Comparative immunohistochemical study of Bcl-2 and CD138 in odontogenic keratocyst, ameloblastoma, and radicular cyst, considering WHO classification

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Background
Odontogenic keratocyst (OKC) is one of the odontogenic lesions that have been recently classified as a developmental cyst in the last WHO odontogenic classification in 2017, despite its clinical aggressiveness and relative rate of recurrence.

Aim
The aim of the current work was to assess and compare OKC with both ameloblastoma (AM) and radicular cyst (RC) in relation to immunohistochemical expression of Bcl-2 and CD138.

Materials and methods
This is a retrospective study that included 15 cases of OKC, 20 cases of AM, and 15 cases of RC. Immunohistochemical technique was applied to detect and compare Bcl-2 and CD138 expressions in these lesions.

Results
For Bcl-2, 86.6% of the OKC cases and all studied AM cases (100%) were positive for Bcl-2, without significant statistically correlation ($P>0.05$). At the same time, OKCs were significantly correlated with RC, which were completely negative to Bcl-2 ($P<0.05$). CD138 was detected in both epithelium and stroma. Epithelial CD138 reactivity was found in 93.4% of OKC and in 86.7% of RC, with significant difference ($P<0.05$), whereas AM showed cytoplasmic and membranous epithelial CD138 staining in 40% of cases. Stromal CD138 expression was detected in 73.4% of OKC, 75% of AM, and 40% of RC, with a nonstatistically significant difference ($P>0.05$).

Conclusion
The results of the current study registered (a) positive Bcl-2 expression in OKC and AM compared with negativity of RC; (b) increased CD138 epithelial expression in OKC and RC than in AM; and (c) increased stromal CD138 expression in both OKC and AM than RC. These results reflect difference in their growth profile and invasive behavior. At the same time, it elucidates that molecular defect in Bcl-2 and CD138 protein is critical for biology of studied odontogenic lesions with consequently effect on their diagnosis and treatment. However, the localization of Bcl-2 expression supports the classification of OKC as a neoplastic one rather than a developmental cyst, in contrary to the new WHO classification. So, further studies with markers panel are recommended for more understanding of the biological nature of OKC.

Introduction

Odontogenic apparatus counting odontogenic epithelia and/or ectomesenchyme gives rise to odontogenic lesions with variable biological behavior and molecular expression (Rajendran, 2009). They have comparable clinical and radiographic highlights and also may have histopathologic overlapping (de Vicente Rodríguez et al., 2010). Odontogenic keratocyst (OKC) is one of these lesions that are as of late reviewed as developmental lesions, agreeing to final WHO odontogenic classification. It was considered as a neoplastic lesion in previous classification (2005) (Barnes et al., 2005; Soluk-Tekkeşin and Wright, 2018).

Clinically, OKC still behaves as a locally aggressive lesion with significant rate of recurrence after excision; at the same time, the considered evidence of its neoplastic nature is insufficient (Hammad et al., 2020), so there is a high interest to study such lesion.

Another notable change in this new classification is concerning. Ameloblastoma (AM), one of the most

common odontogenic tumors, which is known as a slow-growing and a locally invasive tumor with a high recurrence risk. AM is narrowed into conventional AM, unicystic AM, and extraosseous/peripheral types (Bilodeau and Collins, 2017).

Radicular cysts (RC; apical periodontal cysts) are cystic lesions of the jaw with inflammatory reaction within nonvital teeth, keeping its previous categorization as inflammatory lesion in the new classification (Bilodeau and Collins, 2017).

Apoptosis is a distinct form of programmed cell death that is important for embryogenesis and cell homeostasis. The imbalance between proliferation and apoptosis is involved in oncogenesis of various tumors and diseases. B-cell lymphoma 2 (Bcl-2) family proteins constitute the key role in regulating apoptosis process (Brown and Betz, 2015), which consists of proapoptotic proteins (BAX and BAK) and antiapoptotic members (Bcl-2, Bcl-X_L, and BCL-w,). Bcl-2 overexpression has been related to different tumors and many diseases as well as tooth development stages (Baliga and Kumar, 2002).

Bcl-2 is located in the external membrane of mitochondria, and endoplasmic reticulum nuclear membrane; its overexpression, detected in early stages of neoplasm, leads to uncontrolled cell growth with apoptosis resistance (Hockenbery et al., 1990).

