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RESEARCH ARTICLE

COMPARATIVE STUDY OF Bcl-2 PROTEIN EXPRESSION IN THE EPITHELIAL CELLS OF KERATOCYSTIC ODONTOGENIC TUMOUR AND AMELOBLASTOMA

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ABSTRACT

Background: Keratocystic odontogenic tumour and ameloblastoma are classified under tumours by WHO. tumour development and progression involves tumour suppressor genes and down regulation of onco-genes. Apoptosis inhibition is an important event in tumour formation which increases the life span of these cells.

Aims and Objectives: To investigate the involvement of apoptosis inhibition in these lesions, expression of Bcl-2 was examined in 10 cases of KCOT (keratocystic odontogenic tumour) and ameloblastoma each. Our study revealed over-expression of bcl-2 in all the cases in both tumours. The expression was analysed both qualitatively and quantitatively.

Materials and Methods: 10 KCOT cases (sporadic parakeratinized KCOT n-9 and BCCNS KCOT n-1) and 10 Ameloblastoma cases (SMA n-9 and unicystic ameloblastoma n- 1) were selected. Immunohistochemical staining of Bcl-2 was carried out.

- BCCNS(Basal cell carcinoma nevus syndrome)
- SMA(Solid multicystic Ameloblastoma)
- UA(Unicystic Ameloblastoma)

Statistical Analysis: Differences in the mean scores for immunostain among the two types of lesions were analysed by students t test to compare the similarity or dissimilarity in the cell count of mean Bcl-2 expression between ameloblastoma and KCOT and their various layers.

Results: : The intensity varied among the layers from intense to weak in basal / peripheral ameloblast- like cells and parabasal layers respectively and negative staining was seen only in superficial layer of KCOT but not in Ameloblastoma-central stellate reticulum-like cells in qualitative assessment. Quantitatively the basal layer/ peripheral ameloblast- like cells of both tumours had similar over expression while parabasal and central/superficial cells showed considerable differences. Our study indicates similar over-expression of bcl-2 in both the odontogenic lesions specifically basal layer / peripheral ameloblast- like cells which may represent tumorigenesis by escaping apoptosis. However does antiapoptotic activity and proliferative index warrant reclassification to tumour needs to be addressed.

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INTRODUCTION

Apoptosis, also known as cell programmed cell death or physiologic cell death, plays an important role in the development and maintanance of homeostasis within all multicellular organisms (Nazi *et al*., 2015). Bcl-2 is an anti-apoptotic protein that prolongs the survival of cells by blocking apoptosis and promotes development of tumour. (Tekkesin *et al*., 2012) Bcl‑2 belongs to group of antiapoptotic protein that regulates programmed cell death. Bcl‑2 over expression is found in early phase of epithelial carcinogenesis. (Sindura *et al*., 2013) Enhanced expression of Bcl‑2 is seen in almost all human neoplasm. As a critical regulator of apoptosis, Bcl‑2 plays a key role in early stages of oral tumorogenesis. Because Bcl‑2 serves to prevent cell death, its occurrence in odontogenic tissues is helpful in identifying cell population from which odontogenic tumours may arise. (Sindura *et al*., 2013) In KCOT, Bcl‑2 showed a strong positivity in basal layer, which confirmed abnormal control of cell cycle. The Bcl‑2 over expression increased the survival of epithelial cells, which led to peculiar growth pattern of KCOT. (Sindura *et al*., 2013) Bcl‑2 showed a strong positivity for Ameloblastoma. It was detected mainly in the peripheral ameloblast like cells and central stellate reticulum-like cells suggesting that morphological features of peripheral ameloblast-like cells reflect Ameloblastoma growth activity. (Sindura *et al*., 2013) Over expression of Bcl‑2 is associated

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with genesis and development of Ameloblastoma. Studies have been conducted in the past to detect its role in tumourigenesis, a few also to evaluate its role in determining the biological behaviour of odontogenic cysts. (Tekkesin *et al*., 2012) Results of various studies evaluating its role in odontogenic tumours have been conflicting that needs to be explored further. Keeping all this in view, the current study was planned to determine the biological behaviour of the KCOT and Ameloblastoma by evaluating the expression of bcl-2 immunohistochemical marker in them. Thus the aim of the study is to assess, evaluate the pattern of distribution, localization of the positively stained cells and to compare the Bcl2 expression in the epithelial component of KCOT and Ameloblastoma.

