

## **Bcl 2 Proto – oncogene expression correlate with grading of oral carcinoma.**

ظهور الناتج الجيني المنشط للسرطان BCl<sub>2</sub> وعلاقتها بدرجة التمايز في سرطانات الفم الحرشفية

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### **Summary**

**Introduction :-** Bcl-2 is known to belong to a family of apoptosis regulatory gene products that may be death antagonists (eg Bcl-2 , Bcl-X<sub>L</sub> , Mcl-1 , Al) or death agonists (eg. Bax , Bak , Bcl-X<sub>S</sub> , Bad) .

**Aims :-** To identify Bcl-2 oncogene product expression and its correlation to histological grades of oral carcinoma .

**Patients and methods :-** Twenty four patients were presented with oral carcinoma , Formalin-fixed paraffin-embedded sections were stained with H and E and immunohistostaining . Primary antibody kit (Bcl-2 onco-protein ready to use) clone 124 Dako corporation .

**Results:-** Bcl-2 Immunohistochemical expression is confined to the basal cell layers in normal oral mucosa , while Bcl-2 expression in oral carcinoma is peripherally located with in infiltrating tumor cells , which were more intensely stained . Our results reveal that Bcl-2 expression was highly correlated with moderately differentiated (G2) and poorly differentiated (G3) of oral carcinoma and Fischer exact probability test reveals that it is statistically positively significant P=0.0027 .

**Discussion** Our interpretation may be an over expression of Bcl-2 participates in the differentiation of normal oral keratinocytes , or may be that over expression of Bcl-2 oncogene lead to stop the apoptosis and this will lead to increase the cell proliferation of genetically mutated cells which appear as abnormal architecture of cells and disturb normal histological appearance .

**Conclusion :-** Bcl-2 expression in normal oral mucosa and oral carcinoma Bcl-2 immunoreactivity correlated with.Bcl-2 expression was highly associated with moderately differentiated (G2) and poorly differentiated (G3) oral carcinoma .

**Keywords:** grading of oral carcinoma, Bcl -2 proto-oncogene expression .

### **الخلاصة**

BCl<sub>2</sub> هو جين يعود الى عائلة تنظم عملية برنامج انتحار الخلايا و إما ان يكون مقاوم لانتحار الخلايا مثل (BCl<sub>XL</sub> ، BCl<sub>2</sub>) او ان يكون منشط لبرنامج انتحار الخلايا مثل (Al ، MCI-I ، Bax ، Bak ، Bcl<sub>Xs</sub> ، Bad) .  
**الهدف** من هذا البحث اظهار وجود BCl<sub>2</sub> الناتج الجيني في خلايا سرطان الفم الحرشفية وعلاقتها بالدرجة النسيجية او درجة

