Correlation between Antiapoptotic Gene Product Protein Expression in Oral Carcinoma with Age and Gender of Patients

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Abstract
Oral cavity cancer represent a multiplicity of disease, Squamous cell carcinoma represents the most common frequent malignant tumor of the oral cavity.

Aims :- To identify Bcl-2 oncogene product expression of oral carcinoma and its correlation to the age and sex of patients.

Patients and methods :- Twenty four patients were presented with oral carcinoma, Formalin-fixed paraffin-embedded sections were stained with H and E and immunohistostaning. 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. they may be genetically damaged or mutated cells not eliminated by apoptosis, this lead to alter the ratio of Bcl-2 / Bax and hence lead to over expression of Bcl-2. There was no correlation between Bcl-2 expression and the age and sex.

Conclusion :- Bcl-2 expression in normal oral mucosa and oral carcinoma, Bcl-2 immunoreactivity did not correlated neither to the age nor gender of the patients.

Introduction
Oral cancer most commonly occurs in middle-aged and older individuals, although a disturbing number of these malignancies is also being documented in younger adults in recent years(Schantz & Yu, 2002). Cancers of the oral cavity and
oropharynx represent approximately three percent of all malignancies in men and two percent of all malignancies in women in the United States. Squamous cell carcinoma, which arises from the oral mucosal lining, accounts for over 90 percent of these tumors (Silverman, 2001). Despite the great strides that have been made in recent decades to improve the prognosis for a number of cancers throughout the body, the prognosis for oral cancer has not experienced a similar improvement (Swango, 1996). These lesions often present as either white or red patches, known as leukoplakia and erythroplakia. As the cancer develops, the patient may notice the presence of a nonhealing ulcer. Later-stage symptoms include bleeding, loosening of teeth, difficulty wearing dentures, dysphagia, dysarthria, odynophagia, and development of a neck mass (Brad et al., 2002).

The Bcl-2 oncoprotein inhibits apoptosis and is expressed by many tumors including carcinoma such as of breast, cervix and head and neck (Hussain et al., 2003). Bcl2 is known to belong to a family of apoptosis – regulatory gene products that may be death antagonists (eg Bcl-2, Bcl-XL, Mcl-1, A1) or death agonists (eg. Bax, Bak, Bcl-XS, Bad) (Marschitz et al., 2000). 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 Bcl2 family component act as on oncogene and are involved in oral carcinogenesis (Muzio et al., 2003).

Functions of Bcl2 protein: The Bcl-2 protein, block a distal step in an evolutionarily conserved pathway of apoptosis. High concentrations of Bcl2 or Bcl-xL affect the susceptibility of a cell to the induction of apoptosis by altering the ratio of the death promoters to suppressors, providing tumour cells with a survival advantage, and permitting expansion of transformed cells harboring mutations with in their genome (diagram-1). The Bcl2 protein and other family members target intra cellular organelles such as the endoplasmic reticulum and the outer mitochondrial and nuclear members, where they modulate responses to diverse death stimuli. This sub cellular location is important for function (Yang et al., 1997). Some members of the Bcl2 protein family when bound to the mitochondria regulate the function of the large polymeric channel, permeability transition pore complex (Narita & Shimizu, 1998), located at the point of contact between the inner and the outer mitochondrial membranes (Shimizu et al., 1999). The opening of this pore known as mitochondrial permeability transition, is associated with the release of apoptogenic proteins (Kiuck et al., 1997; Green & Reed, 1998).
Diagram (1) Over expression of Bcl-2 inhibits apoptotic cell death (Devita et al 1997)

**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 from January 2008 to March 2009.

**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.

We prepared specially designed case sheet and the clinical staging system proposed by American joint committee on cancer (AJCC) 1992 was used.

**Materials** were used throughout the research. Primary antibody kit [7ML Bcl2 oncoprotein ready to use] clone 124 Dako corporation 6392 via real Fax: 803/566 6688 Dan mark.

