minimal non specific background staining and reduction in total number of assay steps as compared to conventional technique.

Positive and Negative controls

Tonsil sample showing good Bcl2 labeling and Breast carcinoma showing good BAX labeling acted as a positive control. One section from each positive control was used as the negative control by omitting the primary antibody.

Protocol

- Application of peroxidase block: To avoid non-specific staining.
- Application of primary antibody: Binds to specific tissue antigens.
- Application of secondary antibody: Binds to the primary antibody; it is polyvalent antibody that will bind to primary antibodies derived from rabbit, mouse, rat and guinea pig.
- Application of substrate–chromogen: Results in the formation of a coloured precipitate at the tissue antigen sites. Visualization is aided by counter staining with hematoxylin.

Procedure

The tissues were fixed in 10% buffered formalin for 24 hours. The tissues were routinely processed and embedded in paraffin. 4 µm thick sections were prepared.

Adherence of sections to slides

APTES (3-aminopropyl triethoxy silane) (A-3648, Sigma-Aldrich, USA) coated slides (2% APES solution in acetone) were used for the proper adherence of tissue sections to the glass slides. Sections were floated on ATPES coated slides in a 45°C water bath. Sections were dried overnight at 37°C. Then sections were heated at 56°C for one hour.

The slides with tissue section were deparaffinized thoroughly with two changes of xylene for two minutes each.

They were then put in descending grades of alcohol for hydration i.e. 100%, 90% and 70% respectively for two minutes each.

Sections were then dipped in distilled water (one dip). After dipping in distilled water tissue section was transferred to TBS for 5 minutes. Sections were then transferred to coplin jar with antigen retrieval solution.

Antigen Retrival

Sections were then transferred to antigen retrieval solution i.e. Tris EDTA buffer pH 9 and heated in pressure cooker for 10 min (One whistle) for BAX, and at 80% microwave for 7 cycles of 3 minutes each for Bcl2.

- The slides were left to cool down at room temperature for 10-15 minutes.
- After removal from antigen retrieval solution, one dip with distilled water was given and then they were transferred to wash buffer i.e. Phosphate buffer saline (PBS) pH 7.4 for 5 minutes at room temperature.

Blocking of endogenous peroxidase

Slides are properly blotted with blotting paper without touching the tissue section. Peroxidase blocking was done with prediluted peroxidase blocking solution (Dako) for 10 minutes incubated at room temperature. This was to quench endogenous peroxidase activity of cells that would otherwise result in non-specific staining.

The sections were then washed with PBS and incubated at room temperature for 3 minutes.

Application of Primary antibody

Appropriate volume of prediluted primary antibody (BAX or Bcl2) was added to cover the section. Slides were kept in humidifying chamber and incubated for 30 minutes. The sections were then washed with PBS for 3 minutes each and blotted carefully.

EVALUATION

The Bcl-2 and Bax positive and negative controls were included with each set of immunohistochemical analysis.

A minimum of 100 cells from the positive stained area were selected and scored, five fields per slides were selected, and thus total 500 cells per slides were counted. Bcl-2 and Bax expression was estimated by intensity of the immunostaining with both the antibodies that were evaluated by dividing the staining reaction into four groups:

- no cytoplasmic staining intensity,
- weak cytoplasmic staining intensity,
- moderate cytoplasmic staining intensity,
- strong cytoplasmic staining intensity, and
- Very strong cytoplasmic staining intensity.

The quantity of the immunostaining was evaluated as follows

- no positive immunostaining,
- <25% of tumour cells showing cytoplasmic positivity,
- 25±50% of tumour cells showing cytoplasmic positivity,
- 50±75% of tumour cells showing cytoplasmic positivity,
- >75% of tumour cells showing cytoplasmic positivity.

A combined score for the immunostaining, based on both qualitative and quantitative immunostaining, was composed by adding both the qualitative and quantitative scores which were then divided into three main groups:
No immunostaining (±). Score 0,
Weak immunostaining (+). Scores 1±4,
Strong immunostaining (+ +). Scores 5±8.[82]

STATISTICAL ANALYSIS

The data was analysed using following statistical test: Analysis of varience (ANOVA), Student t test & Spermans correlation test – this test was used to compare mean number of cells expressing Bax & Bcl2 in different grades of OSCCS.

