

## Expression of Anti Apoptotic Gene in Exophytic and Endophytic Type of Oral Carcinoma

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### Abstract

**B**ackground: Bcl2 is known to belong to a family of apoptosis regulatory gene products that may be death antagonist eg (bcl- 2, bcl-x<sub>1</sub>, mcl -1) or death agonist ex (bax , bak , bcl -x<sub>s</sub>, bad) , The bcl- 2 oncoprotein inhibits apoptosis and is expressed by many tumors including carcinoma such as breast ,cervix and head and neck .

Aims to identify bcl-2 oncogene product expression of oral carcinoma and its correlation to clinical features (exophytic , endophytic and mixed type) of oral carcinoma

**Patients and methods** Twenty four patients were gathered , 20 patients had squamous cell carcinoma and four patients with adenoid cystic carcinoma . Paraffin blocks were obtained of the study group , specimens of follicular lymphoma paraffin blocks were obtained from pathology department at teaching laboratory of medical city . Normal oral mucosa were obtained from healthy patients who had undergone routine oral surgery following informed consent of the patients . Immunohistochemical procedure of Bcl2 protein were applied .

**Results** our study showed that 11 cases of the exophytic type of oral carcinoma represented a positive bcl2 expression , 4 cases of the endophytic (ulcerative type) showed a positive immunoreactivity with bcl2 antibodies and 3 cases of mixed type (exophytic type and endophytic) showed a positive bcl2 expression . Statistical analysis showed non significant relationship between clinical features (exophytic , endophytic , Mixed type) and bcl2 expression in oral carcinoma .

**Conclusion :-** bcl-2 expression in normal oral mucosa and oral carcinoma ,bcl-2 immunoreactivity was more correlated to mixed type , exophytic and rarely to endophytic oral carcinoma but all didn't reach a statistical significance .

**Keywords** ,anti apoptotic gene product , bcl2 expression , oral carcinoma.

### الخلاصة

ان الاعراض السريرية لسرطان الرأس والرقبة من الصعب معرفته اعتماداً على الفحص النسيجي فقط . لذا ايجاد دلائل او علامات جزيئية تستطيع بدقة معرفة هذا المرض واطهار سلوكه السرطاني والنتيجة السيئة . التسرطن هي عملية متعددة الخطوات تشمل تنشيط الجين المنشط للسرطان وتثبيط الجين المثبط للسرطان .

BCI<sub>2</sub> هو جين يعود الى عائلة تنظم عملية برنامج انتحار الخلايا و إما ان يكون مقاوم لانتحار الخلايا مثل BCI<sub>x's</sub> ، Bak ، Bax او ان يكون منشط لبرنامج انتحار الخلايا مثل (Al ، MCI-I ، BCI<sub>XL</sub> ، BCI<sub>2</sub>) ، (Bad ،

الهدف: من هذا البحث اظهر وجود BCI<sub>2</sub> الناتج الجيني في خلايا سرطان الفم الحرشفية وعلاقتها بالمرحلة او الطور (Stage) وكذلك بالدرجة النسيجية او درجة التمايز النسيجي (grade).

المرضى وطرق العمل: تم اختيار 24 مريض مصاب بسرطان الفم اختيروا في مستشفى الواسطي ومستشفى الجراحات التخصصية . ونماذج من الورم تم حفظها بالفورمالين وبعدها حفظت بالشمع ثم صبغها بالهيماتكسلين

والايوسين (H&E) وكذلك مع صبغة المناعة النسيجية . ان علبة الناتج الجيني Bcl<sub>2</sub> استوردت من شركة داکو واجريت عملية المناعة النسيجية الكيميائية لظهار وجود الناتج الجيني Bcl-2 في خلايا سرطانات الفم الحرفية .

