

Immunohistochemical evaluation of Bcl-2 and Ki-67 in varying grades of oral squamous cell carcinoma

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This study determines predominant mechanism of carcinogenesis in varying grades of oral squamous cell carcinoma (OSCC) by Immunohistochemical evaluation of Bcl-2 gene and Ki-67 antigen. Anti-apoptosis was found dominant in well differentiated lesions than in moderately and poorly differentiated lesions; while cell proliferation dominated in poorly differentiated lesions than in moderately and well differentiated lesions.

Keywords: Bcl-2 protein, Ki-67 antigen, MIB-1 marker, Oral squamous cell carcinoma (OSCC)

Introduction

Oral carcinoma has achieved the status of a disease with high mortality rate. Over 90% of head and neck neoplasms are squamous cell carcinomas¹. Fast-growing tumors may have a high cell turnover, implying that rates of both proliferation and apoptosis are high; but for the tumor to grow, the rate of proliferation should exceed that of apoptosis. Whereas in others, growth occurs due to mutation in the genes regulating apoptosis, resulting in tumor progression due to reduced cell death rather than explosive cell proliferation². Anti-apoptotic proteins comprise of Bcl-2 and Bcl-X_L³. Thus, it is the relative concentration of pro-apoptotic and anti-apoptotic members that decides actual outcome of a cell challenged with an apoptotic stimulus⁴. By promoting cell survival, Bcl-2 facilitates permanent acquisition of mutations and malignant transformation. MIB-1 (Molecular Immunology Borstel) is a monoclonal antibody for detection of Ki-67 antigen; as original Ki-67 antibody shows immunopositivity only in fresh and frozen tissues sections. MIB-1 has been prepared by fusion of spleen cells of BALB/C mice immunized with recombinant parts of Ki-67 antigen, with cells of mouse myeloma cell line X63Ag8.653⁵. Thus, as carcinogenesis may be

associated with both decreased rate of apoptosis and increased cell proliferation, this study determines predominant mechanism of carcinogenesis to correlate expressions of anti-apoptotic marker Bcl-2 and proliferative marker MIB-1 in varying grades of oral squamous cell carcinoma (OSCC), and to find out whether there is any correlation between expressions of Bcl-2 and MIB-1 in OSCC.

Experimental Section

The study sample comprised of histopathologically confirmed 30 cases of OSCC retrieved from archives of various histopathology laboratories in Tamil Nadu, India. In each case, three contiguous 4µm-thick sections were made. Of these, one section was stained with routine hematoxylin and eosin (H&E) stain and other two sections were subjected to immunostaining for Bcl-2 and MIB-1 protein.

H&E staining was done using the following protocol⁶. Sections were deparaffinized in three changes of xylene for 10 min each, then rehydrated through graded concentrations of alcohol and washed in running tap water for 5 min. Sections were then stained with Harris's hematoxylin (8 min), washed in running tap water and differentiated in 1% acid alcohol for 10 s followed by blueing of sections. Also, sections were counterstained with eosin (5 min), washed in running tap water for 1-5 min, dehydrated through three changes of alcohol and

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cleared twice with xylene and finally mounted using a synthetic resinous mounting media (DPX). Of other two sections taken on poly-L-lysine coated slides, one was subjected to immunohistochemistry (IHC) for Bcl-2 staining and the other for MIB-1 staining. During IHC procedure, normal oral mucosa was used as negative control, and reactive lymph node tissue as positive control. IHC was performed using the following protocol⁶.

After overnight incubation at 37°C, sections were heated at 60°C for 1 h before staining. Sections were deparaffinized in three changes of xylene for 10 min each, rehydrated through graded concentrations of isopropanol for 5 min each followed by washing in running tap water for 5 min. Antigen retrieval was performed by heat induced epitope retrieval technique. Slides were placed in slide tank having slots for slides, containing 250 ml of retrieval solution [10 mL⁻¹ sodium citrate buffer (pH 6.0)]. Retrieval solution was prepared by adding 25 ml of citrate buffer to 225 ml of distilled water. Number of slides in the slots was kept same in each run in order to deliver balanced heat to all the slides. Slide tank was placed in pressure cooker and incubated for 1 h, setting upper limit at 115°C for 5 min and lower limit at 90°C for 10 s. Pascal strips were placed into pressure cooker over the stand to check for successful antigen retrieval. Strip changes its color from white to yellow/green on completion of retrieval.

