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ORIGINAL ARTICLE

The Immunohistochemical Evaluation Of The Expression Of Bcl-2 In Different Histological Grades Of Squamous Cell Carcinoma

SURI C

ABSTRACT

The bcl-2 proto-oncogene was first discovered in B-cell lymphomas and is the prototype of cell death regulatory genes. It's gene product, the bcl-2 protein, directly or indirectly inhibits the release of cytochrome C, thus preventing the activation of caspases and hence, inhibiting apoptosis. This leads to cellular immortalization, contributing to formation of the tumour and facilitating the permanent acquisition of mutations.

Studies show increased expression of the bcl-2 protein in about 50% of human cancers. Keeping this in view, our study was undertaken to evaluate the expression, to quantify and to determine the intensity and the pattern of bcl-2 in various histological grades of Oral Squamous Cell Carcinoma.

Method: The present study investigated the immunohistochemical expression of bcl-2 in 38 cases, of which 32 were histologically diagnosed as Oral Squamous Cell Carcinomas and 5 normal lymph nodes along with 1 normal oral mucosa were included as controls.

Each specimen was sectioned at 3 micron thickness, immunostained with the bcl-2 antibody and viewed under a light microscope.

Results: All the 38 cases showed bcl-2 immunopositivity. The number of bcl-2 positive cells was more in poorly differentiated SCC than in well differentiated SCC. The intensity of bcl-2 expression was more in moderately differentiated SCC, while in poorly differentiated SCC, an equal number of cases showed light and dark intensity. When the distribution pattern of bcl-2 expression was assessed, the tumour islands devoid of central keratinization showed bcl-2 expression in all the tumour cells.

Summary and conclusion: In OSCCs, the number of cells expressing bcl-2 increased from well differentiated to poorly differentiated, showing an inverse relationship with the degree of differentiation. Further correlative studies using markers for other members of the bcl-2 family are necessary to elucidate the specific molecular defects critical to the biology of this tumour, which will have an impact on it's diagnosis and treatment.

Key words: Bcl-2, Squamous cell carcinoma (SCC), apoptosis

* (MDS) – Oral & Maxillofacial Pathology
Sri Sai College of Dental Surgery
Vikarabad
Corresponding Author:
Dr. Charu Suri
Address: Dept of Oral & Maxillofacial Pathology,Sri Sai College of Dental Surgery,R.R District,Vikarabad – 01
Phone No.: 9989233003
Emailld:charusuri87&yahoo.com

Introduction

The prevalence of tumours of the head and neck region is rapidly increasing and in particular, Squamous Cell Carcinoma (SCC) represents the most frequent malignant tumour of the oral cavity. It has long been evident that cancer has a multifactorial aetiology and is a multistep process

involving initiation, promotion and tumour progression.

Carcinogenesis involves the activation of oncogenes and inactivation of tumour suppressor genes. The three major antiproliferative pathways recognized are: inhibition of cell growth, induction of cell differentiation and programmed cell death (apoptosis).

Normally, the elimination of genetically damaged cells by apoptosis precludes the development of tumours and also plays a role in the effective response to cancer therapy, including chemo and radiotherapy [1].On the contrary, the imbalance of the apoptotic pathway contributes to the immortalization of replicating cells, thus favouring the accumulation of sequential genetic damage that normally would induce cell death [2].
The bcl-2 proto-oncogene was first discovered in B-cell lymphomas which exhibited the t(14:18) q (32:31) chromosomal aberration and is the prototype of cell death regulatory genes [3]. It's gene product, the bcl-2 protein, blocks a distal step in an evolutionary conserved pathway of apoptosis. It's abnormal expression, usually in terms of overexpression in genetically modified cells such as tumour cells, contributes to the expansion of the damaged cell clone by preventing cell turnover due to programmed cell death, leading to cellular immortalization.