2. Secondary antibody kit.
   A. Biotinlated link (yellow bottle).
   B. Label (red bottle).

3. Target retrieval solution high PH code No. S3307 or 10X concentration code No. S3308.

4. Power block reagent.

5. Buffer phosphate solution.

6. Diamino benzidin (DAB) substrate chromagen solution.

7. Counter stain hemataxilin.

8. Alcohol concentration 70, 80, 90, 100 increased concentration.

9. Ethanol or xylene.

10. D.P.X.

**Methods:** Study group, 24 specimens of excisional biopsies of oral carcinoma were taken 20 specimens of Squamous cell carcinoma and 4 adenoid cystic carcinoma, and 5 specimens–control group–of normal oral mucosa were fixed in 10% buffered...
formalin and embedded in paraffin wax were obtained from the teaching laboratory of medical city.

**Immunohistochemical procedure of Bcl₂ protein (Mikel, 1994)**

1. Dewaxing slides A, B, C and D.
   A. Slides were kept in oven 30 min. at 70 °.
   B. Slides immersed in xylol for 10-20 min.
2. Rehydration slides A, B, C and D were kept in alcohol for 5 min. decreasing concentration 100, 90, 80.
3. 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. Then the jar is kept in water bath at a temperature 90–95 Ce for one hour never above 100Co.
4. Jar with slides A, B, C and D were left to be cooled to room temperature for one hour.
5. Diluted buffer phosphate solution 4% prepared and (4 ml of buffer added to 96 ml of distal H₂O₂).
6. Slides A, B, C and D were kept in buffer solution for one minute the slides were dried by filter papers.
7. Slides A, B, C and D were kept in hydrogen peroxide (H₂O₂) for 5 min.
8. Slides were kept in buffer solution 1-2 min. and left to dry, make a circle by PAP pen on each slide.
9. One drop of Monoclonal primary antibody Bcl₂ ready to use were applied to slides A, B and C for 30 min. but not added to slides group D (negative control).
10. Slides A, B, C and D were kept in buffer solution for 1-2 and dried it by filter papers.
11. 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.
12. Slides group A, B, C and D were immersed in the buffer solution for 1-2 min. then dried it by filter papers.
13. One drop of Label (red bottle) ready to use were applied to all groups A, B, C and D for 20 min.
14. Slides A, B, C and D were kept in buffer solution for 1-2 min. Then dried it by filter papers.
15. One drop of (DAB) substrate chromagen solution was added to each slide A, B, C and D for 5-15 min.
16. Slides were kept in buffer solution 1-2 min. and dried it.
17. Slides A, B, C and D were kept in hematoxylin counter stain for one minute.
18. Slides were washed by distal H₂O for 3 min.
19. Slides were dried and immersed in alcohol with increasing concentration for 70, 80, 90 to 100 for 2 min.
20. Slides were kept in xylol for 5 min.
21. Mounting of the slides few drops of DPX were applied on the cover and put the cover on the slide.
22. 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, score 2 (moderate positive) staining in 25-50% tumor cells, score 3 (strong positive) staining in >50% tumor cells.

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). High frequency was recorded in age groups (60-69) years which constituted 25% of total number of patients Table (1). Total number of cases investigated were 24, 11 males 45.83% with an age ranged 32-80 years and 13 females (54.17%) with an age ranged 19-71 years. Male to female ratio was 0.84:1. Table (1).

Results of Bcl2 expression:
1. Results of Bcl2 expression in normal oral mucosa i.e. 5 cases of (normal control group) displayed Bcl2 immunohistochemical expression to nearly all basal keratinocytes or apical portion of crypts.
2. Result of Bcl2 expression in study group:
Positive expression in oral carcinoma (Squamous cell carcinoma and adenoid cystic carcinoma) consistent Bcl2 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 Bcl2 expression is seen in peripherally located with infiltrating tumor cells where more intensely stained.
Table (1): Clinical and histopathological parameters with Bcl2 expression of oral carcinoma