التمايز النسيجي (grade).  
المرضى وطرق العمل. تم اختيار 24 مريض مصاب بسرطان الفم . العينات النسيجية المثبتة بالفورمالين والمحفوظة في الشمع صبغت بصيغة الهيماتوكسلين والايوسين ان علبة الناتج الجيني BCl<sub>2</sub> استوردت من شركة داکو واجريت عملية المناعة النسيجية الكيميائية لاطهار وجود الناتج الجيني BCL-2 في خلايا سرطانات الفم الحرشفية .  
النتائج ان الناتج الجيني BCl<sub>2</sub> موجودة في خلايا القاعدة (المولدة) لبطانة الفم الحرشفي بينما ظهر في الخلايا السرطانية جانبياً ما بين الخلايا السرطانية وأكثر صبغة في سايٲوبلازم الخلايا السرطانية , اظهرت لنا النتائج ان لظهور BCl<sub>2</sub> الزائد في الخلايا السرطانية مع علاقة درجة التمايز (الدرجة المتوسطة والدرجة الفقيرة) وعند تطبيق اختبار احتمالية فشر للنتائج تبين انها احصائياً معنوية p=0.0027 .  
المناقشة يمكن تحليل ذلك ان زيادة ظهور BCl<sub>2</sub> التي تسيطر في عملية التمايز للخلايا المنقسمة لنسيج الفم الحرشفي قد يكون بسبب ان الزيادة المضطربة في BCl<sub>2</sub> قد تؤدي الى ايقاف عملية برنامج الانتحار للخلايا ومما يؤدي الى زيادة في عملية انقسام الخلايا التي هي جنينياً تشكو من طفرات وراثية مما يؤدي الى ظهور خلايا غير طبيعية وذات مظهر بنائياً وتركيبياً غير طبيعي. ان ظهور الناتج الجيني لـ BCl<sub>2</sub> توجد له علاقة احصائياً معنوية مع التمايز النسيجي ذو الدرجة المتوسط والفقير لسرطان الفم الحرشفي .  
الاستنتاج ان ظهور BCl<sub>2</sub> في الخلايا السرطانية له علاقة احصائياً بدرجة معنوية مع التمايز النسيجي ذو الدرجة المتوسط والفقير لسرطان الفم الحرشفي وهذا يساعدنا في التشخيص والعلاج ودراسة التطورات المستقبلية لسرطان فظهور زيادة في BCl<sub>2</sub> يعني ان السرطان في التمايز النسيجي ذو الدرجة المتوسط والفقير لسرطان الفم الحرشفي.

## Introduction.

Cancer is a disorder of cell growth and behavior , it is a multi step process of both the phenotypic and genetic level <sup>1</sup>.Carcinogenesis is a multi step process involving the activation of oncogenes and the in activation of tumor suppressor genes . Since the majority of human tumors manifest as an imbalance of the regulatory cell-cycle control processes , the critical check-point of cell growth and the identification of molecules involved in such mechanisms could help to elucidate the carcinogenetic process of the head and neck tumors <sup>2</sup>.Bcl<sub>2</sub> was first described in follicular lymphoma that bear t(14:18) (q<sup>32</sup>,q<sup>21</sup>) translocation<sup>3</sup> . This structural chromosomal aberration lead to over production of Bcl<sub>2</sub> messenger RNA and protein . Bcl<sub>2</sub> is localized at outer mitochondrial and the nuclear membrane : as well as at endoplasmic reticulum <sup>4</sup>.Bcl<sub>2</sub> is known to belong to a family of apoptosis – regulatory gene products that may be death antagonists (eg Bcl-2 , Bcl-X<sub>L</sub> , Mcl-1 , A1) or death agonists (eg. Bax , Bak , Bcl-X<sub>S</sub> , Bad) <sup>5</sup> . The Bcl-2 oncoprotein inhibits apoptosis and is expressed by many tumors including carcinoma such as of breast , cervix and head and neck <sup>6</sup> . Apoptosis is a genetically determined process playing an active role in tissue size regulation , morphogenesis and removing damaged cell that could be potentially dangerous for their host . Several agents involved in apoptosis regulation such as Bcl<sub>2</sub> family component act as on oncogenes and are involved in oral carcinogenesis <sup>2</sup> .

The Bcl<sub>2</sub> gene Located at chromosome 18 q(long arm) 21 , it is prototype of cell death regulatory genes and its protein product , the Bcl<sub>2</sub> protein , blocks a distal step in an evolutionarily conserved pathway of apoptosis <sup>2</sup> .

Bcl2 Family . The Bcl<sub>2</sub> protein family plays a central part in the control of apoptosis <sup>7</sup> , <sup>8</sup> . Bcl<sub>2</sub> , a 52 KD protein , is the prototype of this family , and inhibits the induction of apoptosis Bcl-x<sub>L</sub> , Mcl-1 , Bcl-w and A1 are other antiapoptotic members . Where as Bax ,Bad , Bid and Bcl-x<sub>S</sub> are pro-apoptotic <sup>9</sup> .