**النتائج:** تبين من النتائج ان الناتج الجيني Bcl<sub>2</sub> موجودة في خلايا القاعدة (المولدة) لبطانة الفم الحرفي بينما ظهر في الخلايا السرطانية جانبياً ما بين الخلايا السرطانية وأكثر صبغة في سايتوبلازم الخلايا السرطانية . يمكن ان يكون تحليل ذلك الى ان في الحالة الطبيعية الخلية التي يحدث فيها خلل تكويني هناك جين مسيطر على عملية عدم السماح لها بالانقسام او يقوم بعزل الخلية والتخلص منها بعملية الانتحار المبرمج او لاختلاف النسبة او العلاقة بين Bax/Bcl<sub>2</sub> هذين الناتجين الجينيين الذي قد يؤدي الى زيادة ظهور الناتج الجيني Bcl<sub>2</sub> . ان من نتائج الدراسة اظهرت عدم وجود علاقة بين ظهور Bcl-2 الزائد مع الفئة العمرية او الجنس ولكن هناك علاقة بين وجود Bcl-2 الزائد مع نوع من السرطان الحرفي من نوع (النمو السرطاني المنتفخ البارز) وكذلك علاقة Bcl<sub>2</sub> البارز مع المرحلة الثالثة والرابعة ولكن هذه العلاقة لا ترقى الى معنوية احصائياً . كذلك اظهرت لنا النتائج في هذا البحث 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 الحرفي او قد يكون بسبب ان الزيادة المضطربة في Bcl<sub>2</sub> قد تؤدي الى ايقاف عملية برنامج الانتحار للخلايا مما يؤدي الى زيادة في عملية انقسام الخلايا التي هي جينياً تشكو من طفرات وراثية مما يؤدي الى ظهور خلايا غير طبيعية وذات مظهر بنائياً وتركيبياً غير طبيعي . ان الناتج الجيني Bcl<sub>2</sub> يظهر في خلايا الغشاء الفمي الطبيعي وكذلك يظهر بشكل (أكثر تواجداً) في النسيج السرطاني . ان ظهور الناتج الجيني Bcl<sub>2</sub> توجد له علاقة مع المراحل المتأخرة لسرطان الفم الحرفي ولكن هذا لم يصل الى الدرجة احصائياً معنوية ولكن العلاقة احصائياً معنوية مع التمايز النسيجي ذو الدرجة المتوسط والفقير لسرطان الفم الحرفي .

**الاستنتاج:** ان ظهور Bcl<sub>2</sub> يمكن اعتباره مساعد في التشخيص وكذلك يفيدنا في عملية تحسس وجود السرطان في الجوانب والحافات بعد رفع السرطان وكذلك يفيدنا في التحسس المبكر لعودة السرطان ثانية يضاف الى جملة الفحوصات التي نجرها مثل أخذ الخزعة النسيجية ، اخذ الرقائق الشعاعية ، فحص التردد الرنيني ، فحص المفراس ، والمؤشرات او العلامات السرطانية .

## Introduction

Tumor cell growth results from a disturbance in the balance between the rate of proliferation and cell death by apoptosis<sup>(1)</sup>. Carcinogenesis is a multi step process involving the activation of oncogene and the inactivation of tumor suppressor genes ,since the majority of human tumors manifest as an imbalance of the regulatory cell- cycle control process , 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>. There are three classes of normal regulatory genes , the growth promoting proto- oncogene, the growth inhibiting cancer suppressor gene (anti-oncogene) ,and gene that regulate programmed cell death are the

principle targets of genetic damage<sup>(3)</sup> . Apoptosis is a genetically determined process playing an active role in tissues size regulation , morphogenesis and removing damaged cell that could be potentially dangerous for their host .Several agents involved in apoptosis regulation such as bcl2 family component act as oncogene and are involved in oral carcinogenesis<sup>(2)</sup>. Apoptosis can result either from activation of a physiologic cell suicide programs or from cell injury induced by various noxious stimuli such as irradiation , cytotoxic chemical agents and hyperemia<sup>(4)</sup>. bcl2 is known to belong to a family of apoptosis regulatory gene products that may be death antagonist eg (bcl- 2, bcl-x<sub>1</sub>, mcl -1) or death agonist 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 breast ,cervix and head and neck

<sup>(6)</sup>.The principle type of tumor with bcl- 2 over expression is follicular lymphoma .

## Patients and method

**A. Study group.** Twenty four patients 11 males and 13 females their age between

(19 -80) years , 20 patients had squamous cell carcinoma and 4 patients with adenoid cystic carcinoma (selective sample). patients were gathered from Al- Wasitti hospital and specialized surgical hospital .

**B. Control group .** 5 specimens of normal oral mucosa were obtained from healthy patients who had undergone routine oral surgery following informed consent of the patients .

**C . Positive control .**3 specimens of follicular lymphoma paraffin blocks were obtained from pathology department at teaching laboratory of medical city .