<table>
<thead>
<tr>
<th>No.</th>
<th>Gender</th>
<th>Age</th>
<th>Clinical feature</th>
<th>T.N.M System</th>
<th>Stage</th>
<th>Histopathology</th>
<th>Degree of Differentiation.</th>
<th>BCL-2 Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-</td>
<td>F</td>
<td>54y</td>
<td>Exophytic</td>
<td>T3N1M0</td>
<td>III</td>
<td>Adeno cystic car.</td>
<td>Poorly diff. G3</td>
<td>Strong. posit (+ + +)</td>
</tr>
<tr>
<td>2-</td>
<td>F</td>
<td>62y</td>
<td>Exophytic</td>
<td>T3N1M0</td>
<td>III</td>
<td>Sq.cell.car.</td>
<td>Moderately diff. G2</td>
<td>Strong. posit (+ + +)</td>
</tr>
<tr>
<td>3-</td>
<td>M</td>
<td>32y</td>
<td>Exophytic</td>
<td>T2N2M1</td>
<td>IV</td>
<td>Adeno cystic car.</td>
<td>Moderately diff. G2</td>
<td>Strong. posit (+ + +)</td>
</tr>
<tr>
<td>4-</td>
<td>F</td>
<td>28y</td>
<td>Exophytic</td>
<td>T3N1M0</td>
<td>III</td>
<td>Sq.cell.car.</td>
<td>Moderately diff. G2</td>
<td>Mod. posit (+ +)</td>
</tr>
<tr>
<td>5-</td>
<td>M</td>
<td>71y</td>
<td>Exophytic</td>
<td>T3N1M0</td>
<td>IV</td>
<td>Sq.cell.car.</td>
<td>well diff. G1</td>
<td>Weak. posit (+)</td>
</tr>
<tr>
<td>6-</td>
<td>F</td>
<td>63y</td>
<td>Endophytic</td>
<td>T2N1M0</td>
<td>III</td>
<td>Sq.cell.car.</td>
<td>Well diff. G1</td>
<td>Weak. posit (+)</td>
</tr>
<tr>
<td>7-</td>
<td>M</td>
<td>47y</td>
<td>Exophytic</td>
<td>T3N1M0</td>
<td>III</td>
<td>Sq.cell.car.</td>
<td>Moderately diff. G2</td>
<td>Strong. posit (+ + +)</td>
</tr>
<tr>
<td>8-</td>
<td>F</td>
<td>50y</td>
<td>Exophytic</td>
<td>T2N1M0</td>
<td>III</td>
<td>Sq.cell.car.</td>
<td>Moderately diff. G2</td>
<td>Mod. posit (+ +)</td>
</tr>
<tr>
<td>9-</td>
<td>M</td>
<td>41y</td>
<td>Endophytic</td>
<td>T2N0M0</td>
<td>II</td>
<td>Sq.cell.car.</td>
<td>Well diff. G1</td>
<td>Negative (-)</td>
</tr>
<tr>
<td>10-</td>
<td>M</td>
<td>45y</td>
<td>Endophytic</td>
<td>T2N1M0</td>
<td>III</td>
<td>Sq.cell.car.</td>
<td>Moderately diff. G2</td>
<td>Mod. posit (+ +)</td>
</tr>
<tr>
<td>11-</td>
<td>F</td>
<td>71y</td>
<td>Endophytic</td>
<td>T2N1M0</td>
<td>IV</td>
<td>Sq.cell.car.</td>
<td>Poorly diff. G3</td>
<td>Strong. posit (+ + +)</td>
</tr>
<tr>
<td>12-</td>
<td>F</td>
<td>45y</td>
<td>Endophytic</td>
<td>T2N1M0</td>
<td>IV</td>
<td>Sq.cell.car.</td>
<td>Moderately diff. G2</td>
<td>Negative (-)</td>
</tr>
<tr>
<td>13-</td>
<td>F</td>
<td>43y</td>
<td>Endophytic</td>
<td>T2N1M0</td>
<td>IV</td>
<td>Sq.cell.car.</td>
<td>Well diff. G1</td>
<td>Negative (-)</td>
</tr>
<tr>
<td>14-</td>
<td>F</td>
<td>19y</td>
<td>Endophytic</td>
<td>T2N1M0</td>
<td>IV</td>
<td>Sq.cell.car.</td>
<td>Well diff. G1</td>
<td>Negative (-)</td>
</tr>
<tr>
<td>15-</td>
<td>F</td>
<td>58y</td>
<td>Endophytic</td>
<td>T2N0M0</td>
<td>II</td>
<td>Sq.cell.car.</td>
<td>Well diff. G1</td>
<td>Negative (-)</td>
</tr>
<tr>
<td>16-</td>
<td>M</td>
<td>32y</td>
<td>Exophytic</td>
<td>T2N2M0</td>
<td>IV</td>
<td>Adeno cystic car.</td>
<td>Moderately diff. G2</td>
<td>Strong. posit (+ + +)</td>
</tr>
<tr>
<td>17-</td>
<td>F</td>
<td>51y</td>
<td>Exophytic</td>
<td>T2N1M0</td>
<td>III</td>
<td>Adeno cystic car.</td>
<td>Poorly diff. G3</td>
<td>Strong. Posit (+ + +)</td>
</tr>
<tr>
<td>18-</td>
<td>F</td>
<td>37y</td>
<td>Exophytic</td>
<td>T2N1M0</td>
<td>IV</td>
<td>Sq.cell.car.</td>
<td>Moderately diff. G2</td>
<td>Mod. posit (+ +)</td>
</tr>
<tr>
<td>19-</td>
<td>M</td>
<td>60y</td>
<td>Exophytic</td>
<td>T3N0M0</td>
<td>III</td>
<td>Sq.cell.car.</td>
<td>Poorly diff. G3</td>
<td>Mod. posit (+ +)</td>
</tr>
<tr>
<td>20-</td>
<td>M</td>
<td>66y</td>
<td>Endophytic</td>
<td>T2N1M0</td>
<td>IV</td>
<td>Sq.cell.car.</td>
<td>Moderately diff. G2</td>
<td>Mod. posit (+ +)</td>
</tr>
<tr>
<td>21-</td>
<td>M</td>
<td>80y</td>
<td>Exophytic</td>
<td>T2N1M0</td>
<td>IV</td>
<td>Sq.cell.car.</td>
<td>Poorly diff. G3</td>
<td>Mod. posit (+ +)</td>
</tr>
<tr>
<td>22-</td>
<td>M</td>
<td>57y</td>
<td>Exophytic</td>
<td>T2N1M0</td>
<td>IV</td>
<td>Sq.cell.car.</td>
<td>Poorly diff. G3</td>
<td>Strong. Posit (+ + +)</td>
</tr>
<tr>
<td>23-</td>
<td>M</td>
<td>60y</td>
<td>Exophytic</td>
<td>T3N1M0</td>
<td>IV</td>
<td>Sq.cell.car.</td>
<td>Moderately diff. G2</td>
<td>Strong. Posit (+ + +)</td>
</tr>
<tr>
<td>24-</td>
<td>F</td>
<td>60y</td>
<td>Exophytic</td>
<td>T3N1M0</td>
<td>IV</td>
<td>Sq.cell.car.</td>
<td>Well diff. G1</td>
<td>Negative (-)</td>
</tr>
</tbody>
</table>
figure (1) Extra oral photograph of adenocystic carcinoma.

