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Original Article

Immunohistochemical analysis of B-cell lymphoma -2 in pleomorphic adenoma and mucoepidermoid carcinoma

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ABSTRACT

Background: Salivary gland tumors form a major area in the field of oral pathology. B-cell lymphoma -2 (Bcl-2) is an anti-apoptotic gene with up-regulation in various neoplasms. The aim of the present case-control study was to comparatively investigate the expression of Bcl-2 protein in pleomorphic adenoma (PA) and mucoepidermoid carcinoma (MEC) and assess its potential diagnostic role in differentiating these tumors.

Materials and Methods: A total of 28 cases (18 specimens of PA [control] and 10 blocks of MEC [case]) were selected for analysis by streptavidin-biotin peroxidase method with antibody against Bcl-2. The percentage of positive cells was calculated from a minimum of 1000 neoplastic cells and H-score was identified (% positive cells × intensity of staining). Data were analyzed using two-way ANOVA, *t*-test, Spearman, Chi-square and Fisher tests ($\alpha = 0.05$).

Results: bcl-2 expression was shown in 13 cases (71%) of PA and 3 cases (30%) of MEC. In addition, four cases of PA showed strong staining. There was a significant difference between the expression intensity of Bcl-2 in the two tumors ($P = 0.048$) according to ANOVA. No correlation was observed between Bcl-2 expression with the size and location of tumors ($P > 0.05$).

Conclusion: Bcl-2 expression might be used for differentiating these tumors. Bcl-2 protein was overexpressed in PA compared with MEC. Hence, it seems that unlike what was observed in PA, Bcl-2 probably does not participate in the pathogenesis of MEC.

Key Words: B-cell lymphoma-2, mucoepidermoid carcinoma, pleomorphic adenoma, salivary gland tumor

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INTRODUCTION

Salivary gland tumors are the second most common neoplasm in the mouth with widely variable histologic characteristics, which make it difficult to determine the pathogenesis.[1,2] The high prevalence of these tumors and difficulty of diagnosis indicates the need for investigating biomarkers and pathways, which can be used as diagnostic or prognostic factor or can be targeted in cancer molecular-based target therapy.[3-5]

One of such processes is programmed cell death, apoptosis, which occurs in healthy and pathologic tissues. Apoptosis is responsible for deletion of aged, injured and mutated cells in normal and in certain specific pathologies.[1] Changes in apoptosis rates are usually associated with oncogenic or pathologic alterations. The importance of apoptosis inhibition during the process of tumorigenesis has been recognized universally and the ongoing discovery of numerous apoptosis-regulating proteins would provide new potential targets for a molecular cancer therapy.[6] Therefore, screening the expression of apoptotic markers such as P-53 and anti-apoptotic

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markers such as B-cell lymphoma-2 (Bcl-2) is valuable in predicting prognosis of malignancies.[4] Bcl-2 is the first gene proved to be involved in apoptosis and was considered to be a proto-oncogene that exerts its oncogenic effect by suppressing the cell death instead of stimulating cell proliferation.[1,7,8] Bcl-2 oncoprotein is an intracellular, integral membrane protein with a 26-kD molecular weight.[1] It has been proved as a useful investigative tool in oral pathology and in a variety of human malignancies. Its increase can be associated with the increase in resistance to chemotherapy, nodal involvement and remote metastasis.[3,9] Bcl-2 expression has been investigated in oral squamous cell carcinoma, oral epithelial dysplasia and oral submucous fibrosis and its expression increased with the severity of dysplasia and decreased differentiated oral carcinoma.[1,10,11] Recently, Bcl-2 overexpression has been suggested as an important factor in cancers of salivary glands.[1,12] Nonetheless, studies on this matter are limited and to date only a few studies have dealt with salivary gland tumors.[1,13]

The objective of the present study was to comparatively evaluate the immunohistochemical expression of Bcl-2 in pleomorphic adenoma (PA) and mucoepidermoid carcinoma (MEC) and possibly address the probable diagnostic role of Bcl-2 in differentiating these tumors.