By promoting cell survival, bcl-2 facilitates the permanent acquisition of mutations and malignant transformation [4].Moreover, increased bcl-2 expression in cancer cells possibly reflects tumour cell resistance to apoptosis and may have implications for their responsiveness to treatments[5].

Aims And Objectives

Our study aims to explore the bcl-2 protein immunoreactivity in Oral Squamous Cell carcinoma in an attempt to elucidate its influence on the biological behaviour of this neoplasm.

Materials And Method

The material for the study included 38 formalin fixed paraffin embedded tissue blocks. Among these, 10, 12 and 10 blocks of each histologically diagnosed well moderate and poorly differentiated oral squamous cell carcinomas respectively and 5 of normal lymph nodes and 1 of normal mucosa as controls, were considered for immunohistochemical staining for bcl-2.
The antibodies and reagents used for the immunohistochemical technique were obtained from DAKO cytomation (DENMARK) and SIGMA ALDRICH companies (USA).

3 micron thick sections were taken onto poly-L-lysine adhesive coated micro slides and incubated for 1 hour at 48 degree centigrade. The sections were then dewaxed through 3 changes of xylene, hydrated through descending grades of alcohol (100%, 90%, 70%) and brought to water. The micro sections were then dipped in freshly prepared 3% H_2O_2 in methanol for 20 minutes to block endogenous peroxidase activity, followed by a wash in PBS
For antigen retrieval, the micro sections were immersed in preheated 0.01 millimolar sodium citrate buffer and boiled for 8 minutes in a 5 litre domestic stainless steel pressure cooker on an electric heater. This increases the immunoreactivity of the antigen in tissues by enabling the antigen blocked by the cross-linking of the formalin fixative to be uncovered for binding to the relevant antibody. The slides were allowed to cool down in citrate buffer, were then washed in distilled water for 5 minutes and were then washed thoroughly in 3 changes of Phosphate Buffered Saline (PBS)

The sections were then treated with 10% goat serum at room temperature for 30 minutes to block the non–specific antigen sites, followed by incubation with primary antibody (bcl-2) prediluted at 37^0C (1:50)

dilution for 60 minutes in a humid chamber. For reagent control, no primary antibody was added.

The sections were then incubated with biotinylated secondary antibody (Fc conjugated goat anti-mouse IgG) diluted to 1:200, at room temperature for 30 minutes, followed by incubation with the streptavidin-peroxidase conjugate diluted at a concentration of 1:200, for 30 minutes.

For visualization, the sections were incubated with Diaminobenzidine tetra hydrochloride (DAB) at a concentration of 1:50 for 5 minutes. The sections were then lightly counterstained using Meyer's hematoxylin for 95 seconds and were then gently washed in running tap water for 2 minutes. The sections were then dehydrated through ascending grades of alcohol, were mounted with DPX and were covered with coverslips.

Evaluation

Assessment of the antigen expressed cells was performed by using a light microscope at 10X and 40X magnifications.

The criteria used to define the bcl-2 antigen positive cells were

- Brown staining in lymphocytes (intrinsic positive areas)
- Brown staining within the cytoplasm and the nucleus of the tumour cells.

In each case, three fields were selected randomly and 100 cells were counted per field under 40X magnification. The intensity of staining was graded as light /dark by comparing with the internal positive control, which were lymphocytes. In the different grades of squamous cell carcinoma, the pattern of staining was considered as periphery when the expression was seen only in the peripheral cells of the islands, while it was graded as entire when all the tumour cells within the islands expressed bcl-2 reactivity [Table/Fig 1].