figure (2) Entra oral photograph of adenocystic carcinoma.
figure (3) CT scan showed the tumor mass on the floor of mouth and extended to the Para pharyngeal space.

figure (4) chest x-ray showed bilateral lung metastases of the lesion.

Table (2) Distribution of age by sex of oral carcinoma's patients.

<table>
<thead>
<tr>
<th>Age</th>
<th>Males</th>
<th>Females</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>20-29</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>30-39</td>
<td>2</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>40-49</td>
<td>3</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>50-59</td>
<td>1</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>60-69</td>
<td>3</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>70-80</td>
<td>2</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>11</td>
<td>13</td>
<td>24</td>
</tr>
</tbody>
</table>
Table (3) Distribution of age by positive and negative Bcl2 expression of oral carcinoma.

<table>
<thead>
<tr>
<th>Age of the patients</th>
<th>Negative Bcl-2 expression</th>
<th>Weak positive Bcl-2 expression</th>
<th>Moderate positive Bcl-2 expression</th>
<th>Strong positive Bcl-2 expression</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>19-29 y</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>30-39 y</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>40-49 y</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>50-59 y</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>60-69 y</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>70-80 y</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
</tbody>
</table>

Table (4) Distribution of gender of patients by positive and negative Bcl2 expression of oral carcinoma.