MATERIALS AND METHODS

The sample size of this case-control study was consisted of 28 initial oral tumors (18 specimens of PA as control and 10 specimens of MEC as case), which were retrieved from the surgical pathology archive of Imam Khomeini Hospital, Ahvaz, Iran. The study protocol was approved by Ethics Committee of Qazvin University of Medical Sciences.

Sample collection

Histopathological diagnosis was confirmed by an expert pathologist using hematoxylin and eosin stained sections. Clinical data (tumor size and location) were also documented from the patient records. The exclusion criteria were the absence of sufficient patient records and presence of relapse or treatment histories.[5]

Immunohistochemistry (IHC) staining with Bcl-2 markers

The expression of Bcl-2 was determined by IHC assays on paraffin-embedded formalin-fixed sections from tissue blocks using a microwave antigen retrieval method. Bcl-2 (mouse antihuman Bcl-2 oncoprotein clone 124, Dako, Denmark), diluted 1:50 in phosphate buffered saline (PBS) was used as the monoclonal antibody.

For this purpose, 4-μm sections of specimens fixed in 10% formalin were mounted on silicon-coated slides for 24 h. They were then dewaxed in 100% xylene (3 times, 8 min each) and dehydrated in graded ethanol (100%, 100%, 99% [5 min each], respectively). Then, they were stored in 100 methanol (for 2-3 min), 3% hydrogen peroxide in 100% methanol solvent (30 min), 90% ethanol (for 1 min) and 80% ethanol (1 min), respectively. Then, they were rinsed with tap water for 5 min.

Afterward, the specimens were placed in 10 mM citrate buffer (pH = 6) in a plastic container and were heated in a microwave oven for 15 min (100°C, 750 W). After removing from the oven, they were placed in room temperature for 30-60 min and then were rinsed for 10 min with tap water and placed in PBS for 5 min. Non-serum protein was added to the specimens. Then they were placed in a closed container for 30 min.

Afterward, the sections were washed in water (1 min) and PBS (1 min) and were incubated in blocking milk buffer (5% dry skimmed milk in PBS, pH 7.6 for 30 min) with primary antibody at 4°C overnight. 3',3'-diaminobenzidine (DAB) was used as the chromogen. After removing the slides from cold room, they were kept in room temperature for 1 h. Then, they were rinsed with water for 5 min and were stored in PBS for 5 min, respectively. Then, the secondary antibody was added to the slides and after 1 h, the slides were placed in PBS for 5 min. Then, one or two drops of DAB were added to the slides and after 2 or 3 min, they were evaluated under light microscope to assess the consistency of the staining. After the confirming of a proper staining, the slides were rinsed with tap water.

Then the specimens were stained with hematoxylin for 5 min and were water-rinsed for 5 min. Then, they were placed in 95%, 90%, 80%, 70% and 100% ethanol solutions (each for 5 min). Finally, they were stored in xylene for 5 min and then were mounted.

Marker expression

Specimens of follicular lymphoma were also immunostained for Bcl-2 as a positive control slides. For negative control, the primary antibody was replaced by a non-immune serum.

For a definite diagnosis, normal salivary gland tissues were stained with Bcl-2 [Figure 1]. The lymphocytes that infiltrate in the specimens used as internal control. Some representative fields were randomly selected in each immunohistochemically stained section. Ten fields were chosen for each section. Only cytoplasmic staining of neoplastic cells was observed with Olympus CX21 light microscope. The percentage of positive cells was calculated from a minimum of 1000 neoplastic cells and the H-score was expressed (% positive cells × intensity of staining; weak: 1, moderate: 2, strong: 3).[14]

Statistical analysis

Data analysis was performed using the tests: Two-way ANOVA, *t*-test, Spearman correlation coefficient, Chi-square and Fisher's exact in the SPSS (version 17, Chicago, IL, USA). Statistical significance was identified as $P < 0.05$.