Sections were washed with Tris buffered saline (pH 7.6) for 5 min; Endogenous peroxidase activity was blocked by incubating sections in 0.5% hydrogen peroxide in methanol for 10 min, followed by washing in Tris buffered saline. Then power blocking was done for 10 min so as to mask non-specific protein sites. Primary antibody was added, directed against Bcl-2 and Ki-67 antigen (BioGenex) and after 1 h, washed in Tris buffered saline. Then secondary antibody, horseradish peroxidase labeled polymer was added and slides were incubated for 30 min. Sections were then covered with chromogen 3,3'-diaminobenzidine (DAB) for 10 min, as it produces colored reaction end-product, making it suitable for light microscopy. Sections were washed in distilled water, counterstained with Harris's haematoxylin (1-2 dips), again washed in running tap water; dehydrated in alcohol, cleared in xylene and finally slides were mounted with DPX.

Under light microscope, visualization of brown colored areas in cytoplasm of epithelial cells (for Bcl-2 protein) and nuclei of epithelial cell (for Ki-67 antigen)

Table 1—Bcl-2 and MIB-1 positive cells in different grades of oral squamous cell carcinoma (OSCC) (The observation was made from 500 cells of all cases)

Cases	Bcl-2, %			MIB-1, %		
	WD	MD	PD	WD	MD	PD
1	8.4	6.6	3.0	51.8	72.8	86.4
2	8.6	6.8	4.2	61.6	68.4	70.0
3	11.8	7.6	2.8	67.6	79.0	78.2
4	11.8	5.8	2.6	53.2	77.4	81.8
5	7.4	5.6	4.6	71.2	71.0	78.8
6	6.5	6.2	4.6	56.4	72.2	87.4
7	7.6	5.6	3.8	54.0	68.0	71.6
8	13.6	6.8	2.2	67.4	73.6	76.8
9	7.2	4.8	3.2	68.2	77.6	88.6
10	9.8	6.0	4.6	51.8	72.0	76.4

WD, Well differentiated; MD, Moderately differentiated; PD, Poorly differentiated

against the blue background was considered as positive staining. Slides subjected to immunohistochemical staining were observed under 100X and 400X magnifications. Number of Bcl-2 and MIB-1 positive cells was noted in ten different high power fields. 50 cells from each field were counted by making a grid pattern that divided the field into 10 equal divisions and from each division 5 good cells with proper cell outline were chosen in order to reduce the bias. Above data was recorded on the master chart for statistical analysis.

Results and Discussion

The study sample included 30 cases of OSCC (10 cases each of well differentiated, moderately differentiated and poorly differentiated). Sections were cut and stained for routine H&E staining, Bcl-2 and MIB-1 immunostaining. Number of Bcl-2 and MIB-1 positive cells was noted in 10 different high power fields, by counting the number of positive cells per 50 cells in each field. Value (%) for each case was calculated for Bcl-2 and MIB-1 positive cells (Table 1). Mean, standard deviation and Karl-Pearson correlation coefficient was calculated between Bcl-2 and MIB-1 for each group of OSCC. $p < 0.05$ was considered as statistically significant (Table 2). Bcl-2 positivity was observed in all the cases; while its expression decreased from well differentiated (Fig. 1b) to moderately differentiated (Fig. 2b) to poorly differentiated (Fig. 3b) cases, with significant quantitative differences in varying grades of OSCC. MIB-1 positivity was observed in all the cases. Number of MIB-1 positive

Table 2—Mean values $\pm$ standard deviation and co-relation co-efficient of Bcl-2 and MIB-1 expression in well, moderate and poorly differentiated oral squamous cell carcinoma (OSCC).