MATERIALS AND METHODS

A retrospective study included a total 10 KCOT and 10 Ameloblastoma, which were formalin fixed and paraffin embedded. The cases from the department archives were retrieved by random sampling. All the demographic details were noted from the respective case files. Ameloblastoma with no history of malignancy were included and KCOT with orthokeratinised and diagnosed with other odontogenic cyst or tumours were excluded in the study. Institutional review board approval was obtained.

IHC Staining

Sections of 4mm thickness were obtained, deparaffinised and antigen retrieval performed by microwave heating for 8 minutes. Then the slides were later treated with Bcl-2 (Biogenix Life Sciences Limited, CA) for immunohistochemical analysis using the super sensitive polymer HRP detection system. For antigen heat retrieval, deparaffinised sections (xylene 2x10 min; absolute alcohol, 1x5min; 95% alcohol, 1x5 min) were placed in the pressure cooker in 0.01M citrate buffer (pH 6.0 to 6.2) for 2 minutes. After 5 minutes of cooling, they were placed in a humidifying chamber. Hydrogen peroxide (3%) was applied to cover the section and incubated for 10 minutes at room temperature, and rinsed in tris buffer saline (TBS) (pH 7.6). All sections were then incubated with power block for 10 minutes and treated with the primary antiserum (1:25) at room temperature for 1 hour. Incubation with the Super Enhancer was performed for 15 minutes. After washing with TBS for 3 minutes, secondary antibody is added and incubated for 30 minutes; all specimens were then washed with TBS. One drop (38 ml) of liquid diaminobenzidine (DAB) chromogen was added to 1mL of stable DAB substrate buffer in the mixing vial. This DAB chromogen reagent is added and incubated for 5 to 15 minutes until acceptable colour intensity was reached. And then the sections were counter stained with Harris haematoxylin.

Interpretation of Staining

The presence of brown colour at the site of target antigen was considered immunopositive for Bcl-2.The intensity, pattern of distribution, and localization of the immunoreactive cells were determined using conventional light microscopy. The immunoreactivity was assessed both qualitatively and quantitatively by two investigators three times to overcome intra-observer's variability. The qualitative assessment was done by grading the immunoreactivity in a 3 grade scoring system. The BCl2 expression was scored varying from 0-2+. 0 was defined as being negative and 1+ and 2+ as being weakly positive and strongly positive respectively. The epithelium of the KCOT was assessed separately for the basal layer, the parabasal layer (which included single layer of cell above the basal layer), and remaining layers up to the lumen terming them as superficial layer. In Ameloblastoma the same classification was applied for the peripheral ameloblast –like cells and parabasal and the central stellate reticulum like cells. Quantitative assessment was done by counting the number of cells using a 20X20 grid. The grid was divided into 20x20 squares and each square measured 500µm in dimension. At high power magnification three representative areas were selected in the basal layer and superficial/ innermost layer separately for quantitative assessment. The positive cells were calculated for three layers in KCOT – basal layer, parabasal layer and superficial layer and in Ameloblastoma as the peripheral ameloblast- like cells, the parabasal layer and the central stellate reticulum-like cells. The total mean positive cells for all layers were calculated together and independently were calculated for both Ameloblastoma and KCOT. Differences in the mean scores for immunostain among the two types of lesions were analysed by student's t test to compare the similarity or dissimilarity in the cell count of Mean Bcl-2 expression between Ameloblastoma and KCOT and their various layers. A p Value less than 0.05 were considered to be statistically significant.

RESULTS

The qualitative and quantitative assessment when analysed threw some interesting facts. The immunostaining was 100% positive in both KCOT and Ameloblastoma. Qualitatively in KCOT, the Bcl-2 immunostaining was strongly positive in basal cell layer and weakly positive in parabasal layers and negative in superficial layers in 100% of the cases (Table 1). There was no change noticed in either intensity or pattern of staining between the sporadic and BCCNS cases suggesting that Bcl-2 proteins were expressed by both the tumours. The staining of Ameloblastoma showed strongly positive expression in 100% of cases in the peripheral ameloblast like-cells while the parabasal and central stellate reticulum like cells, the intensity of the staining was weakly positive in all cases.(Table 2) There was no difference between the SMA and UA. Quantitatively the results were analysed by using student's t- test (Table 3). The total mean cell count (all layers together) in KCOT was 19.43± 2.577 and Ameloblastoma 20.87±6.46 resulting in p value greater than 0.05 which suggests that the difference between the two study groups was insignificant. The parabasal layers had some differences. The KCOT had mean cell count of 27.50±5.72, while Ameloblastoma had 18.0070±35, the p value lesser than 0.005 suggesting significant difference between the two layers (Table 4a,b,c) In the superficial layer of KCOT the mean cell count in 100% of cases was negative for staining, while central reticulum like cells of Ameloblastoma it was 12.008.50±2. p value is invalid when the mean value of one study group is zero.