## Patients and methods :

A. Study group : Twenty four patients 11 males and 13 females their age between (19-80) years and mean was (52.3) , complaining of oral carcinoma , 20 patients had squamous cell carcinoma and 4 patients with adenoid cystic carcinoma. The patients were gathered from Al-Wasitti hospital and Specialized Surgical Hospital in the Medical City Baghdad .

B. Control group : 5 specimens of normal oral mucosa were obtained from healthy patients who had undergone routine oral surgery , prosthetic , orthodontic purpose were included as control following informed consent of the patient .

C. Positive control : three specimens of follicular lymphoma paraffin blocks were obtained from the pathology department at the teaching laboratory of medical city

D. Negative control : 3 specimens of randomly selected paraffin blocks of the study groups untreated with the primary antibody (mono clonal BCL2 ) were considered as negative control for each set of slides

### **Methods :**

Study group , 24 specimens of excisional biopsies of oral carcinoma were taken 20 specimens of squamous cell carcinoma and 4 adenoid cystic carcinoma

Histo-pathology : Four  $\mu$  m thickness consecutive sections were cut from each paraffin blocks of the (Study group, and the control positive group) . The slides were stained by haematoxylin and Eosin stain in teaching laboratory of medical city to appropriately define tumor grade in a 3 tier scale well differentiated G1 , moderately differentiated G2 , poorly differentiated G3<sup>10</sup>.

Immunohistochemical procedure : A fresh 4 um thickness paraffin embedded tissue slices collected on fisheral glass slides<sup>2</sup> . stained by immunohistochemical procedure at the department of pathology college of medicine as follows .

Twenty-four Slides represented squamous cell carcinoma and adenoidcysitic carcinoma study group (A) .Five Slides represented normal oral mucosa control group (B). Three Slides represented follicular lymphoma positive control (C) .Three slides of study group untreated with primary antibody (Bcl<sub>2</sub> protein) negative control group (D) .

Immunohistochemical procedure of Bcl<sub>2</sub> protein *Mikel UV 1994* .

1. Dewaxing slides A,B,C and D. A Slides were kept in oven 30 min. at 70 °.
2. Slides immersed in xylol for 10-20 min.
3. Rehydration slides A,B,C and D were kept in alcohol for 5 min. decreasing concentration 100 , 90 , 80.
4. Retrieval (high concentration) code No. S3307 diluted 1/9 with distal water and kept the diluted retrieval in special jar which contains all slides A , B , C and D .
5. Then the jar is kept in water bath at temperature 90 – 95 C° for one hour never above 100C°.
6. Jar with slides A , B , C were left to be cooled to room temperature for one hour .
7. Diluted buffer phosphate solution 4% prepared and (4 ml of buffer added to 96 ml of distal H<sub>2</sub>O) .
8. Slides A , B , C were kept in buffer solution for one minute the slides were dried by filter papers .
9. Slides A , B , C were kept in hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) for 5 min.
10. Slides were kept in buffer solution 1-2 min. and left to dry , make a circle by PAP pen on each slide .
11. One drop of Monoclonal primary antibody Bcl<sub>2</sub> ready to use were applied to slides A , B and for 30 min. but not added to slides group D (negative control) .
12. Slides A , B , C and D were kept in buffer solution for 1-2 and dried it by filter papers .
13. One drop of Biotinlated link (yellow bottle) secondary antibody ready to use were applied to all slides A , B , C and D for 20 min.
14. All slides were immersed in the buffer solution for 1-2 min. then dried it by filter papers .
15. One drop of Label (red bottle) ready to use were applied to all groups A , B , C and D for 20 min.
16. Slides were kept in buffer solution for 1-2 min . Then dried it by filter papers .
17. One drop of (DAB) substrate chromagen solution was added to each slide A , B , C and D for 5-15 min.
18. Slides were kept in buffer solution 1-2 min. and dried it .
19. Slides were kept in hematoxylin counter stain for one minute .
20. Slides were washed by distal H<sub>2</sub>O for 3 min .
21. Slides were dried and immersed in alcohol with increasing concentration for 70 , 80 , 90 to 100 for 2 min .
22. Slides were kept in xylol for 5 min .