Group differentiated OSCC (n=10)	Mean $\pm$ SD for Bcl-2	Mean $\pm$ SD for MIB-1	Co-relation between Bcl-2 and MIB-1	P-value	Significance
WD	9.27 $\pm$ 2.39	60.3 $\pm$ 7.72	0.1076	p>0.05	Non significant
MD	6.18 $\pm$ 0.79	73.2 $\pm$ 3.78	0.0797	p>0.05	Non significant
PD	3.56 $\pm$ 0.91	79.6 $\pm$ 6.41	-0.1594	p<0.05*	Significant

WD, Well differentiated; MD, Moderately differentiated; PD, Poorly differentiated

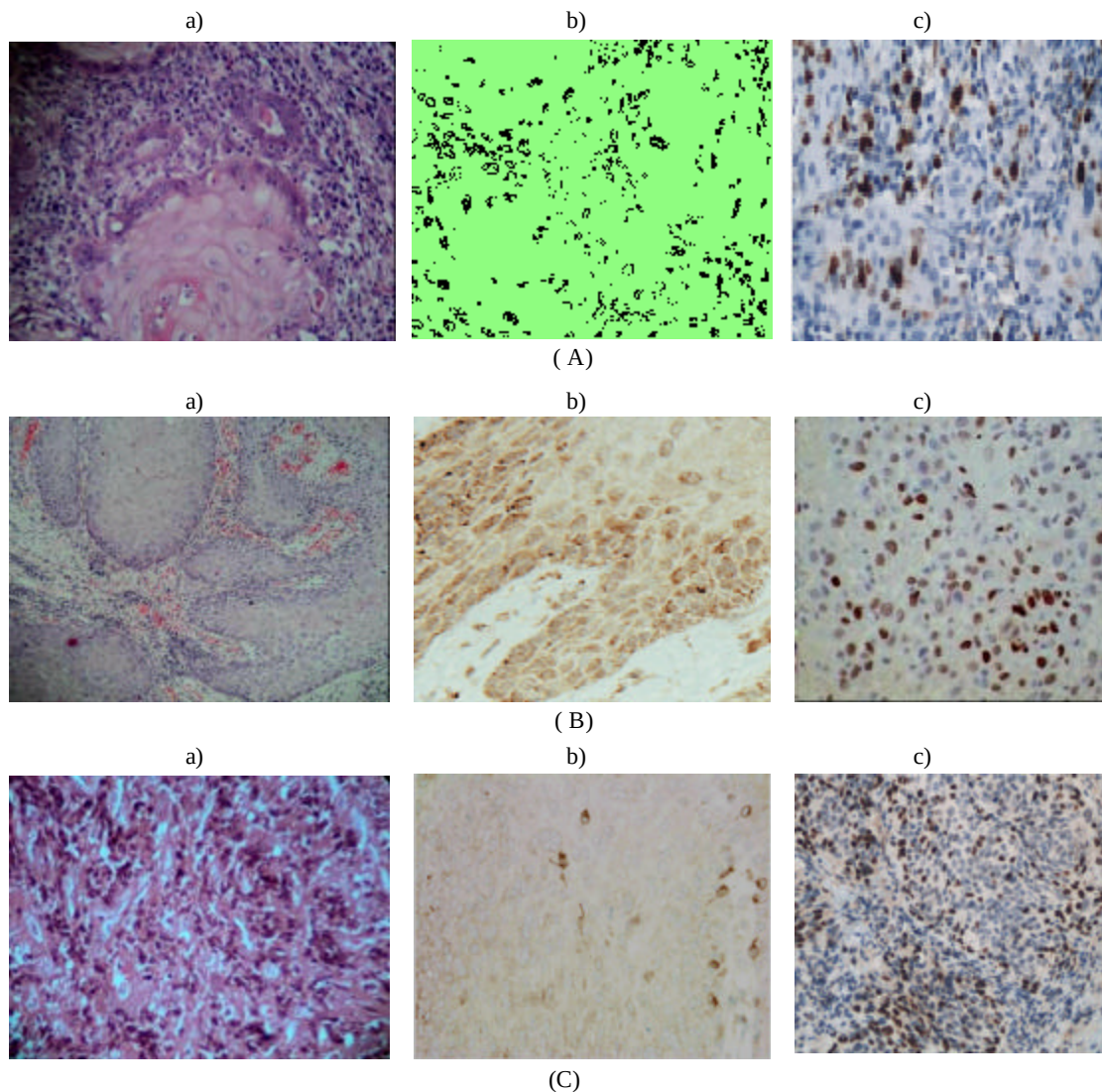


Fig. 1—H & E staining and immunohistochemical expression of Bcl-2 and MIB-1 protein in oral squamous cell carcinoma (OSCC): A) well differentiated; B) moderately differentiated; and C) poorly differentiated [a) H & E staining, b) Bcl-2 staining, and c) MIB-1 staining]

cells increased from well differentiated (Fig. 1c) to moderately differentiated (Fig. 2c) to poorly differentiated (Fig. 3c) OSCC with significant quantitative differences.

Correlation between Bcl-2 and MIB-1 marker expression showed a statistically insignificant correlation in well and moderately differentiated OSCC with p>0.05, but

statistically significant correlation in poorly differentiated OSCC with $p < 0.05$.

This study was conducted for better understanding of the dominant basic pathologic process underlying the development of well differentiated OSCC, as opposed to poorly differentiated lesions. An attempt was made to correlate histologic grade of tumour with pathogenesis. Willis⁷ defined neoplasm as an abnormal mass of tissue, the growth of which exceeds and is uncoordinated with that of the normal tissues and persists in the same excessive manner even after cessation of stimuli, which evoked the change. A tumor is formed by clonal expansion of single precursor cell that has incurred genetic damage. Genetic changes accumulate during carcinogenesis and precede malignant transformation or invasion. Aberrations in cell cycle check points are the major cause of genetic instability in cancer cells. Tumor cells, however, do not necessarily complete cell cycle more readily than normal cells. So, total time of cell cycle for most tumors is either equal to or longer than that of corresponding normal cells.

