

ORIGINAL ARTICLE

Study of immunohistochemical demonstration of Bcl-2 protein in ameloblastoma and keratocystic odontogenic tumor

CS Sindura, Chaitanya Babu¹, Vijaya Mysorekar², Vinod Kumar³

Departments of Oral Pathology, Srinivas Institute of Dental Science, Mangalore, ¹Oral Pathology, Oxford Dental College and Hospital, ³Oral Pathology, Vaidehi Dental College and Hospital, ²General Pathology, M.S. Ramaiah Medical College and Hospital, Bangalore, Karnataka, India

Address for correspondence:

Dr. CS Sindura,
Senior Lecturer, Department of Oral Pathology,
Srinivas Institute of Dental Science, Mangalore
Karnataka, India .
E-mail: sindurarajesh@yahoo.com

ABSTRACT

Background: The Bcl-2 (B-cell lymphoma) gene product also known as apoptotic inhibitor is expressed in many normal and tumor tissues. This Bcl-2 gene protects the cell by blocking postmitotic differentiation from apoptosis, thus maintaining the stem cell pool. **Objective:** To study the expression of Bcl-2 protein in ameloblastoma and keratocystic odontogenic tumor (KCOT) to determine their apoptotic behaviors and to analyze biological nature of KCOT, which has higher proliferative potential and aggressive clinical behavior like odontogenic tumors. **Materials and Methods:** Formalin-fixed paraffin sections of ameloblastoma ($n = 20$) and KCOT ($n = 20$) are considered for immunohistochemical analysis using monoclonal antibody against antihuman Bcl-2 oncoprotein. Lymphomas ($n = 3$) were used as controls. **Statistical Analysis:** The statistical analysis was performed using software package of social science version 16. The data were analyzed using Chi-square test and Student's t test. In all the above tests, $P < 0.05$ was accepted as statistically significant. **Results:** The positive ratio of Bcl-2 was 85% (17/20) in ameloblastoma, 85% (17/20) in KCOT and 100% (3/3) in lymphomas. Bcl-2 was expressed in peripheral cells and few scattered cells of stellate reticulum in ameloblastoma. KCOT showed strong positivity for Bcl-2 mainly in the basal layer. **Interpretation and Conclusion:** The present study demonstrates the aggressive nature of KCOT and intrinsic growth potential of its lining epithelium. This study clearly demonstrates that KCOT like ameloblastoma demonstrates aggressive clinical and noticeable invasive behavior. Therefore, it is now considered as no longer a developmental cyst but as odontogenic tumor.

Key words: Ameloblastoma, B-cell lymphoma-2, keratocystic odontogenic tumor, lymphomas

INTRODUCTION

Ameloblastoma is a true neoplasm of enamel organ-type, which does not undergo differentiation to the point of enamel formation.^[1] The solid or multicystic ameloblastoma (SMA) is traditionally considered a benign epithelial neoplasm with virtually no tendency to metastasize. It is of utmost importance to understand that the local biologic behavior of an SMA is that of a low-grade malignant tumor.^[2]

The term "Odontogenic Keratocyst" was introduced by Philipsen in 1956. Since Odontogenic Keratocyst has a higher proliferative potential compared with other cystic lesions and has aggressive clinical behavior like that of tumors, a lot of discussion is going on regarding the change in terminology of Odontogenic Keratocyst.^[3-7] In the year 2005, World Health Organization (WHO) designated Odontogenic Keratocyst as a "Keratocystic Odontogenic Tumor" (KCOT). It is now defined as "a benign uni- or multicystic, intraosseous tumor of odontogenic origin, with a characteristic lining of parakeratinized stratified squamous epithelium and potential for aggressive, infiltrative behavior." WHO recommends the term Keratocystic Odontogenic Tumor as it better reflects its neoplastic nature.^[8]

Bcl-2 belongs to group of antiapoptotic protein that regulates programmed cell death. Bcl-2 overexpression is found in early

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phase of epithelial carcinogenesis.^[9] Enhanced expression of Bcl-2 is seen in almost all human neoplasms. As a critical regulator of apoptosis, Bcl-2 plays a key role in early stages of oral tumorigenesis. Because Bcl-2 serves to prevent cell death, its occurrence in odontogenic tissues is helpful in identifying cell population from which odontogenic tumors may arise.^[10]

Bcl-2 showed a strong positivity for ameloblastoma. It was detected mainly in the outer layer tumor cells and intermediate cells suggesting that morphological features of outer layer tumor cells reflect ameloblastoma growth activity. Overexpression of Bcl-2 is associated with genesis and development of ameloblastoma.^[11]

In KCOT, Bcl-2 showed a strong positivity in basal layer, which confirmed abnormal control of cell cycle. The Bcl-2 overexpression increased the survival of epithelial cells, which led to peculiar growth pattern of KCOT.^[12] In our study, we analyzed the expression of Bcl-2 in ameloblastoma and KCOT.