(Table/Fig 1) Reports The Number Of Cases With Expression Of Bcl-2 In Different Grades Of Oscc

GRADES OF OSCC →		WDSCC	MDSCC	PDSCC	TOTAL
NUMBER OF CASES		10	12	10	32
MEAN NO. OF POSITIVE CELLS		187.6000	223.7500	264.7000	225.2500
STAINING INTENSITY	DARK	2	10	5	17
	LIGHT	8	2	5	15
STAINING LOCATION	ENTIRE ISLAND	9	11	1	21
	PERIPHERY	1	1	-	2
	SHEETS	-	-	9	9

Statistical Analysis

The data was analyzed using t-test for the number of bcl-2 positive cells and the by using the Kendall's tau–b test for staining intensity and location to eliminate the interobserver bias. Comparisons within the different grades of OSCCs were subjected to the Fischer test for the number of positive cells and to the Chi Square test for staining intensity and location. For the intragroup comparison, Tukey HSD was applied for the number of bcl-2 positive cells, Fischer Exact test and Chi Square test were applied for staining intensity and the Fischer Exact test was applied for staining location.

Results And Observation

In the present study, the control sections of the lymph nodes showed bcl-2 expression in the mantle zone lymphocytes and most interfollicular lymphocytes, whereas only occasional germinal centre lymphocytes expressed bcl-2. In the normal oral mucosa, bcl-2 expression was observed in a few cells of the basal layer. The suprabasal spinous layers were devoid of the bcl-2 antigen expression.

It was observed that all the 32 squamous cell carcinoma cases tested for the expression of bcl-2, showed immunopositivity. The immunohistochemical staining of the tumour cells showed granular cytoplasm and the nuclear envelope staining was positive. The sites of expression (periphery/entire island) in tumour islands/sheets were noted and the intensity of the expression of bcl-2 was also graded as light or dark when compared to the internal positive control

lymphocytes. To eliminate subjective bias, two observers independently evaluated the expression of bcl-2 and to minimize the interobserver bias, t- test was applied and the p value was found to be non-significant Thus, the mean number of bcl-2 expressing cells counted by observer 1 was then subjected for further statistical analysis.

When the cells were evaluated for bcl-2 expression in well, moderately and poorly differentiated OSCCs, the mean number of cells were found to be 187.600, 223.750 and 264.700, respectively. Fischer test was applied on the results to compare the number of cells expressing bcl-2 in different grades of OSCCs and the p value was found to be very highly significant (p= 0.001) [Table/Fig 2].

(Table/Fig 2) Observer significance-Number of bcl-2 positive cells in different grades of OSCCs by two observers and comparison between different grades.

OBSERVER		N	MEAN	STD. DEVIATION	t – TEST
WDSCC positive staining	1.00	10	187.6000	9.10677	0.28500
	2.00	10	188.8000	9.69307	p = 0.779 ns
MDSCC positive staining	1.00	12	223.7500	12.65001	1.12900
	2.00	12	218.5833	9.55804	p = 0.271 ns
PDSCC positive staining	1.00	10	264.7000	7.08755	0.98800
	2.00	10	261.3000	8.26035	p = 0.336 ns
positive staining	1.00	32	225.2500	32.49020	0.33300
	2.00	32	222.6250	30.61704	p = 0.741 ns

F = 145.87, p = 0.001 vhs

When bcl-2 expression was compared between the different grades of OSCCs, it was found that;

- Well vs moderately differentiated - mean difference of 36.150 cells was observed;
- Well vs poorly differentiated – mean difference of 77.100 cells was observed;
- Moderately vs poorly differentiated – mean difference of 40.950 cells was observed

These inter comparison results when subjected to the Tukey HSD test, showed p values which were very highly significant (p=0.001) in all the inter comparisons [Table/Fig 3].

(Table/Fig 3) Intragroup comparison of number of bcl-2 positive cells in different grades of OSCCs

Tukey HSD

(I) GRP	(J) GRP	Mean Difference (I-J)	p
Moderate	Poor	-40.9500	.001 vhs
	Well	36.1500	.001 vhs
Poor	Well	77.1000	.001 vhs

After ruling out the interobserver bias [Table/Fig 4] for the staining intensity of bcl-2 positive cells, it was observed that only 2 out of 10 cases of the well differentiated OSCCs showed a dark intensity of expression as compared to 10 out of 12 cases of the moderately differentiated and 5 out of 10 of the poorly differentiated OSCC cases, i.e. out of 32 samples , 17 showed dark staining and 15 showed light staining The results obtained were compared using the Chi Square "χ^2" test and the value obtained was 8.843, with the p value being significant (p= 0.012) [Table/Fig 5].