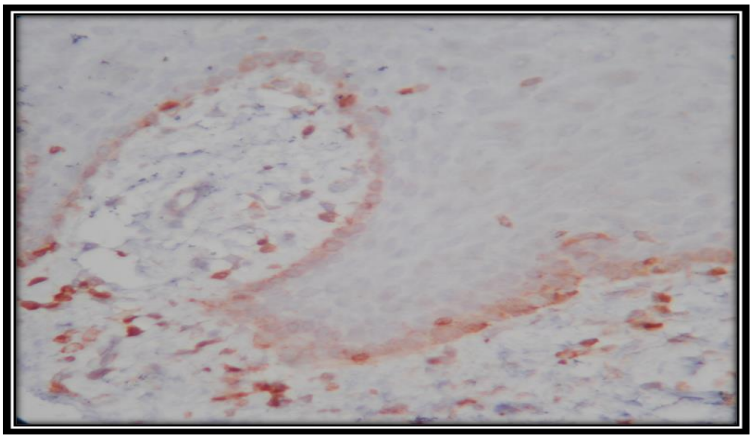

Fig-1: Photomicrograph showing Bcl2 staining in normal epithelium in basasl and supra basal cell layers(40X magnification)

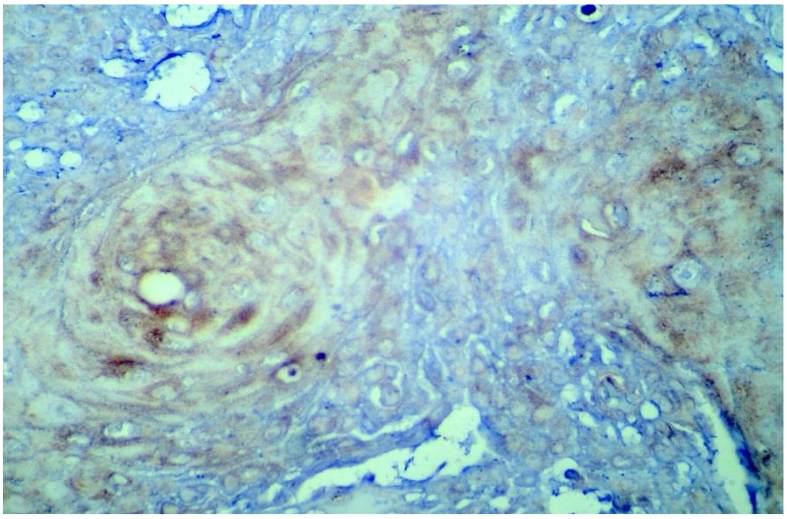

Fig-2: Photomicrograph showing Bax staining more at centre in MDSCC

RESULTS

Table-1: Distribution of the number of cases with expression of Bax in different grades of OSCCs

Grades of cases		WDSCC	MDSCC	PDSCC	TOTAL
NUMBER OF CASES		10	10	10	30
MEAN NUMBER OF POSITIVE CELL		71.80	75.40	49.60	65.60
STAINING INTENSITY	DARK	7	7	4	18
	LIGHT	3	3	6	12
STAINING LOCATION	Periphery/individual cell in center	7	2	0	9
	Full island	2	1	3	6
	sheet	1	7	7	15
	negative	0	0	0	0

Table 1 shows distribution of the number of cases with expression of Bax in different grades of OSCCs. Thirty cases of Oral squamous cell carcinoma that comprised of ten cases for each grade of OSCC i.e. well, moderate and poorly differentiated. Mean number of BAX positive cells for WDSCC, MDSCC and for PDSCC were 71.80 75.4 and 49.60 respectively. Total mean of 65.60 for positive stained cells was obtained. According to staining intensity 7 cases in WDSCC, 7 cases in MDSCC and 4 cases in PDSCC were darkly stained and 3 cases in WDSCC, 3 cases in MDSCC and six cases each in PDSCC were lightly stained. Based on staining location 7 case in WDSCC, 2 cases in MDSCC and none of the case in PDSCC was stained for cells in centre. 2 cases in WDSCC, 1 case in MDSCC and 3 in PDSCC were stained for fullisland. One case in WDSCC, seven cases in MDSCC and seven cases in PDSCC were stained for complete sheet. No case exhibited negative staining.

Table-2: Distribution of the number of cases with expression of Bcl2 in different grades of OSCCs

Grades of cases		WDSCC	MDSCC	PDSCC	TOTAL
NUMBER OF CASES		10	10	10	30
MEAN NUMBER OF POSITIVE CELL		41.60	43.20	36.40	40.40
STAINING INTENSITY	DARK	2	6	5	13
	LIGHT	7	0	0	7
STAINING LOCATION	Periphery/individual cell in center	1	0	1	2
	Full island	8	4	0	12
	sheet	0	2	4	6
	negative	1	4	5	10

Table 2 shows distribution of the number of cases with expression of Bcl2 in different grades of OSCCs. Thirty cases of Oral squamous cell carcinoma that comprised of ten cases for each grade of OSCC i.e. well, moderate and poorly differentiated. Mean number of Bcl2 positive cells for WDSCC, MDSCC and for PDSCC were 41.60, 43.20 and 36.40 respectively. Total mean of 40.40 for positive stained cells was obtained. According to staining intensity 2 cases each in WDSCC, 6 each in MDSCC and 5 cases in PDSCC were darkly stained and 7 cases in WDSCC, none of the case in MDSCC and PDSCC was lightly stained. Based on staining location 1 case in WDSCC, no case in MDSCC and PDSCC were stained for cells at periphery. 8 cases in WDSCC, 4 cases in MDSCC and no case in PDSCC were stained for full Island. No case in WDSCC, two cases in MDSCC and four cases in PDSCC were stained for complete sheet. One case in WDSCC, four cases in MDSCC and five cases in PDSCC showed negative staining.