The expression of Bcl-2 gene products has been studied in tooth germs and AM, suggesting an important role in tumor growth and odontogenesis (Kumamoto and Ooya, 1999).

Syndecan-l, known as CD138, is a transmembrane sulfate proteoglycan that is expressed in endothelial cells, and stromal fibroblasts (Stepp et al., 2015). Syndecan-1 is essential for many biological processes, including the regulation of cellular differentiation, the maintenance of epithelial morphology and cell adhesion, as well as for epithelial–mesenchymal interactions (Averbeck et al., 2017). Its expression has been varied from complete loss to overexpression in relation to tumors behavior (Wang et al., 2018).

In the current work, OKC was studied in comparison with AM (neoplastic category) and RC (inflammatory category) in relation to the immunohistochemical Bcl-2 and CD138 expression to shed more light on its biological behavior, aggressive nature, and its compatibility with the new classification as a development cyst.

Materials and methods

This is a retrospective selective controlled study approved by the Institutional Review Board of Faculty of Medicine, Benha University. Overall, 15 tissue specimens of OKC, 20 of histopathologically confirmed cases of AM, and 15 cases of RC were included in this study. The cases were collected from histopathologic archive of files of Pathology Department, Faculty of Medicine, Benha University, in the period January 2015 to December 2019. Paraffin-embedded tissue sections were obtained from archival tissue blocks. Hematoxylin and eosin sections were reviewed to confirm diagnosis.

Immunohistochemistry staining

Two sections of 4-μm thickness of each block were processed for immunohistochemical analysis using avidin–biotin complex technique according to manufacturer's guidelines (Genemed, South San Francisco, California, USA) (Hsu et al., 1981). Antigen retrieval was applied with 10-mM sodium citrate solution (pH=6) using a pressure cooker for 4 min. The primary antibodies were applied for 60 min of Bcl-2 (clone 124, ready to use; Dako, Dali, Produktionsvej, Denmark) at room temperature, and CD138 (CD138: Clone MIB 15, 1 : 100, monoclonal mouse anti-human; Dako) at 4°C overnight.

Sections of tonsil and normal skin tissue were used as a positive control for Bcl-2 and CD138, respectively; the incubation with the primary antibodies was omitted for negative controls.

Immunostaining interpretation

The expression was calculated by light microscopy, and photomicrographs were obtained using a digital camera (Olympus C-7070).

For Bcl-2, brown cytoplasmic staining of epithelial cells was defined as positive staining and negative staining was considered with the complete absence of the stain. The staining distribution was classified as follows: 0, absence of immunostaining; 1 (low), 1–10% positive cells; 2 (intermediate), 11–50% positive cells; and 3 (high), more than 50% positive cells (Tenório et al., 2018).

For CD138, syndecan-1 expression was considered positive with membranous and/or cytoplasmic immunoexpression of more than 5% of the epithelial or stromal cells using the following criteria: negative: less than 5% positive, low expression: 5–50% positive, and high expression: more than 50% of cells positive.

The intensity of staining was classified as weak (+), and strongly positive (++) (Nadalin *et al.*, 2011).

Statistical analysis

Statistical analysis is performed using the SPSS (statistical program for social science) software, version 16.0 (SPSS Inc., Chicago, Illinois, USA). The significant P value was considered to be less than 0.05.

Results

Histopathological results

The histopathologic diagnosis was established according to WHO classification (2017) (Wright and Vered, 2017).

(1) OKC (15 cases): it showed uniform stratified squamous epithelial lining without rete ridges. They have palisaded and hyperchromatic basal cells with often a corrugated surface layer of keratin (Fig. 1a).

(2) AM (20 cases): acanthomatous type and follicular variants with discrete islands of tumor cells showing peripheral nuclear palisading besides central loose stellate reticulum-like cells (Fig. 2a).

(3) RC (15 cases): it showed cystic lesion lined by epithelium, and numerous inflammatory cells beneath the lining with occasional cholesterol clefts within fibrous wall and/or inside the cavity (Fig. 3a).

Immunohistochemical results

Interpretation of Bcl-2

Concerning Bcl-2 staining within the studied three groups, 13 (86.6%) of the 15 studied cases of OKC and all the 20 studied AM cases were positive for Bcl-2, whereas none of studied RC showed positive expression, as shown in Table 1.