MATERIALS AND METHODS

A retrospective study was done on 40 archival specimens, which were formalin fixed, processed, and paraffin embedded. It comprised of 20 cases of ameloblastoma (Group 1), 20 cases of KCOT (Group 2), and lymphomas as controls. These were retrieved from archives of Department of Oral Pathology, of our college by random sampling. All the demographic data pertaining to the blocks were retrieved from the case files of our college.

Inclusion criteria

All the archival specimens that had been previously diagnosed as ameloblastoma and KCOT with no history of malignancy either orally and systemically were included in the study group.

Exclusion criteria

All the cases of KCOT and ameloblastomas diagnosed with other odontogenic cysts or tumors were excluded from the study.

Ethical clearance

The study was approved by the institutional review board of M.S. Ramaiah Dental College and Hospital, Bangalore.

Antibody kit (NOVOCASTRA, UK)

Primary antibody—anti-Bcl-2 clone

Secondary kit:

- Hydrogen peroxidase (3% H₂O₂ in water)
- Link secondary antibody (biotinylated antirabbit,

antimouse, and antigoat immunoglobulin in phosphate-buffered saline containing carrier protein on 15 mM azide)

- Streptavidin peroxidase (streptavidin conjugated horse radish peroxidase in phosphate-buffered saline containing carrier protein and antimicrobial agent)
- Buffered substrate (buffered substrate solution, pH 7.5, containing hydrogen peroxide and a preservative)
- 3,3-diamino benzidine chromogen solution (DAB)
- Protein block.

Technique proper

Sections of 4 µm thickness were taken on silanized slides for immunohistochemistry. Sections were incubated at 50°C overnight prior to immunostaining.

The slides were then deparaffinized by placing on the slide warmer at 60°C for 10-20 min. The sections are treated with three changes of xylene for 10 min each. It is then treated with absolute alcohol, 70% alcohol, and 50% alcohol for 10 min each. The slides are then washed in running tap water followed by distilled water for 3 and 2 min, respectively. The slides are later treated with Tris–buffer solution for 2 min and the excess is wiped with tissue paper.

Peroxidase block is applied for 30 min (kept in moist chamber). It is then washed with Tris–buffer twice for 5 min each.

Heat-induced epitope retrieval (HIER) using microwave oven method is used for antigen retrieval. Slides were placed in plastic jar containing Tris–ethylene diamine tetraacetic acid buffer 1000 mL, pH 9. Heated for 10 min and allowed to cool for 5 min. This is repeated three times in three cycles. The slides are then washed in running tap water for 1 min. It is then washed with Tris–buffer twice for 5 min each.

Protein block is applied for 5 min after which the primary antibody is applied for 45-60 min. The slides are then washed with Tris–buffer twice for 5 min each. Post-primary block is now applied 30 min after which they are washed with Tris–buffer twice for 5 min each.

Secondary antibody is then applied for 30 min followed by washing with Tris–buffer twice for 5 min each. DAB is then applied onto the slides for 5 min following which it is treated with distilled water for 2-3 min. The slides were stained with Harris hematoxylin for 20 s and washed gently under running water for 10 min. They were dehydrated and dipped in xylene and mounted in DPX, a nonaqueous permanent mounting medium using coverslips.

Determination of Bcl-2 positivity

All the stained slides were examined at ×40. All the cells showing cytoplasmic immunostaining were taken as Bcl-2

positive. At least 10 representative areas of the epithelium were selected by random sampling. The criterion for selection of those areas was that these representative areas should have had a minimum of 100 cells thickness in the full length of the epithelium. Only those samples that were immunostained along with positive Bcl-2 expression with the control sections of lymphoma were considered, and lymphocytes within the section were taken as internal controls.

Grading of Bcl-2 positivity

The intensity of Bcl-2 positivity was estimated as previously described.^[13]

- (-) fewer than 5% positive cells or no staining;
- (+) 5%-24% positive;
- (++) 25%-50% positive; and
- (+++)

Statistical analysis

The statistical analysis was performed using software package of social science version 16. The mean age distribution of patients, the intensity of Bcl-2 expression, and positivity of cases in ameloblastoma and KCOT were analyzed using Chi-square test and Student's *t* test. In all the above tests, $P < 0.05$ was accepted as statistically significant.

RESULTS

The mean age distribution in ameloblastoma (Group 1) was 31.6 years and in KCOT (Group 2) was 37.8 years. In Group 1 out of 20 cases, 11 were males and 9 were females. In Group 2 out of 20 cases, 10 were males and 10 were females.