Table-3: Comparison of number of cells expressing Bax in different grades of OSCCS

Grades of cases	N	Mean	Std. Deviation	F	Sig
Well	10	71.80	13.871	3.297	0.05
Moderate	10	75.40	18.957		
Poor	10	49.60	34.999		
Total	30	65.60	26.192		

$p \leq 0.05$= statistically significant

Table 3 shows distribution of cells expressing Bax in different grades of OSCC. The mean number of cells expressing Bax in WDSCC was 71.80 with standard deviation of 13.87, in MDSCC was 75.40 with standard deviation of 18.957 and in PDSCC was 49.60 with standard deviation of 34.99 were According to ANOVA there was statistically significant difference (P=0.05) in number of cells expressing Bax in different grades of OSCC.

Table-4: Comparison of number of cells expressing Bcl2 in different grades of OSCCs

Grades of cases	N	Mean	Std. Deviation	F	Sig
Well	10	41.60	27.913	.099	.906
Moderate	10	43.20	38.230		
Poor	10	36.40	39.811		
Total	30	40.40	34.583		

$p \leq 0.05$= statistically significant

Table 4 shows distribution of cells expressing Bcl2 in different grades of OSCC. The mean number of cells expressing Bcl2 in WDSCC was 41.60 with standard deviation of 27.91, in MDSCC was 43.20 with standard deviation of 38.23 and in PDSCC was 36.40 with standard deviation of 39.81. According to ANOVA there was statistically non-significant difference (P=0.906) in number of cells expressing Bcl2 in different grades of OSCC.

DISCUSSION

Understanding the inherent mechanism of carcinogenesis with involved molecular marker can help us in initiation of better treatment. Among the molecular mechanisms involved in carcinogenesis, defects in the regulation of programmed cell death (apoptosis) may contribute to the pathogenesis and progression of cancer. Programmed cell death is a critical step in cell differentiation and turnover and in tissue homeostasis. The best studied regulatory protein is Bcl2 family proteins. Bcl-2 family members are apoptosis regulatory proteins, which can homo- or heterodimerize with one another. The Bcl-2 family includes both anti-apoptotic (e.g. Bcl-2, Bcl-X) and pro-apoptotic proteins (e.g. Bax and Bak), and the balance between them determines the cell's fate [4].

Overexpression of Bcl2 can inhibit cell apoptosis and initiate carcinogenesis. Expression of Bcl2 in OSCC has been reported to vary. According to our present study Bcl2 positivity was reported in 20 out of 30 cases i.e. 66.6%.It was 57%, 47.27%, 20%, 86.8% , 27.1% and 100% according to Dijkema *et al*.[5]

The mean number of cells expressing Bcl2 was 41.60 ± 27.91, 43.20 ± 38.23 and 36.40 ± 39.81 in WDSCC, MDSCC and PDSCC respectively with an overall mean of 40.40±34.58. The number of cells expressing Bcl2 in different grades of OSCC was statistically non significant. (p=0.906)

Our finding of statistically non significant correlation of Bcl2 expression with histologic grade was similar to that observed by authors such as Teni *et al*. [6]

We observed slightly higher positivity in MDSCC as compared to PDSCC. Similar increase in Bcl2 positivity in MDSCC and less in PDSCC tumors have been observed by Teni *et al.* [6] and Malheiros *et al*. [7]. Further, it is found increased expression in certain precancerous lesions and corresponding decrease in carcinomas arising from these lesion e.g. uterine cervix, endometrium, stomach and large bowel.

The increased expression in moderately differentiated carcinomas could be because of the increased activity of the Bcl-2 protein during the early stages of tumor progression. Although intensity of staining in different grades of OSCC statistically significant for dark staining (13:7). There is a functional interaction between different members of Bcl-2 and also, because of the binding of this molecule to other cellular proteins, their intrinsic activity is modulated during the later stages of tumorigenesis. The differential staining obtained in poorly differentiated OSCC in our study could be because of elective caspase activation, resulting in the proteolytic degradation of bcl-2 in tumor cells [8].

CONCLUSION

No significant correlation could be established between Bax and Bcl2 in the study performed. Bcl2 family proteins decide the fate of lesion towards carcinogenesis and prognosis with advanced pathologic and histologic grade of the disease, still it remains uncertain because of variable factors such as due to the limitations of immunohistochemistry, in particular that it is not a functional assay, loss of Bcl-2 and Bax in OSCC progression could not be a attributed to mutations in the coding regions of these genes but may be the result of transcriptional or post-transcriptional regulation, geographic specificity, difference in etiologic factors involved, sample size and genetic background.

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