RESULTS

Of the 18 patients with PA, 8 were males (mean age = 33.1 ± 16.8 [range: 18-66]) and 10 were females (mean age = 42.3 ± 15.1 [range: 16-63]). Of those 10 with MEC, 3 were men (mean age = 67 ± 17.4 [range: 47-79]) and 7 were women (mean age = 47.3 ± 17.2 [range: 20-66]). Chi-square test showed that there was no difference in the gender distributions in the two tumors ($P = 0.66$) and in both tumors, females were involved more than males (but not significantly higher [$P = 0.25$]).

The mean age for PA and MEC were 38.2 ± 16.1 and 53.2 ± 18.8 years, respectively. The difference between the age of the two groups was significant ($P = 0.035$).

The most common site for PA was parotid and for MEC was minor salivary gland. PA was seen in 12 parotids, 2 submandibulars and 4 minor glands. MEC was seen in 3 parotids, 1 submandibular and 6 minor glands. According to Fisher's exact test, there was no significant difference between distribution of the two tumors in different glands ($P = 0.122$). Chi-square test showed that there was significant differences between the glands in the case of PA ($P = 0.009$), but not for MEC ($P = 0.150$).

Thirteen of 18 cases (71%) of PA were positive for Bcl-2 (cytoplasmic expression). From this positive group, 4 cases demonstrated strong staining reaction (22%) while 5 cases were negative (27%). The localization of Bcl-2 was in neoplastic ductal and myoepithelial cells [Figures 2 and 3]. In MEC samples, Bcl-2 protein expression was positive in 3 of 10 cases (30%), and the localization of Bcl-2 was in neoplastic epidermoid cells [Figure 4].

The mean percentage of Bcl-2 stained cytoplasm was 47.78 ± 40.56% (42 ± 43.98 for females, 55 ± 37.42 for males) in PA and 19.5 ± 36.55% (27.86 ± 41.62 for females and 0.0 ± 0.0 for males) in MEC tumors [Figure 3]. Two-way ANOVA showed that there was a significant difference between the expression of Bcl-2 in the two tumors ($P = 0.048$). However, the difference between the genders was not significant ($P = 0.658$). *t*-test showed that there was a close to the significant difference between the expression of Bcl-2 in the two tumors ($P = 0.074$). In males, this difference was significant ($P = 0.004$), but in females,

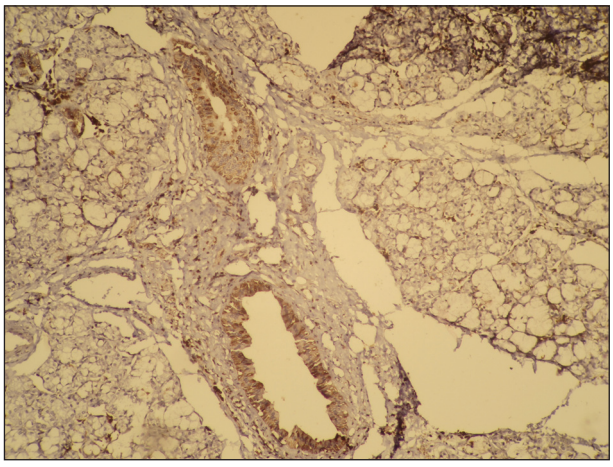

Figure 1: Photomicrograph showing B-cell lymphoma-2 expression in normal salivary gland ducts (×200)