**D .Negative control .**3 specimens of randomly selected paraffin blocks of the study group untreated with the primary antibody ( mono clonal Bcl<sub>2</sub> ) were considered as negative control

### Materials

Primary antibody kit (7ML Bcl<sub>2</sub> oncoprotein ready to use) clone 124 Dako corporation 6392 via real :803/566 6688 Denmark , secondary antibody kit , biotinlated link , label red bottle ,target retrieval solution high PH code No. S3307 .

### Methods

1 .Histopathology . Four µm thickness consecutive sections were cut from each paraffin blocks of the (study group , Control group ,and Positive control group) , the slides were stained by Hematoxylin and Eosin stain in the teaching laboratory of medical city .

2 . immunohistochemical procedure .

A fresh 4 µm thickness paraffin embedded tissues slices collected on

fisheral glass slides **Muzio et al 2003** , 24 slides represented squamous cell carcinoma and adenoid cystic carcinoma (**study group A**), 5 slides represented normal oral mucosa (**control group B**) , 3 slides represented follicular lymphoma (**Positive control group C**) , 3 slides of study group untreated with primary antibody (bcl2 protein)(**negative control group D**).

### Immunohistochemical procedure of Bcl2 protein <sup>(7)</sup> :

1. Dewaxing slides A, B , C and D , slides were kept in oven 30 minute at 70 C<sup>0</sup>.
2. Slides immersed in xylol for 10 -20 minute .
3. Rehydration of the slides A, B , C , and D were kept in alcohol for 5 minutes , 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 a temperature 90 – 95C<sup>0</sup> for one hour never above 100 C<sup>0</sup>.
6. Jar with slides A ,B , C and D 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 water , slides were kept in buffer solution for one minute .
8. Slides were dried by filter paper .
9. All slides were kept in hydrogen peroxide for 5 minutes.
10. Slides were kept in buffer solution 1 -2 minutes then left to dry .
11. One drop of monoclonal primary antibody Bcl2 ready to use were applied to slides A , B , and C for 30 minute but not added to slides group D ( negative control ) .

12. Slides A , B , C , and D were kept in puffer solution for 1 – 2 minute and dried by filter papers .
13. One drop of biotinlated link secondary antibody ready to use were applied to all slides A , B , C and D for 20 minute.
14. All slides were immersed in the buffer solution for 1 -2 minutes ,then dried it by filter papers .
15. One drop of label ready to use were applied to all groups A , B , C and D for 20 minute.
16. Slides were kept in buffer solution for 1 2 minutes ,then dried it by filter papers .
17. One drop of (DAB) substrate chromagen solution was added to each slides A , B ,C and D for 5 -15 minute .
18. Kept all slides in buffer solution for 1 -2 minutes and dried it .
19. Slides kept in Hematoxylin counter stain for one minute .
20. Slides were washed by distal water for 3 minute .
21. Slides were dried and immersed in alcohol with increasing concentration for 70 ,80 ,90 to 100 for 2 minute .
22. Slides were kept in xylol for 5 minute.
23. Mounting of the slides few drops of DPX were applied on the cover and put cover on the slide

#### Score of Bcl2 immunohistochemical expression.

Bcl2 immunohistochemical expression was semi quantitatively evaluated in at least 1000 cells examined at 40x 10 magnification ,and recorded as the percentage of Bcl2 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 % tumor cells , score 2 (moderate positive) staining in 25 -50 % tumor

cells score 3 (strong positive) staining in <50 % tumor cells<sup>2</sup> .

#### Results

Twenty four patients 11 males and 13 females their age between (19 -80 ) years, 20 patients had squamous cell carcinoma and 4 patients with adenoid cystic carcinoma ,high frequency was recorded in age groups (60 -69) years which constituted 25% of total number of patients ,table (1)

Type of clinical features of oral carcinoma :

Our study showed that 12 cases (50%) of oral carcinoma represented as exophytic type ( 8 cases 33.4% were squamous cell carcinoma and 4 cases 16.6 of adenoid cystic carcinoma). 9 cases 37.55 of squamous cell carcinoma presented as ulcerative (entophytic) type and 3 cases (12.5%) of squamous cell carcinoma represented as mixed type ,table 2 .