Figure 1
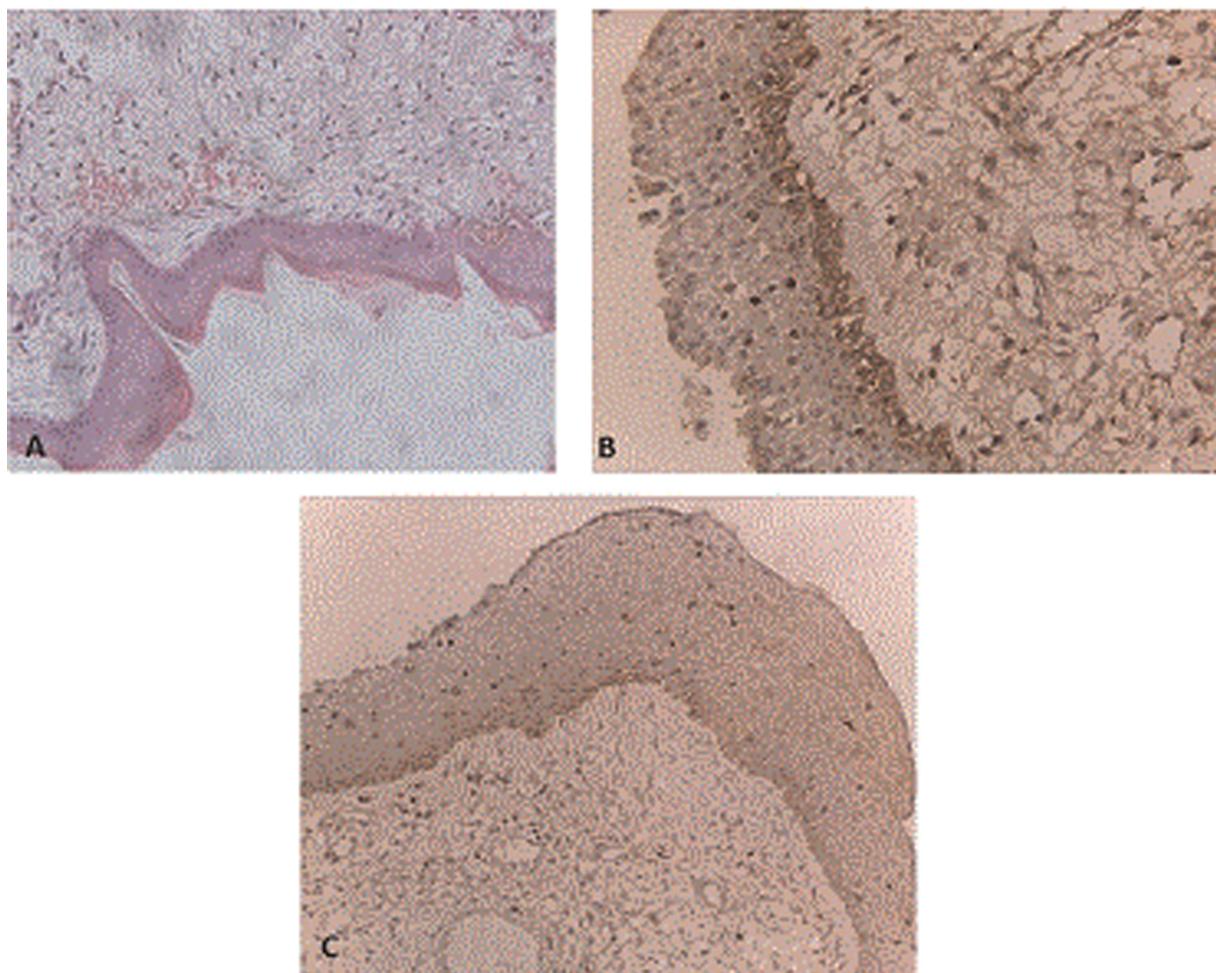
Odontogenic keratocyst. (a) Uniform stratified squamous epithelium with basal palisading, and overlying corrugated parakeratin (hematoxylin and eosin, ×100). (b) Showing positive cytoplasmic staining of Bcl-2 in the epithelial layers with basal layer showing accentuation of the staining (immunohistochemical, ×200). (c) Showing positive membranous and cytoplasmic staining of CD138 of all epithelial layers (immunohistochemical, ×200).