23. Mounting of the slides few drops of DPX were applied on the cover and put the cover on the slide , Examination of the slides by light microscope .

**Score of Bcl-2 Immunohistochemical expression of oral carcinoma**

Bcl-2 Immunohistochemical expression was semi quantitatively evaluated in at least 1000 cells examined at 40x10 magnification , and recorded as the percentage of Bcl-2 positive tumor cells over the total number of neoplastic cells present in the same area , Score 0(negative) no staining or staining in <5% tumor cells , score 1(weak positive) staining in 5-24%tumour cells,score2(moderate positive) staining in 25-50% tumor cells , score 3 (strong positive) staining in >50% tumor cells<sup>2</sup> .

**Statistical Analysis:**

Statistical analysis had been made using the fischer exact probability test with a P value of < 0. 05 considered statistically significant .

**Results :**

Twenty four cases with oral carcinoma [20 cases of squamous cell carcinoma and 4 cases of adenoid cystic carcinoma] The age range was between (19-80) years and the mean was (52.3) .

Results of Bcl<sub>2</sub> expression :

**1. Negative control group :** Three cases of study group without application of primary antibody (Bcl<sub>2</sub> oncoprotein) represented no cytoplasmic immuno reactivity .

2. All three cases of follicular lymphoma (**positive control group**) were positive for Bcl<sub>2</sub> expression detected in lymphocytes cells . (figure – 4 )

3. Result of Bcl<sub>2</sub> expression in **study group** :

Positive expression in oral carcinoma (squamous cell carcinoma and adenoid cystic carcinoma) consistent Bcl<sub>2</sub> cytoplasmic immunoreactivity was detected in 14 cases of squamous Cell Carcinoma and 4 cases of adenoid cystic carcinoma . It means 18 cases was positive out of 24 cases Bcl<sub>2</sub> expression is seen in peripherally located with infiltrating tumour cells where more intensely stained (figure – 5 ,6, 7). Bcl-2 expression and grading of oral carcinoma .

Two cases (8.34%) of the total number of well differentiated oral carcinoma (G1) showed a positive Bcl-2 expression , 16 cases (66.66%) of moderately and poorly differentiation ( G2 , G3 ) oral carcinoma showed a positive Bcl-2 expression .

The Fischer exact probability test reveals that statistically positively significant P=0.0027.Bcl-2 expression was highly correlated with moderately and poorly differentiated oral carcinoma (Table – 5, Fig-9) .



Figure (1)

Photograph of the clinical features of squamous cell carcinoma



Figure (2)  
Photograph of the clinical features of adenoid cystic carcinoma

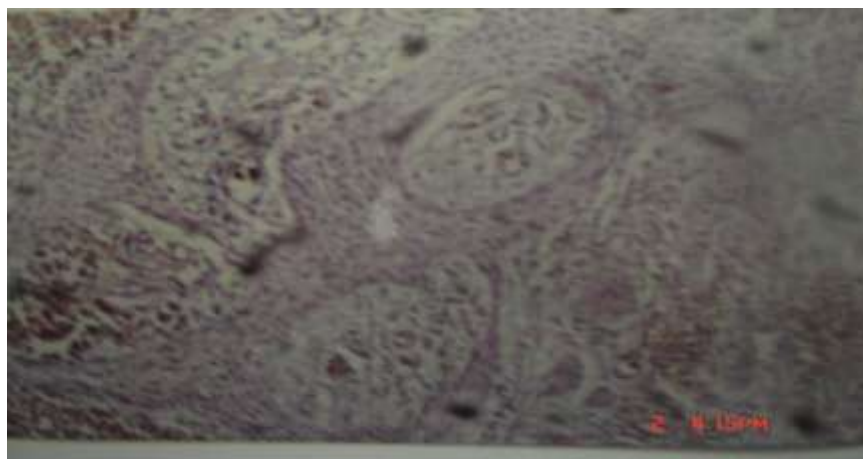


Figure (3)  
Follicular lymphoma ( H & E ) ( 40x 10) .