(Table/Fig 5) Comparison of intensity of staining in different grades of OSCCs

			GRP			
			WDSCC	MDSCC	PDSCC	Total
Staining Intensity	DARK	Count	2	10	5	17
		%	20.0%	83.3%	50.0%	53.1%
	LIGHT	Count	8	2	5	15
		%	80.0%	16.7%	50.0%	46.9%
Total		Count	10	12	10	32
		%	100.0%	100.0%	100.0%	100.0%

a. χ^2=8.843 p=.012 sig

(Table/Fig 4) Observer significance- Staining intensity of bcl-2 in different grades of OSCCs by two observers.

STAINING INTENSITY			OBSERVER 1	OBSERVER 2	TOTAL
WDSCC	DARK	COUNT	2	2	4
		%	20.0 %	20.0 %	20.0%
	LIGHT	COUNT	8	8	16
		%	80.0 %	80.0 %	80.0%
	TOTAL	COUNT	10	10	20
		%	100.0 %	100.0 %	100.0%
MDSCC	DARK	COUNT	10	10	20
		%	83.3 %	83.3 %	83.3%
	LIGHT	COUNT	2	2	4
		%	16.7 %	16.7 %	16.7%
	TOTAL	COUNT	12	12	24
			100.0 %	100.0 %	100.0%
PDSCC	DARK	COUNT	5	5	10
		%	50.0 %	50.0 %	50.0%
	LIGHT	COUNT	5	5	10
		%	50.0 %	50.0 %	50.0%
	TOTAL	COUNT	10	10	20
			100.0 %	100.0 %	100.0%

Kendall's Tau-b Test - p=1

On comparing the intensity of the expression between individual grades

- 2 of the 10 well differentiated cases showed dark staining as compared to 10

out of the 12 moderately differentiated cases. The "χ2 "value was found to be 8.824 and the p value as 0.003, the difference being highly significant [Table/Fig 6] (Table 6a).

- Likewise, on comparing well differentiated (2 of 10 cases) vs poorly differentiated cases (5 of 10 cases) using the Fischer Exact test, the p value obtained was found to be 0.35, the difference being non-significant [Table/Fig 6] (Table 6 b).
- Similarly, on comparing moderately differentiated (10 of 12 cases) vs poorly differentiated cases (5 of 10 cases), the p value obtained by using the Fischer Exact test was found to be 0.172, which showed no significant difference [Table/Fig 6] (Table 6 c).

(Table/Fig 6) Intragroup comparison of staining intensity in different grades of OSCCs

6A

			GRP		
			MDSCC	WDSCC	Total
Staining Intensity	DARK	Count	10	2	12
		%	83.3%	20.0%	54.5%
	LIGHT	Count	2	8	10
		%	16.7%	80.0%	45.5%
Total		Count	12	10	22
		%	100.0%	100.0%	100.0%

a. χ2=8.824 p=.003 hs

6B

			GRP		
			PDSCC	WDSCC	Total
Staining Intensity	DARK	Count	5	2	7
		%	50.0%	20.0%	35.0%
	LIGHT	Count	5	8	13
		%	50.0%	80.0%	65.0%
Total		Count	10	10	20
		%	100.0%	100.0%	100.0%

6C

			GRP		
			MDSCC	PDSCC	Total
Staining Intensity	DARK	Count	10	5	15
		%	83.3%	50.0%	68.2%
	LIGHT	Count	2	5	7
		%	16.7%	50.0%	31.8%
Total		Count	12	10	22
		%	100.0%	100.0%	100.0%