Figure 2
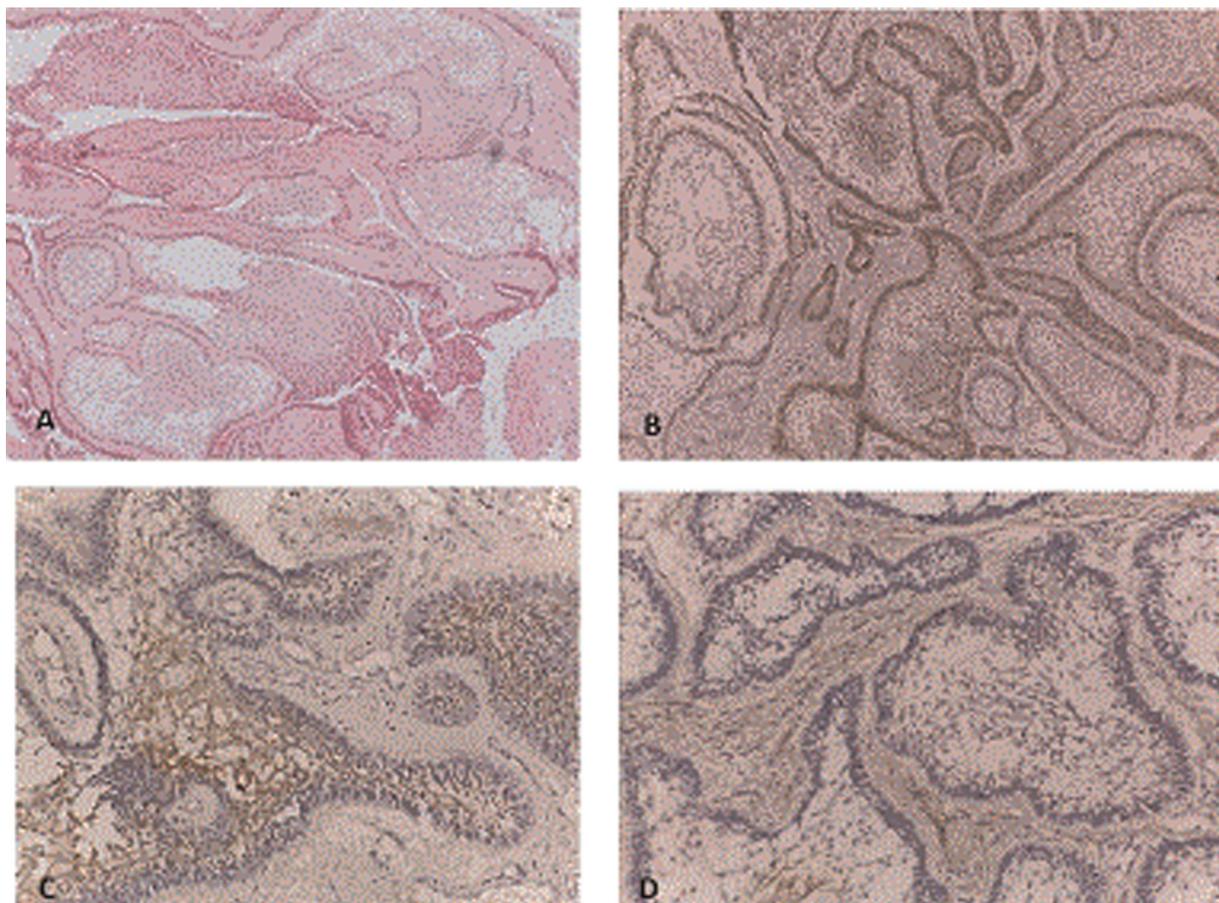
Ameloblastoma. (a) Acanthomatous ameloblastoma showing islands of epithelium with peripheral palisading, reverse nuclear polarity (hematoxylin and eosin, ×100). (b) Showing positive cytoplasmic staining of Bcl-2 in the epithelium (immunohistochemical, ×100). (c) Showing positive stellate reticulum cells of CD138 and negative epithelium (immunohistochemical, ×200). (d) Showing positive membranous and cytoplasmic staining of CD138 in the stroma with negative epithelial cells (immunohistochemical, ×200).

Bcl-2 was positive in 86.6% of OKC and 100% in studied AM cases, with no significant statistical difference ($P>0.05$), whereas OKC was significantly correlated with RC, which were completely negative to Bcl-2 ($P<0.05$).

Concerning the distribution, in studied OKC cases, it was detected mainly in basal and suprabasal cells of OKC cases (Fig. 1 and 2b). In AM, the peripheral cell layers showed more accentuation than the central layers, as shown in Fig. 2b.