Rapidity of growth of tumor depends on the doubling time of tumor cells, fraction of cells that are in replicative pool, and the rate at which they are shed and lost in the growing lesion. The proportion of cells within tumor population that are in proliferation pool is the growth fraction. As tumor continues to grow, cells leave proliferation pool in ever increasing numbers owing to shedding, lack of nutrients or apoptosis and after differentiation enter into G_0 phase. Most tumor cells remain in G_0 or G_1 phase. By the time tumor becomes clinically detectable, most of the cells have completed major portion of cell-cycle². Cell proliferation is increase in cell numbers resulting from completing the cell cycle and is fundamental to both embryonic and postembryonic existence. Hyper-proliferation is thought to be an early marker of disorderly growth. Increased proliferation is associated with more advanced lesions and that the distribution of proliferating cells in tissue may tell more about the regulatory mechanism that become dysfunctional during multistep process of carcinogenesis⁸. Apart from cell growth and hyper-proliferation, cell survival is controlled by the genes that regulate apoptosis. There are present large families of genes that regulate apoptosis in normal as well as cancer cells. Defective DNA repair genes, activation of growth promoting proto-oncogenes, which affect cell proliferation or survival indirectly by influencing the ability of organism to repair non-lethal damage in other genes, inhibition of tumor

suppressor genes and genes that regulate apoptosis are the principal targets of DNA damage. Although both anti-apoptosis and increased cell proliferation may play a role in the pathogenesis of carcinogenesis, it is a well established fact that in case of slow growing and well differentiated cases, the rate of anti-apoptosis is predominant than in moderately and poorly differentiated cases; while in rapidly growing and poorly differentiated cases, the rate of proliferation is predominant than in well and moderately differentiated cases.