Qualitative Assessment:

Table 1. Localization of bcl-2 expression in the lesions

KCOT	Basal layer	+ ve
	parabasal layer	+ve
	Superficial layer	-ve
Ameloblastoma	Peripheral ameloblast- like cells	+ve
	Central stellate reticulum- like cells	+ve

Table 2. Strength of expression of bcl-2 in the lesions

KCOT	Ameloblastoma				
	Basal	Parabasal	Superficial	peripheral	Central stellate reticulum-like cells
0	-	-	100%	-	-
1+	-	100%	-	-	100%
2+	!00%	-	-	100%	

0- negative staining, 1+ weakly positive, 2+ strongly positive.

Table 3:

Trial 1:

Parameter	Observer	Mean	Std dev	SE of Mean	Mean difference	T	P-Value
KCOT - Basal Layer	Observer 1	30.80	4.78	1.51	-7.300	-2.166	0.052
	Observer 2	38.10	9.53	3.01			
KCOT - Parabasal Layer	Observer 1	14.40	4.48	1.42	-0.200	-0.062	0.951
	Observer 2	14.60	9.08	2.87			
KCOT - Superficial Layer	Observer 1	0.00	0.00	0.00	0.000	---	---
	Observer 2	0.00	0.00	0.00			
Ameloblastoma - peripheral Ameloblast - like cells	Observer 1	32.60	8.40	2.65	-3.000	-0.947	0.356
	Observer 2	35.60	5.46	1.73			
Ameloblastoma - Parabasal Layer	Observer 1	18.00	7.35	2.32	-0.700	-0.215	0.832
	Observer 2	18.70	7.20	2.28			
Ameloblastoma – central stellate reticulum - like cells	Observer 1	12.00	8.52	2.70	-1.600	-0.453	0.656
	Observer 2	13.60	7.21	2.28			

Trial 2:

Parameter	Observer	Mean	Std dev	SE of Mean	Mean difference	t	P-Value
KCOT **- Basal Layer**	Observer 1	30.10	7.69	2.43	-1.100	-0.359	0.724
	Observer 2	31.20	5.90	1.87			
KCOT **- Parabasal Layer**	Observer 1	15.30	4.90	1.55	-1.800	-0.475	0.643
	Observer 2	17.10	10.94	3.46			
KCOT **- Superficial Layer**	Observer 1	0.00	0.00	0.00	-1.000	-1.500	0.151
	Observer 2	1.00	2.11	0.67			
Ameloblastoma **Peripheral ameloblast-like cells**	Observer 1	31.30	8.58	2.71	-3.500	-1.094	0.289
	Observer 2	34.80	5.37	1.70			
Ameloblastoma **- Parabasal Layer**	Observer 1	20.10	10.66	3.37	0.100	0.027	0.979
	Observer 2	20.00	4.94	1.56			
Ameloblastoma **central stellate reticulum–like cells**	Observer 1	17.30	5.76	1.82	3.300	1.347	0.195
	Observer 2	14.00	5.19	1.64			

Trial 3:

Parameter	Observer	Mean	Std dev	SE of Mean	Mean difference	t	P-Value
KCOT - Basal Layer	Observer 1	31.90	6.79	2.15	-1.800	-0.707	0.488
	Observer 2	33.70	4.32	1.37			
KCOT - Parabasal Layer	Observer 1	16.30	4.32	1.37	0.100	0.300	0.976
	Observer 2	16.20	9.44	2.98			
KCOT - Superficial Layer	Observer 1	0.00	0.00	0.00	0.000	---	---
	Observer 2	0.00	0.00	0.00			
Ameloblastoma- peripheral ameloblast- like cells	Observer 1	34.80	11.98	3.79	-0.400	-0.090	0.930
	Observer 2	35.20	7.48	2.37			
Ameloblastoma -Parabasal Layer	Observer 1	20.90	9.99	3.16	-1.800	-0.486	0.633
	Observer 2	22.70	6.09	1.93			
Ameloblastoma-central stellate reticulum-like cells	Observer 1	20.00	11.37	3.60	2.800	0.706	0.490
	Observer 2	17.20	5.31	1.68			