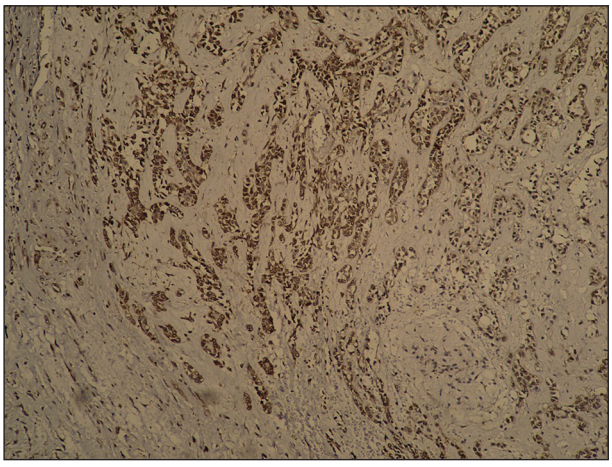

Figure 2: Photomicrograph showing B-cell lymphoma-2 expression in pleomorphic adenoma (×200)

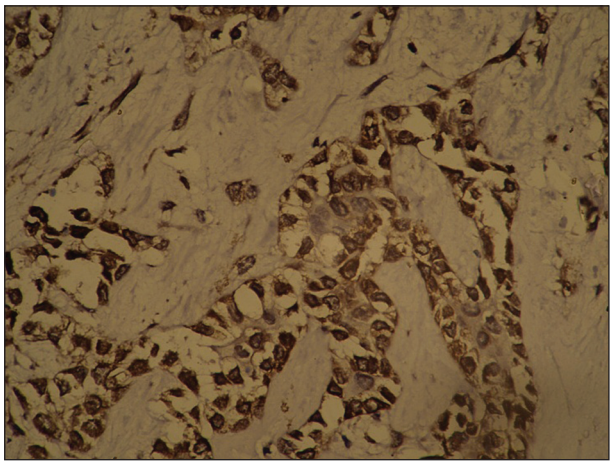

Figure 3: Photomicrograph showing B-cell lymphoma-2 expression in pleomorphic adenoma (×400)

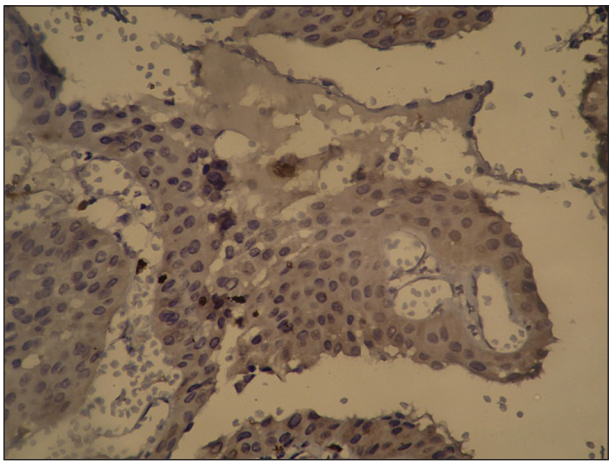

Figure 4: Photomicrograph showing B-cell lymphoma-2 expression in epidermoid cells of mucoepidermoid carcinoma (×400)

this was not significant ($P = 0.51$). There were no differences between males and females in either of tumors ($P > 0.2$) according to *t*-test.

Fisher's exact test showed that there was no significant difference between the patterns of staining intensity of the two tumors ($P = 0.17$) [Table 1].

The mean percentage of H-score was 1.04 ± 1.18% for PA (1.08 ± 1.65 for women and 1 ± 1.02 for men) and 0.11 ± 0.25% for MEC (0.41 ± 0.72 for women and 0.0 ± 0.0 for men) [Figure 4]. Two-way ANOVA showed a close to the significant difference between the tumors ($P = 0.071$), but not between genders ($P = 0.584$). There was a statistically significant difference between the tumors ($P = 0.004$), according to *t*-test. There were no significant differences between the two genders in each tumor ($P > 0.3$).

Tumor size and location did not correlate with Bcl-2 expression in any of the evaluated groups ($P > 0.05$) according to Spearman correlation coefficient.

Overall, there was a close-to-significant difference between the pattern of Bcl-2 expression and between the H-scores of the two tumors. There was a significant difference between the expression intensity in the tumors.