Tumors with high Bcl-2 immunoreactivity are generally slow growing as compared to those with high immunoreactivity for proliferation markers like Ki67, PCNA, Cyclin D and CENP-F. Studies have shown poor overall survival rates in low Bcl-2 as compared to tumors with moderate or high Bcl-2 immunoreactivity⁹⁻¹¹. Both MIB-1 and Ki-67 antibodies react with the same epitope. In present study, MIB-1 antibody was used in order to detect Ki-67 expression, as Ki-67 has a major drawback that it is used only for fresh and frozen sections¹². Ki-67 human nuclear antigen is expressed during G_1 , S, G_2 and M phases in cell cycle, but is absent in quiescent G_0 phase¹³. In tissue sections, Ki-67 antibody is used to localize Ki-67 protein. Antibody against Ki-67 antigen has been used as a simple, rapid and reliable means of evaluating growth fraction of normal and neoplastic cell populations. Ki-67 protein, detected by immunolocalisation of Ki-67 antigen, is located in nucleus^{14,15}. In present study, Bcl-2 and MIB-1 activity was quantified in 10 different high power fields, by counting the number of positive cells per 50 cells in each field. Expression of anti-apoptotic marker Bcl-2 showed a statistically significant decrease from well to moderate to poorly differentiated OSCC, and was in accordance to reported studies^{16,17}, where dysregulation of Bcl-2 and loss of its expression was noted with increasing grades of carcinoma. However, in contrast to these findings, study done by Solomon *et al*¹⁸ showed an inverse relationship between Bcl-2 and level of differentiation of OSCC, because of over-expression of Bcl-2 and loss of function of p53 that resulted in defective apoptosis and subsequent progression of tumor. Present study demonstrated a deregulation of Bcl-2 oncoprotein, which may be a reason for many genetic aberrations in the progression of OSCC.

Bcl-2 is related to cancer development in initial stages of carcinogenesis up to the appearance of metastasis¹⁹. Function of Bcl-2 is to inhibit apoptosis in different stages, increasing genetically altered cell survival rate and

facilitating appearance of new mutations²⁰. Down-regulation of Bcl-2 expression with increasing grades of squamous cell carcinoma indicates that this oncoprotein may play a role in relatively early events in the development and progression of OSCC. This down-regulation of Bcl-2 expression in advanced stage of squamous cell carcinoma with metastatic potential suggests that this oncoprotein may not be required for cell survival. In present study, expression of proliferating marker MIB-1 showed a statistically significant increase from well to moderate to poorly differentiated OSCC. Other reports^{21,22} suggested that proliferative fraction progressively increase with the grade of carcinoma and supported the hypothesis that these may be the result of abnormal proliferation and maturation of epithelial cells.

In present study, a statistically non significant correlation was found between Bcl-2 and MIB-1 expression in well and moderately differentiated OSCC cases. While statistically significant correlation was found between Bcl-2 and MIB-1 expression in poorly differentiated OSCC cases and a similar finding was observed by Piattelli *et al*²³ who concluded that proliferative activity was highest while anti-apoptosis was lowest in case of invasive carcinoma. Studies on apoptotic markers and MIB-1/Ki-67^{24,25} had also shown a correlation between high values of Ki-67 expression with poor prognosis in squamous cell carcinoma of tongue. As opposed to these findings, a recent study²⁶ has shown significantly higher expression of Ki-67 in well differentiated OSCC versus poorly differentiated OSCC, and in certain non-proliferative tumors, Ki-67 cells are certain to be quiescent because Ki-67 is expressed in all cell cycles except for G₀. Staibano *et al*²⁷ found a low positivity for proliferating marker combined with high positivity for Bcl-2 protein correlated with a better clinical outcome in OSCC. Hindermann *et al*²⁸ found that there was a decrease in number of cells undergoing apoptosis in well-differentiated tumors resulting in an increase in cell accumulation and thus tumor cells causing tumor to grow.

Conclusions

Although both cell proliferation and anti apoptosis play an important role in pathogenesis of OSCC, present study imply that proliferation was predominant in well, moderately and poorly differentiated OSCC and the rate of proliferation was maximum in poorly differentiated OSCC. Whereas anti apoptosis was the dominant mechanism in well differentiated lesions than in

moderately and poorly differentiated lesions. A significant correlation between Bcl-2 and MIB-1 was also observed in poorly differentiated OSCC.

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