1. Results of bcl2 expression in normal oral mucosa 5 cases of ( **normal control group**) displayed bcl2 immunohistochemical expression to nearly all basal Keratinocyte or apical portion of crypts (Figure – 1) .
2. All three cases of follicular lymphoma (**positive control group**) were positive for bcl2 expression detected in lymphocytes cells (figure -3) .
3. **Negative control group** ,three cases of study group without application of primary antibody (Bcl2 oncoprotein) represented no cytoplasm immunoreactivity .
4. Bcl2 expression in **study group**, positive expression of bcl2 cytoplasm immunoreactivity was detected in 18 cases of oral carcinoma , 14 cases squamous cell carcinoma and 4 cases adenoid cystic carcinoma (figure 4,5,6) .

5. Bcl2 expression and clinical feature , our study showed that 11 cases (45,84%) of the Exophytic type of oral carcinoma represented a positive bcl2 expression and only one case didn't show any immunoreactivity with bcl2 antibodies , 4 cases (16,66) of the endophytic (ulcerative type) presented a positive immunoreactivity with bcl2 antibodies and 3 cases (12.5 %) of mixed type (exophytic type and endophytic) presented a positive bcl2 expression. Table 3. Statistical analysis had been using the Fischer exact probability test with a P value of <0.05 considered statistically non significant between clinical features (exophytic, endophytic , mixed

type) and bcl2 expression in oral carcinoma.

## Discussion

Results showed that Bcl2 immunoreactivity in normal oral mucosa is confined to the basal cell layers , Bcl2 proto-oncogene possibly is involved in the preservation of an adequate reservoir of proliferating stem cells , our results agreed with **Muzio et al 2003** <sup>(2)</sup> reported that normal oral epithelium displayed Bcl2 immunohistochemical expression limited to nearly all basal Keratinocyte , in agreement with **Backus et al 2002** <sup>(1)</sup> reported that Bcl2 staining was limited to apical to area in normal colonic mucosa cells .

Table 1. Distribution of oral carcinoma by age and gender

Age	Males	Females	Total
20-29	0	2	2
30-39	2	1	3
40-49	3	2	5
50-59	1	4	5
60-69	3	3	6
70-80	2	1	3
	11	13	N=24

Table 2. Distribution of clinical features with type of oral carcinoma

Clinical features	Squamous cell carcinoma		Adenoid cystic carcinoma		Total	
	No.	(100%)	No.	(100%)	No.	(100%)
Exophytic type	8	(33.4%)	4	(16.6%)	12	(50%)
Endophytic type	9	(37.5%)	0	(0%)	9	(37.5%)
Mixed type	3	(12.5%)	0	(0%)	3	(12.5%)
Total	20	(83.4%)	4	(16.6%)	24	(100%)

Table 3. Distribution of clinical features by positive and negative Bcl2 expression of oral carcinoma

Clinical features	Positive Bcl2 expression		Negative Bcl2 expression	
	No.	(100%)	No.	(100%)
Exophytic type	11	(45.84%)	1	(4.16%)
Endophytic type	4	(16.66%)	5	(20.84%)
Mixed type	3	(12.5%)	0	(0%)
Total	18	(75%)	6	(25%)



figure 1. Bcl-2 expression of normal oral mucosa (10x10)

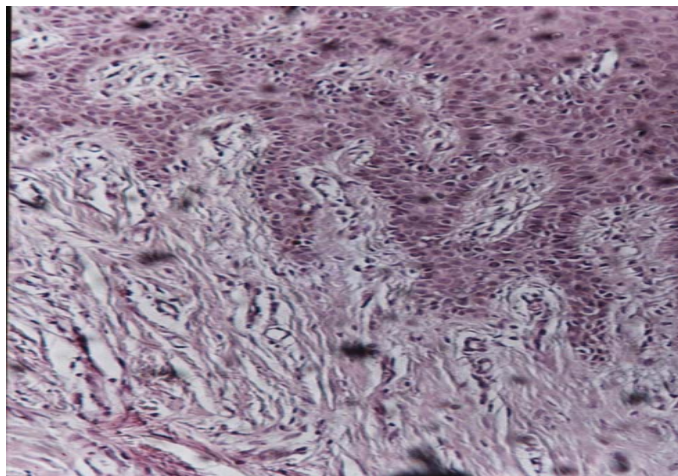


Figure 2. Norma oral mucosa (Hematoxylin and eosin) (10x10)