Table 1: Intensity of Bcl-2 expression in PA and MEC

Serial no.	Lesion	Staining intensity			
		0	Mild	Moderate	Strong
1	PA	5	6	3	4
2	MEC	7	2	1	—
Total cases		12	8	4	4

PA: Pleomorphic adenoma; MEC: Mucoepidermoid carcinoma; Bcl-2: B-cell lymphoma-2

DISCUSSION

Overexpression of Bcl-2 is common in many types of cancer including prostate, colorectal, lung, gastric, renal, neuroblastoma, non-Hodgkin lymphoma and both acute and chronic leukemia. This factor contributes to increased resistance to chemotherapy. Nevertheless, the prognostic value of Bcl-2 overexpression seems to be depended upon the tumor type and progression of the disease was not observed to be correlated with Bcl-2 overexpression in some of the previous cases.[6] In recent years, Bcl-2 protein has been shown to play an important role in the regulation of apoptosis. In addition, the over expression of Bcl-2 as an apoptosis suppression marker has been reported to protect salivary gland tumor cells from this process.[15]

According to the results of a study by Yáñez *et al*.[12] which resembled our results, all PA cases expressed Bcl-2 protein, especially in the tubuloductal, solid and trabecular areas. Nonetheless, 2 of 3 MEC subjects were positive for this marker and only in the epidermoid areas.[12] Aoki *et al*.[16] detected a high percentage of Bcl-2 positive cases, with immunoreactivity being observed in 33 (94.3%) of 35 analyzed cases. Their study showed that Bcl-2 was positive in basal cells of ductal structures in normal salivary glands. In addition, in PA cases, the localization of Bcl-2 was in the neoplastic myoepithelium including myxomatous stromal cells and plasmacytoid cells.[16] In the research of

Genetzakis *et al*.,[17] Bcl-2 was detected in 23.1% of patients with malignant parotid tumor and 55.6% of patients with Warthin's tumor. On the other hand, normal parotid gland tissues were found universally negative for Bcl-2.[17] Their study suggested that Bcl-2 expression predicted an unfavorable treatment outcome in patients with locally advanced or histologically aggressive tumors.[17] In the study of Abd-Elhamid and Elshafei,[18] 90% of Warthin's tumors showed positive expression of Bcl-2. Hence, they suggested a protective role of tumor cells from apoptosis to maintain their survival, but not to increase their malignant potentiality.[18] According to a study by Soini *et al*.,[19] Bcl-2 expression was observed in all PA and Warthin's tumors in which most cases showed strong expression. However, 9 out of 25 cases of malignant salivary gland tumors showed no expression. In the rest of the cases, Bcl-2 expression was often weak except for the cases of adenoid cystic carcinoma (ACC).[19] In Manjunatha *et al*.'s[1] research, 55% of PA, 45% of MEC and 100% of ACC samples were positive for this marker. Among the positive cases of MEC, all but one was negative in the mucous cells. In this study, Bcl-2 expression was observed in both benign and malignant salivary gland tumors except canalicular adenoma.[1] The authors concluded that the lack of Bcl-2 expression in these cells might be associated to the degree of differentiation. The expression of this marker was found negative in terminal differentiated cells such as the mucous cells, normal acinar cells and salivary gland duct cells.[1] According to Pammer *et al*., Bcl-2 was expressed in basal cells of striated and excretory ducts of salivary glands, which may show that these cells are reserving sites. Bcl-2 was not expressed in acinar cells, myoepithelial cells and most luminal cells. They found that Bcl-2 expression in PA was mainly in basal cells of tubuloductal structures.[20]

In the present investigation, Bcl-2 staining was positive in most cases of PA (71%) which is in accordance with previous reports.[12,16,18] The localization of Bcl-2 in PA was mostly in ductal and myoepithelial cells. Four cases demonstrated strong staining reaction (22%), whereas 5 cases were negative (27%). This variable expression may be suggestive of a different susceptibility rate of tumor cells to apoptosis. Furthermore, Bcl-2 is localized in the luminal and myoepithelial cells of the normal salivary gland tissue, which are considered to be the origin of PA. 3 out of 10 (30%) cases of MEC were positive for this marker. The localization of Bcl-2 was in neoplastic epidermoid cells. Tumor size and location were not correlated with Bcl-2 expression in this study.