Table 4a. Comparison of Basal layer values between the KCOT and peripheral ameloblast like –cells of ameloblast lesions

Lesion	Mean	Std dev	SE of Mean	Mean difference	t	P-Value
KCOT	30.80	4.78	1.51	-1.800	-0.589	0.563
Ameloblastoma	32.60	8.40	2.65			

Table 4b. Comparison of Parabasal layer values between the two lesions

Lesion	Mean	Std dev	SE of Mean	Mean difference	t	P-Value
KCOT	27.50	5.72	1.81	9.500	3.226	0.005*
Ameloblastoma	18.00	7.35	2.32			

*denotes significant difference

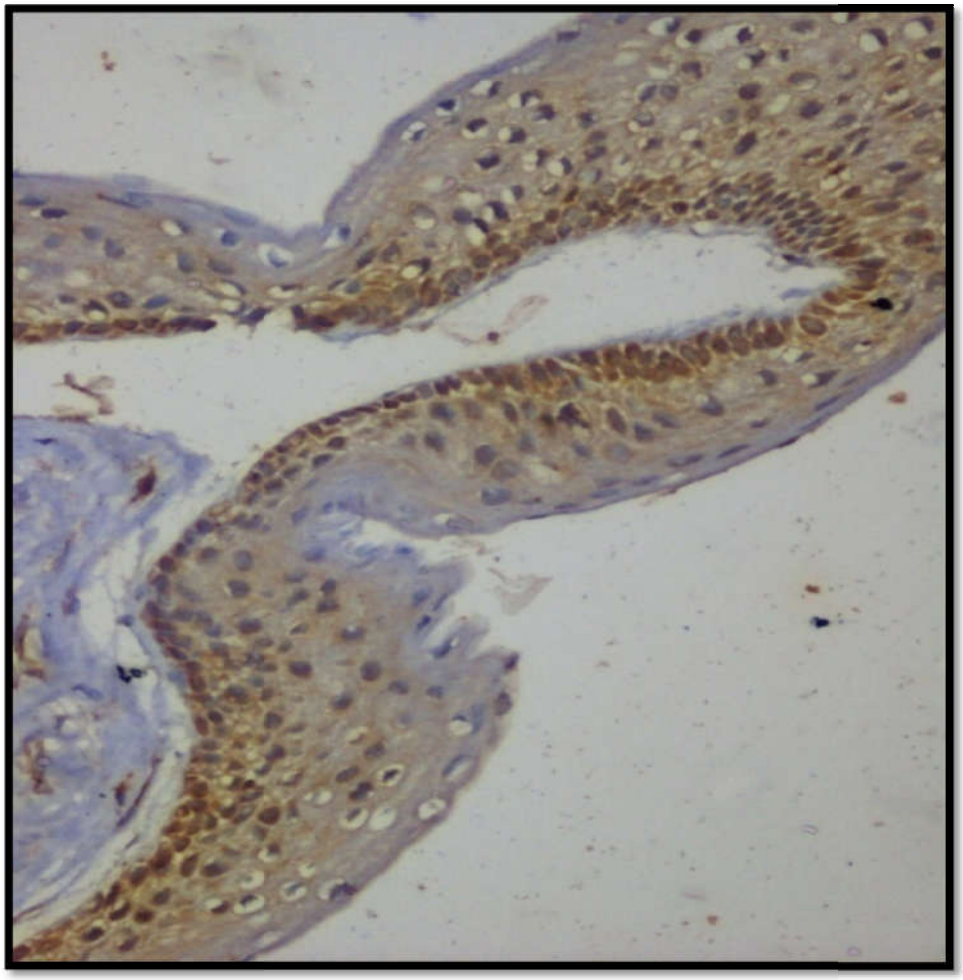
The difference in mean no. of cells in parabasal layer was found to be high in KCOT compared to Ameloblastoma and the difference between them was found to be statistically significant (P<0.01).