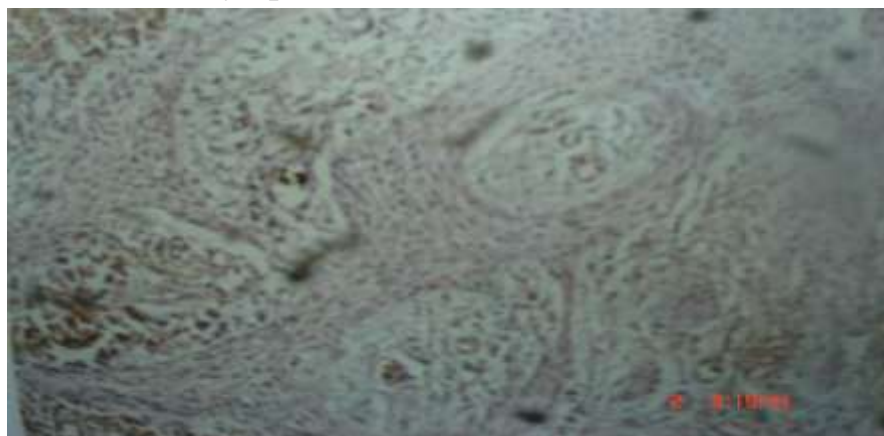


Figure (4)  
Bcl 2 expression of Follicular lymphoma ( strong positive ) ( 40x 10).



Figure (5)  
Bcl 2 expression of moderately differentiation squamous cell carcinoma  
( weak positive score 1 ( 10 x 10 )



Figure (6)  
Bcl 2 expression of moderately differentiation squamous cell carcinoma  
( moderately positive score 2 (10 x 10 ).



Figure (7)  
Bcl 2 expression of poorly differentiation adeoid cystic carcinoma  
( strongly positive score 3 (10 x 10 ).

Table (1) grading of different type of oral carcinoma.

Grading	Squamous cell carcinoma		Adenoid cystic carcinoma		Total	
	No.	(100%)	No.	(100%)	No.	(100%)
Well differentiation G1	7	(29.16%)	0	(0%)	7	(29.17%)
Moderately differentiation G2	9	(37.5%)	2	(8.34%)	11	(45.83%)
Poorly differentiation G3	4	(16.66%)	2	(8.34%)	6	(25%)
Total	20	(83.4%)	4	(16.6%)	24	(100%)

Table (2) Distribution of grading (degree of differentiation) by positive and negative Bcl2 expression of oral carcinoma

Grading	Positive Bcl2 expression		Negative Bcl2 expression		Total	
	No.	(100%)	No.	(100%)	No.	(100%)
G1	2	(8.34%)	5	(20.83%)	7	(29.17%)
G2+G3	16	(66.66%)	1	(4.17%)	17	(70.83%)
Total	18	(75%)	6	(25%)	24	(100%)