<table>
<thead>
<tr>
<th>Gender of the patients</th>
<th>Negative Bcl-2 expression</th>
<th>Weak positive Bcl-2 expression</th>
<th>Moderate positive Bcl-2 expression</th>
<th>Strong positive Bcl-2 expression</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>1</td>
<td>1</td>
<td>4</td>
<td>5</td>
<td>11</td>
</tr>
<tr>
<td>Female</td>
<td>5</td>
<td>1</td>
<td>3</td>
<td>4</td>
<td>13</td>
</tr>
</tbody>
</table>

Discussion

The bcl2 proto-oncogene was first discovered in B cell lymphomas exhibiting the t(14; 18)(q32;q31) chromosomal translocation(Tsujimoto et al., 1985) , the bcl2 gene product is associated with the outer mitochondrial membrane(Hockenbery et al., 1990) and located on the endoplasmic reticulum and the nuclear envelope(Krajewski et al., 1993) . Bcl2 expression has been demonstrated in several human tumors such as gastrointestinal carcinoma(Bronner et al., 1995), lymphoma(Krajewski et al., 1995), breast(Lipponen et al., 1995) . In fact, increased bcl2 immunoreactivity was also detected in sever epithelial dysplasias in comparison with mild and moderate dysplasias and squamous cell carcinomas, suggesting a role for this oncoprotein in relatively early stages of oral tumor progression(Singh et al., 1998).

The result of the current study show that bcl2 immunoreactivity in normal oral mucosa is confined to the basal cell layers and possibly is involved in the preservation of an adequate reservoir of proliferating stem cells . This studies showed similar findings to that of Muzio et al (2003) they reported that normal oral epithelium displayed Bcl-2 immunohistochemical expression limited to nearly all basal keratinocytes , while the study of Backus et al (2002) reported that Bcl2 staining was limited to apical area in normal colonic mucosa cells(Backus et al., 2002).

Bcl2 expression in oral carcinoma.

Cytoplasm immunoreactivity was represented in peripherally located with infiltrating tumor cells where more intensely stained .This could 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.
These results are in agreement with Backus et al (2002), They reported that diffuse expression was more common in malignant colon cells, also there finding sported by the finding of Singh et al (1998) they reported that increased Bcl2 immunoreactivity was also detected in sever epithelial dysplasia in comparison with mild and moderate dysplasias and squamous cell carcinoma, also the finding was in agreement with Krajewski et al, (1995) they 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), endometrial Chan et al (1995), and gastrointestinal carcinoma Bronner et al (1995).

**Patient’s age**

In our study 19 cases (79.16%) of the total number of cases in both sexes were over 40 years which was in agreement with Flower et al (1980) stated that 90% of patients with oral cancer are over 40 years of age. Five cases (20.84%) in both sexes were below 40 years of age, this percentage is higher than that found by Flower G et al (1980). This high incidence in our study may be explained that in Iraq there was a shift in the age group, this may be due to malnutrition, bad oral hygiene, immune deficiency, or may be related to the use of chemicals agents like depleted uranium or may be due to severe pollution in addition to the stress.

There was no statistically significant correlation between Bcl2 immunoreactivity and age which was in agreement with Muzio (2003), they reported that no statistically significant correlation could be demonstrated between bcl-2 immunoreactivity and age of the patient.

**Male to female Ratio**

Our study has shown that females were more commonly affected than males with M:F ratio 0.84 : 1 our finding was disagreement with Rich et al (1984) they stated that ratio of males affected to females was 1.3:1 in Sweden. In England and Wales the ratio was 1.7:. There is striking increase in the number of women that are affected by oral cancer, this is due to increased cigarette smoking among women which was in agreement with Christopher et al (1999), or may be due to stress, malnutrition and increase number of women working in factories.

Our study reveals that there was no statistically significant correlation between Bcl2 immunoreactivity and sex of the patient, which was in agreement with Muzio L. Lo 2003 they reported that there was no statistically significant correlations could be demonstrated between bcl-2 immunoreactivity and the sex of the patients. Our results in agreement with Orest Gallo et al (1999) they reported that there was no association of bcl2 expression with gender.

**Conclusions**

1. There was no association of bcl2 expression with the age of the patients.
2. There was no statistically significant correlation between Bcl2 immunoreactivity and sex of the patient.

**References**


Christopher F; Holsinger: Management metastasis in squamous cell of the oral Tongue, from the grounds archive at Baylor. the babby R Al-ford, Department of otolaryngology and communicative sciences August 26, 1999 (internet).