Table 4c:

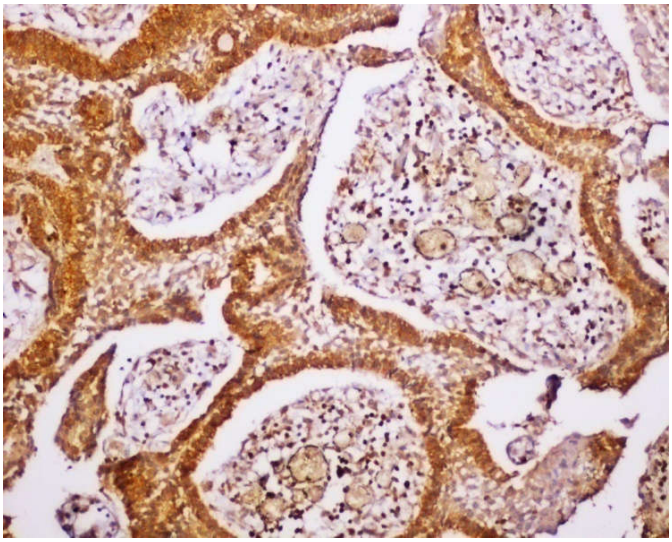
Layer	KCOT (Mean±SD)	Ameloblastoma (Mean±SD)	Mean Difference	t	P-Value
Basal/peripheral ameloblast- like cells	30.80±4.78	32.60±8.40	-1.8	-0.589	0.563
Parabasal	27.50±5.72	18.00±7.35	9.5	3.226	0.005*
Innermost/central stellate reticulum- like cells	0.00±0.00	12.00±8.52	-12	-4.452	0.002*

*denotes significant difference

The difference in no. of cells between KCOT and Ameloblastoma at Basal Layer/peripheral ameloblast –like cells is not statistically significant (P>0.05).
The difference in no. of cells between KCOT and Ameloblastoma at Parabasal Layer is found to be statistically significant (P<0.01).
The difference in no. of cells between KCOT and Ameloblastoma at superficial /central stellate reticulum-like cells is found to be statistically significant (P<0.01).
No comparison is carried out between the two lesions for superficial/central stellate reticulum –like cells as the 32values for KCOT was all zero.



Photomicrograph of KCOT showing Bcl-2 expression at 20x objective



Photomicrograph of Ameloblastoma showing Bcl-2 expression at 20x Objective

In 25% of KCOT there was intense inflammation in the underlying connective tissue capsule. It was further noticed that the basal layer of the epithelial lining above the inflamed capsule, the staining was weakly positive.

While in Ameloblastoma the inflammation did not seem to have any effect in staining pattern of peripheral ameloblast like- cells or in central stellate reticulum like--cells of Ameloblastoma.

DISCUSSION

Ameloblastoma represent the most common odontogenic neoplasia of the mandible, being included by the WHO in the group of tumours derived from odontogenic epithelium, with mature fibrous stroma. (Kramer *et al*., 1992) In the follicles, the peripheral cells of Ameloblastoma have mitotic potential; in addition they have inherited a long life span via Bcl–2-related pathways. Dysregulated expression of Bcl-2 oncoprotein in tumours suggests its possible role in tumerogenesis. (Sindura *et al*., 2013) In the present study strongly positive immune staining at the basal layer whereas parabasal layers revealed weak intensity. Similar finding has been reported by Kumamoto H *et al*. In contrast there is significant difference in Bcl-2 expression between central and peripheral cells of tumour islands has been reported by various authors. Strongly positive BCl2 expression at peripheral areas being considered proliferative areas. Bcl-2 showed a strong positivity for Ameloblastoma. It was detected mainly in the peripheral ameloblast like- cells and parabasal layer suggesting that morphological features of peripheral ameloblast like cells reflect Ameloblastoma growth activity. Overexpression of Bcl-2 is associated with genesis and development of ameloblastoma. (Jie *et al*., 2006) Sandra *et al* correlated the expression of Ki67 & PCNA index with age, the lowest index value was noted in the young patient compared to elderly age group. (Sandra *et al*., 2001) Whereas Jaaskelanin *et al*. could not find the correlation of proliferative marker and clinical parameter. (Jaaskelainen *et al*., 2002) Migaldi *et al.* demonstrated low proliferation index irrespective of histological type of ameloblastoma. (Migaldi *et al*., 2008) Similarly, Bello IO *et al* studied proliferative activity in Ameloblastoma carcinoma and Ameloblastoma; he found Ki 67 index was almost same in both. (Bello *et al*., 2009)