When compared for the location of the positive cells (periphery/entire island) in tumour island/sheets after interobserver bias was ruled out [Table/Fig 7] , it was observed that 9 out of 10 cases of well differentiated OSCCs showed staining in entire tumour islands as compared to 11 out of 12 cases of moderately differentiated and 1 out of 10 cases of poorly differentiated OSCCs. The results obtained were compared using the Chi Square "χ^2" test. The "χ2 "value obtained was 27.594, with the p value being very highly significant (p= 0.001) [Table/Fig 8].

(Table/Fig 7) Observer significance-Staining location of bcl-2 in different grades of OSCCs by two observers.

STAINING LOCATION			OBSERVER		TOTAL
			1	2	
WD SCC	ENTIRE ISLAND	COUNT	9	9	18
		%	90.0 %	90.0 %	90.0%
	PERIPHERY	COUNT	1	1	2
		%	10.0 %	10.0 %	10.0%
	TOTAL	COUNT	10	10	20
		%	100.0 %	100.0 %	100.0%
MD SCC	ENTIRE ISLAND	COUNT	11	11	22
		%	91.7 %	91.7 %	91.7 %
	PERIPHERY	COUNT	1	1	2
		%	8.3 %	8.3 %	8.3%
	TOTAL	COUNT	12	12	24
			100.0 %	100.0 %	100.0%
PD SCC	ENTIRE ISLAND	COUNT	1	1	2
		%	10.0 %	10.0 %	10.0%
	SHEETS	COUNT	9	9	18
		%	90.0 %	90.0 %	90.0%
	TOTAL	COUNT	10	10	20
			100.0 %	100.0 %	100.0%

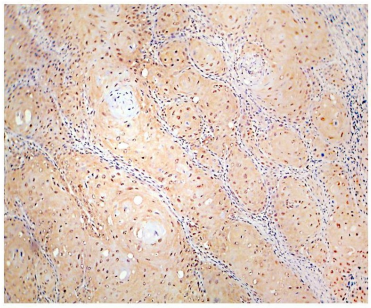
Kendall's Tau-b Test - p=1

On comparing the location of the positive cells between individual grades, it was seen that 9 of the 10 well differentiated cases showed staining in the entire island as compared to 11 out of 12 of moderately differentiated cases. The p value obtained with the Fischer Exact test was 1, the difference being non- significant [Table/Fig 8].

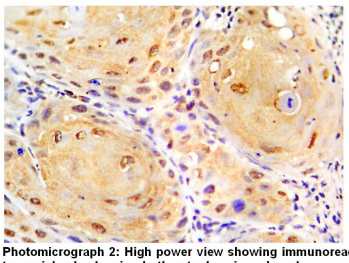
(Table/Fig 8) Comparison of expression of bcl-2 in different locations in different grades of OSCCs

		GRP			
		WDSCC	MDSCC	PDSCC	Total
ENTIRE ISLAND	Count	9	11	1	21
	%	90.0%	91.7%	10.0%	65.6%
PERIPHERY	Count	1	1	0	2
	%	10.0%	8.3%	.0%	6.3%
SHEETS	Count	0	0	9	9
	%	.0%	.0%	90.0%	28.1%
Total	Count	10	12	10	32
	%	100.0%	100.0%	100.0%	100.0%

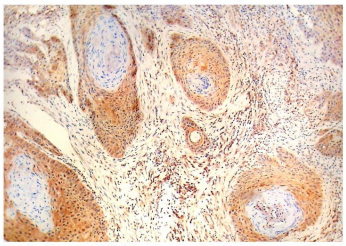
a. χ^2=27.594 p=.001 vhs



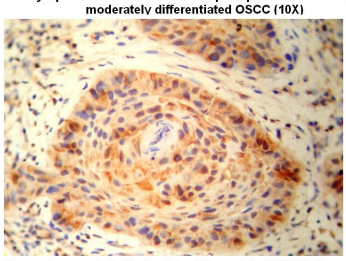
Photomicrograph 1: Well differentiated OSCC showing bcl-2 expression in the cytoplasm and nuclear envelope of tumor islands (10X)