When intensity of Bcl-2 was analyzed, most of both OKC and AM cases showed moderate intensity (46.1 and 60%, respectively), whereas strong immunostaining was found in 15.5% of OKC and 25% of AM cases, as illustrated in Table 2.

Interpretation of CD138
CD138 immunostaining was observed in both epithelial and stromal cells of the studied lesions.

Epithelial CD138 reactivity was found in 93.4% of OKC (in all lining epithelium) (Fig. 1c) and in 86.7% of RC cases (mainly in superficial layers) (Fig. 3c), with significant difference ($P<0.05$), whereas it was detected in 40% of AM as a cytoplasmic and membranous staining, as listed in Table 3.

Stromal CD138 expression was detected in 73.4% of OKC, 75% of AM, and 40% of RC, with nonsignificant statistically difference, as described in Table 4.

Regarding stromal expression of studied AM, there was a significant increased CD138 expression, which was recorded in 75% of stellate reticulum cells in comparison with 40% of epithelial cell (Fig. 2c), as shown in Table 4 ($P<0.05$).

Discussion
Odontogenic lesions are a heterogenous group that are classified into developmental lesions, cysts, and benign

Figure 3
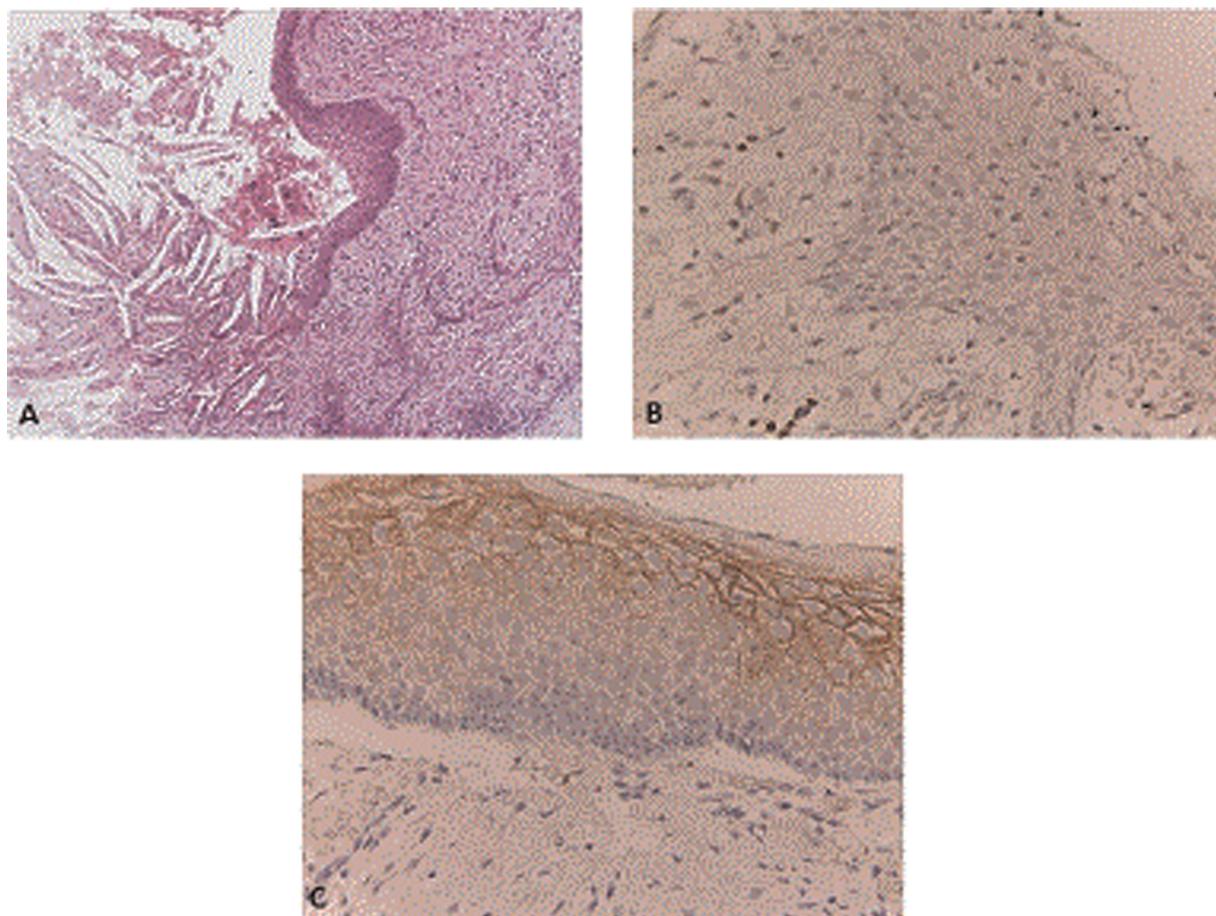
Radicular cyst. (a) The epithelial lining assumed eroded pattern with numerous inflammatory cells beneath its surface and cholesterol clefts in the epithelial surface (hematoxylin and eosin, ×100). (b) Showing negative staining of Bcl-2 in both the epithelium and the stroma (immunohistochemical, ×200). (c) Showing positive cytoplasmic and membranous staining CD138 with accentuation in the superficial epithelial layers (immunohistochemical, ×200).