The intensity of Bcl-2 expression in Group 1, which consisted of 20 archival specimen showed >50% positivity in 11 cases, 25%-50% positivity in six cases, and three cases

showed complete negative expression for Bcl-2. In Group 2 out of 20 cases, 9 cases showed >50% positivity and eight cases showed 25%-50% positivity for Bcl-2 expression and three cases showed complete negative expression for Bcl-2 ($P = 0.78$, value 0.7843) as indicated in Graph 1.

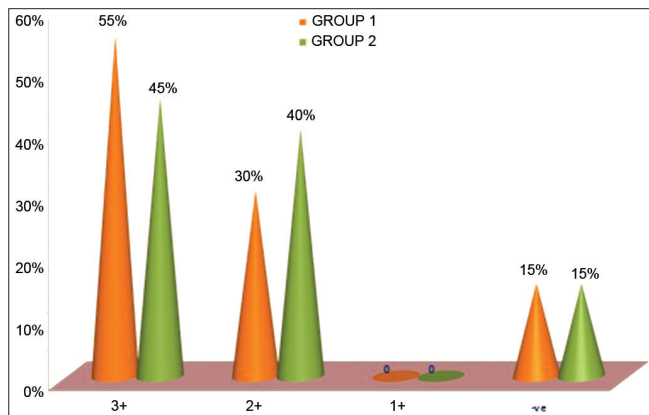
Out of 20 cases, the total positivity for Bcl-2 among Group 1 was 85% and among Group 2 the total positivity for Bcl-2 expression was 85%. Fifteen percent showed negative expression for Bcl-2 in both Group 1 and Group 2 ($P = 1.000$) as indicated in Graph 2.

In all 17 of 20 ameloblastomas, Bcl-2 positivity was seen in the outer layer of the tumor cells and some cases in intermediate cells as shown in Figure 1. In all 17 of 20 KCOTs, positively stained cells were observed in the basal layer as shown in Figure 2.

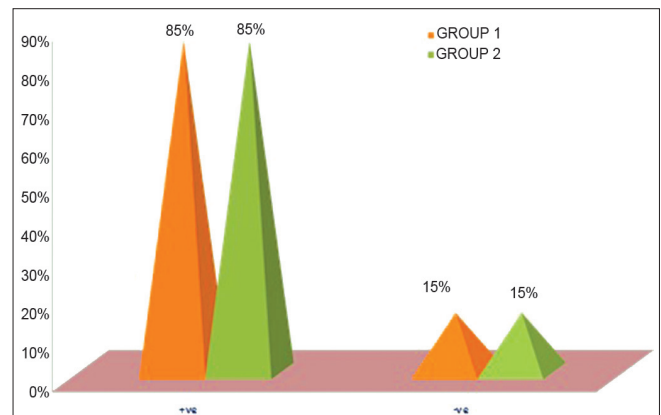
DISCUSSION

Bcl-2, an antiapoptotic protein, was selected for the present study because it is strongly and consistently expressed by all basal cells of KCOTs but not in other odontogenic cysts. Studies have shown that Bcl-2 is rarely expressed by some basal keratinocytes of normal epithelium. Hence, it was likely that Bcl-2 could be used to differentiate keratocyst lining from normal epithelium.

The peripheral cells of ameloblastoma have mitotic potential; in addition they have inherited a long life span via Bcl-2-related pathways. Therefore, residual tumor epithelium containing cells with long life span and high mitotic potential are likely to display an enhanced capacity for latent recurrence. Dysregulated expression of Bcl-2 oncoprotein in oral preneoplastic and neoplastic lesions suggests its possible role in the progression of oral cancer. Therefore, Bcl-2 plays a key role in the early stages of oral tumerogenesis. Hence going by these reasons, Bcl-2 was considered for the present study.



Graph 1: Intensity of Bcl-2 expression in the study groups; Group 1 (ameloblastoma) $n = 20$; Group 2 (keratocystic odontogenic tumor) $n = 20$; $n =$ number of samples



Graph 2: Total positivity and negativity of Bcl-2 expression in the study groups; Group 1 (ameloblastoma) $n = 20$; Group 2 (keratocystic odontogenic tumor) $n = 20$; $n =$ number of samples

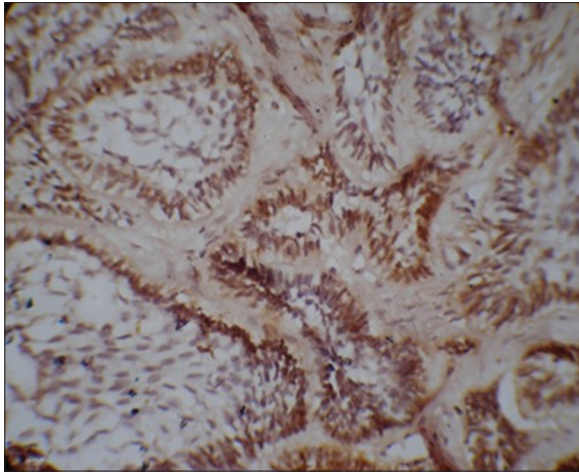


Figure 1: Bcl-2 expression in the outer layer of ameloblastoma (IHC stain, $\times 400$)