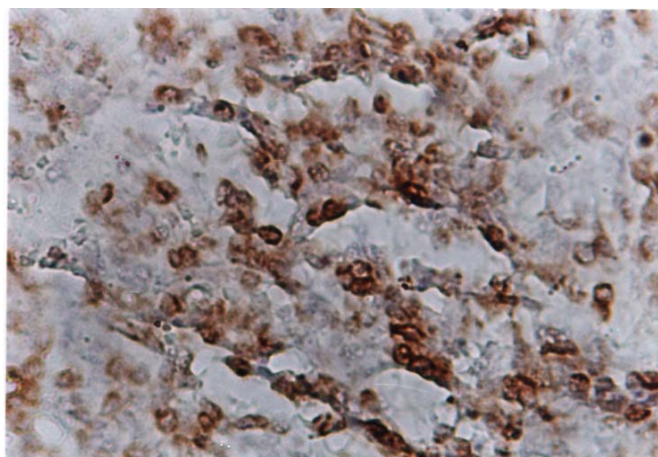


Figure 3. Bcl-2 expression of follicular lymphoma (strong positive) (40x10)

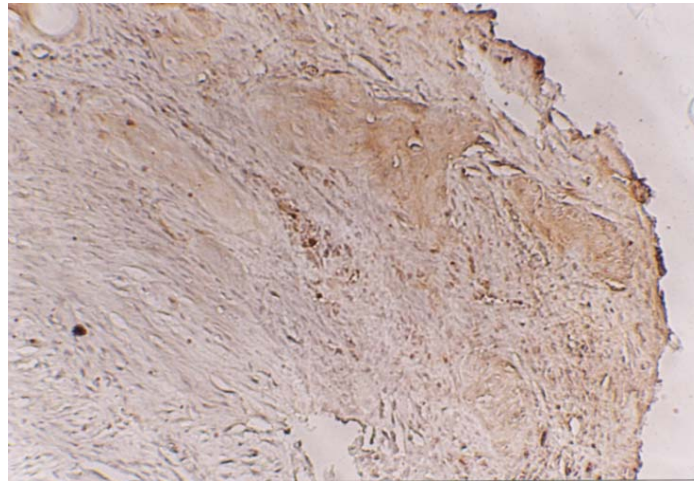


figure 4. Bcl-2 expression of moderately differentiated squamous cell carcinoma (weak positive score 1(10x10))

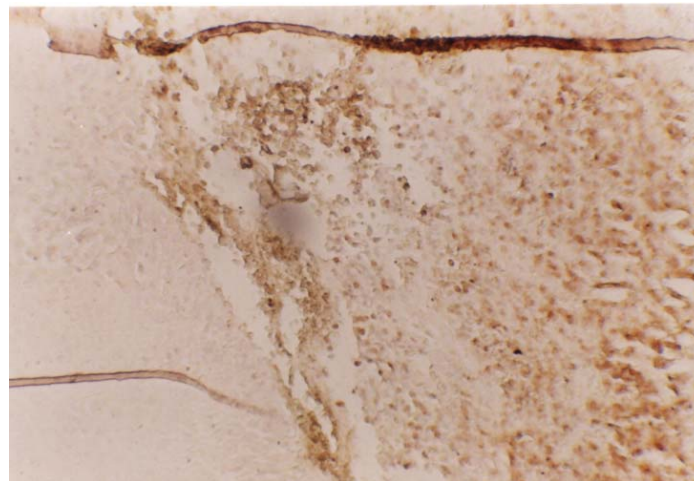


figure 5. Bcl-2 expression of moderately differentiation squamous cell carcinoma (moderately positive score 2) (10x10)

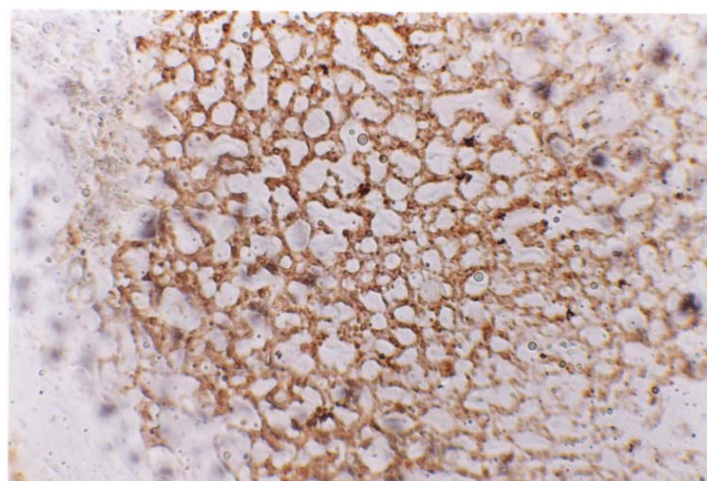


figure 6. Bcl-2 expression of poorly differentiated adenoid cystic carcinoma (strongly positive score 3) (10x10)