KCOT

In the present study KCOT showed, strong and consistent expression of Bcl-2 in the basal layer, with mild staining in the parabasal layer and negative staining in the superficial layer. This is in concordance with the study by Jahanshahi *et al*, where the positive ratio of Bcl-2 was 95% in KCOT. (Jahanshahi *et al*., 2006) In this study the immunoreactivity for Bcl□2 was predominantly seen in the basal layer. This observation holds good for our study as well where in the positive ratio of Bcl-2 was seen in 100% of KCOT and was predominantly localized in the basal cell layer. The proliferative status has not always been directly associated with biological behaviour or true neoplastic nature. (Souza *et al*., 2000) Carolina *et al.* have opined that the biology of tumour is beyond the cell proliferation and that cell cycle regulation plays an important role. Non–neoplastic proliferative lesions also demonstrated high expression of some proliferative markers. (Gomes *et al*., 2009) An example was the expression of ki-67 in mucoepidermoid carcinoma which was significantly lower than in glandular odontogenic cyst. (Kaplan *et al*., 2005) The aggressive behaviour might be attributed to the increased life span of cells; where Bcl-2 seemed to have a role in the extension of cell survival. (Piattelliab *et al*., 1998) It's over expression has been reported in most low-grade tumours and this inhibition of apoptosis was regarded as one of the common pathways of tumorigenesis. (Shear, 2002) Hence bcl-2 expression was compared between Ameloblastoma a common well known odontogenic tumour and KCOT a cystic tumour.

In KCOT, the exact reason for the layers of cells to change from intense staining to weakly staining and finally turning negative was unknown. However Kichi *et al*. 2005 tried to explain this expression and concluded that Bcl-2 inhibited apoptosis to facilitate cellular proliferation in the basal layer whereas apoptosis maintained the homeostasis of the thickness of the lining epithelium and allowed the synthesis of keratin on the surface. This finding they suggested, indicated that although active cell proliferation occurred in the lining of KCOT, as evidenced by p53, PCNA and ki-67 positivity demonstrated in number of studies, this proliferation was regulated by the apoptotic activity so that this lesion manifest as cyst and not as tumour masses. (Mervyn Shear and Paul M. Speight, 2007) The Bcl2 strongly and consistently expressed by all basal cells of KCOTs. Studies have shown that Bcl-2 is rarely expressed in other odontogenic cysts. Hence, it was likely that Bcl-2 could be used to differentiate keratocyst lining from normal epithelium and other odontogenic cysts.

In the present study KCOT showed, 100% strong and consistent expression of Bcl-2 in the basal layer, with mild staining in the parabasal layer and negative staining in the superficial layer. This is in concordance with the study by Jahanshahi *et al*. where the positive ratio of Bcl-2 was 95% in KCOT. (Jahanshahi *et al*., 2006) 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 100% of KCOT and was predominantly localized in the basal cell layer, parabasal weak staining was observed and negative in the superficial 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. (Sandra *et al*., 2001) 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 lead to peculiar aggressive pattern of KCOT. (Piattelliab *et al*., 1998) Both Ameloblastoma and KCOT showed significant and consistent positive, intense staining at the basal layer with mean value of 30.80±4.78 and 32.60±8.40 respectively and did not show statistical significant difference. Whereas, the difference in number of cells between KCOT and Ameloblastoma at Parabasal Layer and superficial layer in KCOT and parabasal layer and peripheral ameloblast like- cells showed statistically significant difference ($P<0.01$). However does antiapoptotic activity and proliferative index warrant reclassification to tumour needs to be addressed.

Conclusion

The consistent intense expression of Bcl2 in the basal cell of KCOT and peripheral ameloblast like- cells in Ameloblastoma indicates that, the KCOT behaviour is similar to Ameloblastoma and also this factor can be well correlated with the clinical behaviour of tumour and recurrence. However the difference in the central and superficial layer of cells may be the reason for one lesion to turn out to be a cyst while the other turns into a solid mass of cells resulting in a tumour.

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