In ameloblastoma, Bcl-2 is intensely expressed in peripheral cells (outer layer of tumor cells) and in intermediate cells. This was in concordance with the study of Jie *et al.*^[11] In their study the positive ratio of Bcl-2 was 88% in ameloblastoma, wherein the Bcl-2 protein was expressed in peripheral cells and in stellate-shaped cells with equal intensity. This was true with regard to our study as well where the positive ratio of Bcl-2 was 85% in ameloblastoma and was expressed predominantly in the peripheral cells. The expression of Bcl-2 in the outer layer of the tumor cells suggests that the morphological features of the outer layer cells reflect ameloblastoma growth activity. The outer layer not only has proliferative activity but also has inhibited cell death in the same manner as an epithelial component of the tooth germ.^[14] In addition, Bcl-2 expression appears to be inversely related to the degree of epithelial cell differentiation.^[10]

In KCOT, Bcl-2 is primarily expressed in the basal layer. This is in concordance with the study by Jahanshahi *et al.*^[15] where the positive ratio of Bcl-2 was 95% in KCOT. In this study the immunoreactivity for Bcl-2 was predominantly seen in the basal layer. This observation holds good for our study as well wherein the positive ratio of Bcl-2 was seen in 85% of KCOT and was predominantly localized in the basal cell layer. Because the basal layer of normal mucosal epithelium is also positive for Bcl-2, the lack of expression in the upper layers may be due to decrease in the dividing ability and the termination of cells' life span. Considering the fact that Bcl-2 expression may lead to increased survival of the epithelial cells, there is a definite relationship between aggressive nature of KCOT and intrinsic growth potential of its lining epithelium.^[16] The epithelium of KCOT is thought to have intrinsic growth potential and has been shown to present higher rate of proliferation as compared with other type of cysts. The Bcl-2 positivity in the basal layer of KCOT could point to an abnormal control of cell cycle. The Bcl-2 over-expression could then produce an increase in survival of epithelial cells and this increased life span could in turn

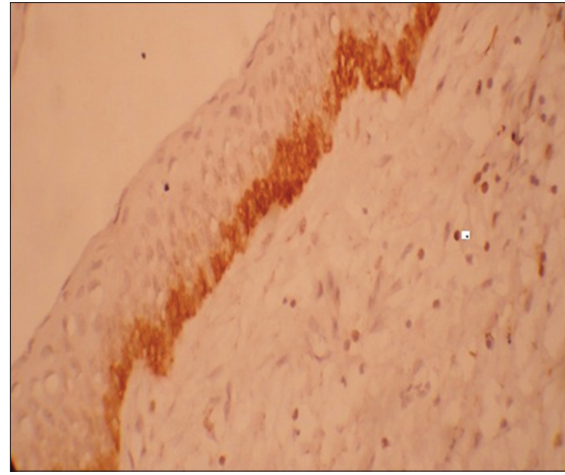


Figure 2: Bcl-2 expression in the basal cells of keratocystic odontogenic tumor (IHC stain, $\times 400$)

lead to peculiar aggressive pattern of KCOT.^[12] In our study 85% of the cases showed positive immunoreactivity and 15% showed negative immunoreactivity for Bcl-2 in both ameloblastoma and KCOT.

CONCLUSION

In our study, the antiapoptotic protein Bcl-2 was expressed in equal intensity in both ameloblastoma and KCOT.

KCOT has an equally aggressive potential like ameloblastoma because the intensity of Bcl-2 expression was the same in both.

The intense expression of Bcl-2 demonstrated a frank difference in the biological character of KCOT. The fact that Bcl-2 positivity was found in the differentiated cells of the basal layer is indeed surprising because Bcl-2 is expressed normally in the progenitor or the stem cells. This unusual proliferative characteristic of KCOT lining may represent an epithelial disorganization similar to dysplasia in oral squamous epithelium. The main difference between this lesion and other cystic lesions is the growth pattern, which suggests that the KCOT has a higher proliferative potential than the other cystic lesions.

Our results provide further support to the assumption that KCOT can be of neoplastic rather than of a cystic nature thus justifying the WHO classification of KCOT as "keratocystic odontogenic tumor." Further efforts in this area are needed to help in our understanding of the Bcl-2 role in the regulation of cell proliferation of KCOT.

The aggressive nature of KCOT warrants an aggressive treatment strategy and its recent reclassification by WHO as a neoplasm should further motivate clinicians in this direction. Hope this study will spawn further investigations in this regard and the novel designation of KCOT as a tumor will serve as a compass by which clinicians can navigate further treatment plans.

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