**P= 0.0027 statistically positively significant**

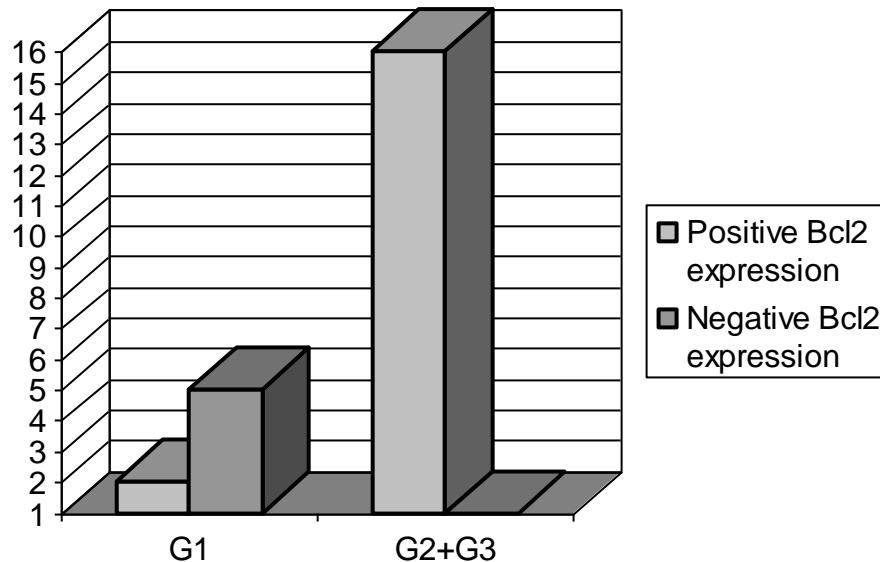


Fig. (8) Distribution of grading ( degree of differentiation ) by positive and negative Bcl2 expression of oral .

## **Discussion**

Bcl-2 expression was highly correlated with moderately and poorly differentiated (G2+G3) of oral carcinoma, the Fischer exact probability test reveals that statistically positively significant  $P=0.0027$ . Our interpretation may be an over expression of Bcl-2 participates in the differentiation of normal oral keratinocytes, or may be that over expression of Bcl-2 oncogene lead to stop the apoptosis and this will lead to increase the cell proliferation of genetically mutated cells which appear as abnormal architecture of cells and disturb normal histological appearance. This finding similar to the finding of Casse *et al* 1998 they reported that increased Bcl-2 expression in cancer cells possibly reflects tumour cell resistance to apoptosis and it agree with Chen *et al* 2000, they reported that a correlation between altered Bcl-2 and bax expression and tumour differentiation. Wilson *et al* 2001, reported that Bcl-2 over expression has been reported in several different human cancers and correlated with tumour differentiation. Our results in agree with Muzio *et al* 2003 they reported that Bcl-2 immunoreactivity in G2 and G3 oral squamous cell carcinoma was statistically significant and also with Hussain *et al* 2003 they reported that Bcl-2 over expression is associated with high grade of bladder carcinoma. Our study showed (2 cases) of exophytic type of adenoid cystic carcinoma. The primary lesions presented as stage III and Bcl2 expression was positively weak. Recurrence of oral carcinoma was presented as stage IV was received chemotherapy and radiation and Bcl2 expression was strongly positive. Failure in inducing programmed cell death may due to over expression of Bcl2 gene products which act as anti apoptotic effect or may be due to altered to the Bcl2 / Bax ratio. Apoptosis occurs in response to many different stimuli, including UV, ionizing radiation, chemotherapy, hypoxia, oncogene expression, tumour – suppressor gene in activation and growth factors Jameson 1998. Similar finding of Hussain *et al* 2003 they reported that Bcl2 expression in bladder tumours adversely effects chemotherapy and radiosensitivity. Additional finding of Kong *et al* 1998 they reported that improved survival of patients with Bcl2 negative tumours with chemotherapy or radiotherapy. In agreement with Casse *et al* 1998 they reported that increased Bcl2 expression in cancer cells possibly reflects tumour cell resistance to apoptosis and may have implication for their responsiveness to treatment. In Kerr *et al* 1994, reported that the possible role of increased Bcl2 proto – oncogene expression in the development of resistance of tumours to anti – cancer drugs also this finding supported by the finding of Fisher *et al* 1993, Miyashita *et al* 1992, Collins *et al* 1992; they reported that Bcl2 gene products inhibits apoptosis occurring in a variety of circumstances, can greatly increase their resistance to the apoptosis – inducing effect of anti cancer drugs.

**Conclusions** Bcl-2 immunoreactivity in moderately differentiated (G2) and poorly differentiated (G3) oral carcinoma was statistically significant. Bcl-2 immunoreactivity was directly correlated with grading of oral carcinoma and Bcl-2 expression can be consider as tumor marker for diagnosis and detect prognosis of lesions.

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