These findings were in contrast with the results of a study by Gordón-Núñez *et al*. that found most PA cases negative for Bcl-2 protein which made them conclude that this protein was not involved in the pathogenesis of PA.[21] Also in Al-Rawi *et al*. research 70% of PA samples did not show Bcl-2 expression.[2] They found that all cases of malignant salivary gland tumors were positive for Bcl-2. The highest recorded score was observed in ACC and the lowest in both low grade carcinoma ex-PA and low grade MEC.[2] This finding is in accordance with a study by Nagler *et al*.[22] that found markedly positive Bcl-2 staining in salivary gland malignancies but not in the surrounding normal tissues. Jia *et al*.[15] found that Bcl-2 expression in ACC (51.9%) was slightly higher in the solid and cribriform types. Perhaps this finding is attributed to Bcl-2 expression in myoepithelial rather than ductal cells.[15] According to an investigation by Carlinfante *et al*.,[13] high expression of Bcl-2 was noted in 90% of ACC cases. However, no relation was identified between Bcl-2 expression and the histologic type, clinical staging and survival rate of the cases. Therefore, they suggested that Bcl-2 may be involved since the early stage of carcinogenesis.[13] Our results are in contrast with the Sakamoto *et al*.[23] study that found Bcl-2 overexpression in MEC compared with PA.[23] Furthermore, Yin *et al*.[4] found that MEC grade I tumors had the highest expression of Bcl-2 in all investigated groups. Our results that Bcl-2 was expressed more in the benign PA cases compared with the malignant MEC tumors might first look inconsistent. However, it should be noted that the significant results observed ruled out any artifacts and that there are several known and unknown factors involved in cancer environment and it is possible that certain types of MEC adopt other pathways of carcinogenesis rather than anti-apoptotic approach of Bcl-2. For example, it is shown that Bcl-2 expression might be downregulated in malignant transformation of ameloblastoma,[1,24] indicating the possibility of such inconsistencies. Also, finding that Bcl-2 expression do not change in different stages of MEC,[1] might indicate the weakness of its role in MEC malignancy. Another study on Warthin's tumor as well showed that although Bcl-2 might play a protective role for

cancerous cells, it can not be associated with the level of malignancy.[1,18] On the other hand, it is shown that Bcl-2 expression in different levels of differentiation of PA is significantly different[1] and it is probable that Bcl-2 is more expressed in low-differentiated PAs. The absence of documenting of the stages, grades and levels of differentiation of tumors in this study disallowed the authors to evaluate the results further and there might be poorly differentiated PA tumors in the control group, which could result in more expression of Bcl-2 in this sample. Furthermore, the higher age of the group with MEC might be another confounding variable, since Bcl-2 expression might be affected by age.[25]

Since the number of studies is small, further studies are warranted to rule out the effect of confounding factors and draw a clearer conclusion. It also is relevant that Bcl-2 is not necessarily associated with the level of malignancy, which this can be as well assessed when the confounders are controlled. Future research should take into account two factors: Collecting a larger sample, which is balanced in terms of age, gender and tumor types and controlling the degree of differentiation and/or staging and grading, in order to become able to draw more accurate yet more comprehensive findings.

CONCLUSION

These results suggest that Bcl-2 protein was overexpressed in PA compared with MEC. Hence, it seems that unlike what was observed in PA, Bcl-2 probably does not participate in the pathogenesis of MEC. The lack of this protein expression in this neoplasm may be correlated with the type of the cells and degree of differentiation. Bcl-2 seems to be a suitable marker for differentiation of these salivary gland tumors from each other. More comprehensive studies are necessary to evaluate this in larger samples with more subgroups.

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