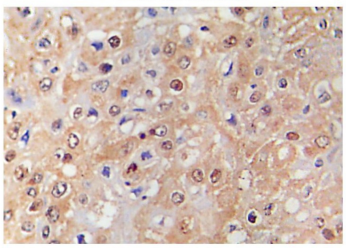
Photomicrograph 2: High power view showing immunoreactive tumor islands showing both cytoplasmic and nuclear envelope staining in well differentiated OSCC (40X)



Photomicrograph 3: Islands and strands of tumor cells showing cytoplasmic and nuclear envelope expression of bcl-2 in moderately differentiated OSCC (10X)



Photomicrograph 4: Moderately differentiated OSCC showing island of tumor cells showing bcl-2 expression in cytoplasm and nuclear envelope (40X)



Photomicrograph 5: Poorly differentiated OSCC showing sheets of tumor cells with bcl-2 immunopositivity of both cytoplasm and nuclear envelope (40X)

Observations were also noted for the expression of bcl-2 in the connective tissue stroma and positive staining was observed in a few of the endothelial cells lining the blood vessels and secretory ducts of the minor salivary glands

Discussion

Programmed cell death or apoptosis is a critical step in cell differentiation and turnover and in tissue homeostasis. Perhaps the best studied regulatory proteins are the bcl-2 family of molecules. Recent advances on cancer biology have shown that the process of carcinogenesis may involve not only increased cell proliferation, but also decreased cell death or increased cell survival. Mutations of any of the genes encoding anti-apoptotic proteins or any changes in the levels of their expression can lead to increased cell survival.

In this immunohistochemical study, Bcl-2 expression was observed in 32 cases of squamous cell carcinoma, which was also seen by ***Ravi et al (1999)*** in their study on squamous cell carcinoma [6]. The expression of bcl-2 was in the form of granular cytoplasmic staining with an accentuation around the nuclear membrane. This is due to the cellular localization of bcl-2 in the outer membrane of the mitochondria, endoplasmic reticulum and the nuclear membrane.

In this study, the number of bcl-2 positive cells was more in the poorly differentiated squamous cell carcinoma (mean 264.700) than in well differentiated squamous cell carcinoma (mean 187.600). An increase of bcl-2 expression in poorly differentiated squamous cell carcinoma was also seen by ***Yu chen et al (2000***) and ***Muzio et al (2003***) [7],[8]. This observation of an increase in the number of bcl-2 positive cells with a decrease in the differentiation, probably reflects that bcl-2 is expressed in keratinocytes that have an increased capacity for survival.

However, in earlier studies by ***Loro et al (1999),*** bcl-2 oncoprotein expression was suppressed in poorly differentiated squamous cell carcinoma. This variation in the expression pattern which was detectable by the bcl-2 antibody, could be because of elective caspase activation, resulting in the proteolytic degradation of bcl-2 in tumour cells [9].

Studies done on squamous cell carcinoma of the oesophagus and the oral cavity by ***Jordan et al (1996),*** showed a higher percentage of immunoreactivity in poorly differentiated than in well differentiated carcinomas.[10] However, this uniform immunoreactivity was not observed in carcinomas of the lung in studies by ***Francesso Pezella et al (1993)*** [11]. This variation suggests that bcl-2 expression may be organ specific and may depend on the biological behaviour of individual tumours.