Table 1 Total Bcl-2 immunostaining in odontogenic keratocyst, ameloblastoma, and radicular cyst studied cases

Groups	Bcl-2 staining [n (%)]		Total
	Negative	Positive	
OKC	2 (13.4)	13 (86.6)	
AM	0	20 (100)	20
RC	15 (100)	0	15

AM, ameloblastoma; OKC, odontogenic keratocyst; RC, radicular cyst.

or malignant neoplasm with variable biological and clinical behavior. The molecular pathogenesis of them is still speculative (Wright and Vered, 2017). OKC was considered as a benign tumor rather than a developmental cyst owing to its locally aggressive behavior in the previous WHO classification (2005) (Leite *et al.*, 2011).

Recently, the newest WHO odontogenic lesion classification reclassified OKC as a developmental cyst (Wright and Vered, 2017). Despite the established new reclassification of OKC, its nature is still controversial issue, especially 85% of OKC is related to the nevoid basal cell carcinoma syndrome, and 30% of nonsyndromic cases reported mutated PTCH gene. Besides, OKC still invades the surrounding tissue and has a relative recurrence rate (Martin and Speight, 2015).

There is a significant advance in the oral pathology practice, especially with the considerable change of recent WHO odontogenic tumor and cyst classification. The assessment of tumor markers is the center point in this advance for better understanding their nature and behavior (Yoithapprabhunath *et al.*, 2019).

This study investigated the expression of Bcl-2 (antiapoptotic) and CD138 (cell adhesion molecules) to highlight the biological aspect of OKC and comparing that with AM (neoplastic category) and RC (inflammatory category).

Table 2 Comparative analysis of Bcl-2 staining score and intensity in odontogenic keratocyst, ameloblastoma, and radicular cyst studied cases

Bcl-2 immunoreactive score	Negative	OKC [n (%)]	AM [n (%)]	RC [n (%)]	P value		
					OKC vs. AM	OKC vs. RC	AM vs. RC
		2 (13.4)	0	15 (100)	>0.05	<0.05*	<0.05*
	1	1 (7.8)	2 (10)	0			
	2	6 (46.1)	11 (55)	0			
	3	6 (46.1)	7 (35)	0			
Total		15	20	15			
Bcl-2 intensity	Negative	2 (13.4)	0	15 (100)	>0.05	<0.05*	>0.05
	Faint	5 (38.4)	3 (15)	0			
	Moderate	6 (46.1)	12 (60)	0			
	Strong	2 (15.5)	5 (25)	0			
Total		15	20	15			

AM, ameloblastoma; OKC, odontogenic keratocyst; RC, radicular cyst. *Significant.

Table 3 Comparative analysis of CD138 staining score in odontogenic keratocyst, ameloblastoma, and radicular cyst studied cases

Epithelial cell CD138 score	OKT [n (%)]	AM [n (%)]	RC [n (%)]	P value		
				OKC vs. AM	OKC vs. RC	AM vs. RC
Negative	1 (6.6)	12 (60)	2 (13.3)			
Low	1 (6.6)	7 (35)	2 (13.3)	<0.05*	>0.05	<0.05*
High	13 (86.8)	1 (5)	11 (73.4)			
Total	15	20	15			
Stromal cell CD138 score						
Negative	4 (26.6)	5 (25)	9 (60)			
Low	8 (53.4)	3 (15)	6 (40)	>0.05	>0.05	>0.05
High	3 (20)	12 (60)	0			
Total	15	20	15			

AM, ameloblastoma; OKC, odontogenic keratocyst; RC, radicular cyst. *Significant.