Cytoplasm immunoreactivity was represented in peripherally located with infiltrating tumor cells where more intensely stained, may be due to that genetically damaged or mutated cells can not be eliminated by apoptosis, which leads to alter the ratio of Bcl2 / Bax lead to over expression of Bcl2, in agreement with **Backus et al 2002**<sup>(1)</sup>, they reported that diffuse expression was more common in malignant colon cells, in agreement with **Single et al 1998**<sup>(8)</sup> they reported that increased Bcl2 immunoreactivity was also detected in sever epithelial dysphasia in comparison with mild and moderate dysphasia and squamous cell carcinoma, also in agreement with **Krajewski et al 1995**<sup>(9)</sup> reported that Bcl2 over expression has been demonstrated in several human tumors lymphoma and neuroblastoma, Bcl2 over expression was reported in breast **Lipponen et al 1995**<sup>(10)</sup>, endometrial **Chan et al 1995**<sup>(11)</sup> and gastrointestinal carcinoma **Bronner et al 1995**<sup>(12)</sup>.

Bcl2 expression in different type of clinical presentation of oral carcinoma, our results represented 11 cases (45, 84 %) of oral carcinoma which was shown as Exophytic positively of Bcl2 expression, over expression of proto-oncogene Bcl2 which may lead to blockage apoptosis and promote cell proliferation and lead to Exophytic type of growth, in agreement with **Yang et al 1997**<sup>(13)</sup> reported that Bcl2 response to diverse death stimuli, also similar finding carried **Devita et al 1997**<sup>(14)</sup> reported that high concentration of Bcl2 or Bcl-x<sub>1</sub> affect the susceptibility of a cell to the induction of apoptosis by altering the ratio of the death promoters to suppressors, providing tumor cells with a survival advantage, in agreement with **Muzio et al 2003**<sup>(2)</sup>

reported that the Bcl2 protein, blocks a distal step in an evolutionarily conserved pathway of apoptosis.

Our results showed three cases (12.5%) of oral carcinomas represented as mixed type (Exophytic and Endophytic) was positive Bcl2 immunoreactivity, which reflects the disturbance between Bcl2 promote apoptosis and others that promotes cell proliferation.

Our results showed that Statistical analysis non significant between clinical features (Exophytic, Endophytic, Mixed type) and Bcl2 expression in oral carcinoma, **Raja Kummoona et al 2008**<sup>(15)</sup> reported that there was a strong correlation between the degree of Bcl2 expression and grade of malignancy, **Header D Al Muala et al 2009**<sup>(16)</sup> reported that tumor size and TNM staging system were a good clinical predictors for treatment and prognosis of oral squamous cell carcinoma.

Our results showed that four cases (16.66%) presented as ulcerative (Endophytic type) showed a positive Bcl2 expression and five cases (20.84%) were Bcl2 negative, may be due to disturbance between proliferation and apoptosis might be due to not only over expression of Bcl2 that block apoptosis which was used in our study but may be accompanied by over expression of other types of Bcl2 family that promotes the apoptosis, in agreement with **Merry et al 1997**<sup>(17)</sup>, **Adams et al 1998**<sup>(18)</sup> they reported that Bcl2 protein family plays a central part in control of apoptosis, in agreement with **Okaro et al 2001**<sup>(19)</sup> they reported that Bcl-x<sub>1</sub>, Mcl-1, Mcl-w and Al inhibits the induction of apoptosis where as Bax, Bad, Bid and Bcl-x<sub>s</sub> are pro-apoptosis, **Muzio et al 2003**<sup>(2)</sup> reported that Bcl2 facilitate the permanent acquisition of mutations and malignant transformation.



## Conclusions

Bcl-2 immunoreactivity within normal oral mucosa is confined to the basal cell layers, it controls the terminal differentiation of normal oral Keratinocyte, Bcl-2 immunoreactivity present within peripherally located infiltrating tumor cells which were more intensely stained. Bcl-2 over expression could be considered as a diagnostic aid and to detect the free margin and the recurrence of oral cancer in addition to histopathology and conventional X-ray and recent modalities as C T scan and MRI. Bcl-2 over expression in primary oral carcinoma can be considered as predictive and prognostic indicator in treatment decision.

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