The activity of the bcl-2 family is partly regulated by the formation of homo and heterodimers and the relative concentration of the pro-apoptotic and anti-apoptotic members which would decide the outcome of a cell challenged by an apoptotic stimulus. In this study, the intensity of bcl-2 expression was dark in fewer cases of well differentiated carcinomas (2 cases) as compared to moderately differentiated squamous cell carcinomas (9 cases). In poorly differentiated SCC, an equal number of cases (5 each) showed light and dark intensities. The increased number of cases showing a dark intensity of expression in moderately differentiated carcinomas could be because of the increased activity of the bcl-2 protein during the early stages of tumour progression. However, as 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.

Terminal differentiation is a form of programmed cell death which occurs in the oral epithelium where the basal cells proliferate, mature and differentiate to form keratin squames. When the distribution pattern of bcl-2 expression was assessed, it was observed that the tumour islands which were devoid of central keratinization showed bcl-2 expression in all the tumour cells. However, those islands which had keratinized neoplastic cells showed diminished or no immunoreactivity in the centre with staining which was restricted to only the peripheral cells. These observations are similar to those of ***Teni et al (2002)*** [11].However, ***Loro et al (1999)*** observed that the tumour islands devoid of any keratin in the central parts of the islands showed a progressive loss of bcl-2 immunoreactivity[13]. which suggests that the central cells are differentiated and further, have the potential to terminally differentiate. In poorly differentiated tumours, immunoreactivity for bcl-2 was seen throughout the tumour cell population. This overexpression possibly reflects the resistance of these tumour cells to apoptosis and increased cell survival.

Bcl-2 immunoreactivity in the normal oral mucosa is confined to the basal cell layer and possibly is involved in the preservation of an adequate reservoir of proliferating stem cells, whereas the other apoptotic proteins like bax predominate in the superficial layers[13].In all the cases examined for the overlying dysplastic epithelium, bcl-2 expression was observed even in the superficial layers, suggesting an alteration in the normal expression of bcl-2. Non-neoplastic epithelium adjacent to OSCCs observed in a few cases, showed expression of bcl-2 in a few basal cells as was also seen in normal epithelium which was taken as control.

Bcl-2 protein upregulation in tumour microvascular endothelial cells can be induced by vascular endothelial growth factor (VEGF) which enhances the availability of oxygen and nutrients for tumour cells. Bcl-2 expression was also observed in the endothelial cells lining the

blood vessels. ***Nor JE et al (2001)*** observed bcl-2 expression in endothelial cells and suggested that these cells that overexpress bcl-2 secrete increased amounts of chemokine IL-8 which functions as an endogenous inducer of tumour angiogenesis [14].

Bcl-2 expression was also observed in a few duct cells of the minor salivary glands, which is consistent with the findings of ***Qi Long et al (1993)*** who examined the bcl-2 expression in embryonic non-haematopoietic tissues, wherein bcl-2 expression was seen in all the duct cells of all exocrine glands including salivary, pancreas and sweat glands. The expression seen in few of the duct cells, possibly suggests that these cells are undifferentiated reserve cells [15].

The findings from our study show that inhibition of apoptosis is a frequent event in squamous cell carcinomas. The bcl-2 family of proteins appears to be involved in regulating the terminal differentiation of keratinocytes. The downregulation of bcl-2 expression was concomitant with terminal differentiation and an increased over expression of this protein protects the tumour cells from undergoing apoptosis, thus facilitating their survival.

However, additional studies by using a larger sample size and by assessing the family of bcl-2 proteins may provide useful information for a more accurate tumour classification based on a biological basis.

Summary And Conclusion

In OSCCs, the number of cells expressing bcl-2 increased from well differentiated to poorly differentiated, showing an inverse relationship with the degree of differentiation of the tumour. As the onco-protein bcl-2 regulates programmed cell death by allowing the tumour cells to escape apoptosis, it promotes their survival, thereby facilitating the acquisition of further mutations.

Further correlative studies using markers for other members of the bcl-2 family are necessary to elucidate the specific molecular defects critical to the biology of this tumour, which will have an impact on diagnosis and treatment.

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