Table 4 Comparative analysis of CD138 score in ameloblastoma studied cases

	Ameloblastoma (%)		P value
	Negative	Positive	
Epithelial cells	60	40	<0.05*
Stellate reticulum cell	25	75	

*Significant.

Bcl-2 was expressed in lining epithelium of 86.6% of studied OKC with slight increase in studied AM cases (100%), including intermediate score in 50% and moderate intensity in 60%, whereas all of studied RC showed negative Bcl-2 expression. Tenório et al. (2018) reposted moderate Bcl-2 expression in the most of AM cases with insignificant decrease in OKC, which is almost same compared with our results. Bcl-2 was analyzed in AM, OKC, and RC in the study of Tekkesin et al. (2012), with results that were almost compatible with ours. Our findings are also similar to those found by Piattelli et al. (1998) and Kichi et al. (2005).

However, Amaral et al. (2012) disagrees with us, as they found same Bcl-2 expression in AM and OKC.

This is may be owing to the different TUNEL method that was used in their study, besides the diverse staining protocols.

A distinctive finding was observed about the localization of Bcl-2 expression. It was found in the epithelial cells of tumor islands with more expression in the peripheral layers.

Our findings are similar to other studies using various proliferating markers, like PCNA (Kim and Yook, 1994) and Ki67 (Florescu et al., 2012; Rodriguez-Archilla and Barragan-Muñoz, 2018), who found higher expression in the peripheral neoplastic cells of AM than central cells. Such localization reflects the growth potential of AM, as peripheral cells are the proliferative components with high potency of cell vitality. Hence, Bcl-2 has an important role as an apoptotic marker on these cells in the survival and growth of AM (Tenório et al., 2018).

Concerning OKC, Bcl-2 was detected in the basal and suprabasal layers of lining epithelium. This explains the potency of the basal cell layer as epithelial cell source

and decreases the ability of superficial layer to divide. In addition, this characteristic immunoexpression of Bcl-2 in studied AM and OKC declares the abnormal control of their cell cycle.

Mitsuyasu *et al.* (1997), Kumamoto and Ooya (1999), and Sandra *et al.* (2001) are in compliance with our observation.

Moreover, Vered *et al.* (2009) recorded similar results of Bcl-2 and other immunohistochemical profile in OKC compared with AM, whereas they recorded different results from other odontogenic cysts (radicular and dentigerous cysts). These results support the neoplastic condition of OKC.

Syndecan proteoglycan, one of cell adhesion molecules, is essential for cell cytoskeleton and extracellular matrix interaction, so loss of its expression contributes in aggressive and invasive behavior of different lesions (Leocata *et al.*, 2007).

CD138 immunostaining was found in both epithelial and stromal cells of studied three lesions, which was in harmony with Leocata *et al.* (2007). The present study found higher epithelial CD138 expression in OKC (93.4%) than that of AM (40%), with a significant difference, whereas 73.4% of stromal cells of OKC and 90% the stromal cells of AM were CD138 positive. Concerning the RC, similar findings like these of OKC were found, with higher epithelial CD138 expression (100%) compared with 40% of stromal cells, with a significant difference compared with AM ($P<0.5$).

The study by Brito-Mendoza *et al.* (2018) was in agreement with our finding, registering higher epithelial CD138 expression in OKC in comparison with AM, with a significant difference ($P<0.5$).

Al-Otaibi *et al.* (2013) found a significant higher CD138 epithelial score in OKC than that of AM. In the same study, stellate-reticulum cells showed more CD138 expression than epithelial cells of AM, with nonsignificant statistically difference with other studied lesions, which support our findings.

Similarly, in the study of Nadalin *et al.* (2011), CD138 was mainly expressed in epithelial lining of OKC (94%) and RC (85.7%) with similar score, but AM was not included in their study.

Shifting of CD138 reaction from epithelia to stroma was reported in invasive tumors, including AM. Its expression was significantly higher in stroma and stellate reticulum cells more than epithelial cells of AM. It can be explain by that transmembrane Syndecan protein interacts with growth factors leading to its accumulating in the tumor stroma, supporting the invasiveness and neoangiogenesis (Al-Otaibi *et al.*, 2013). That explains why CD138 expression is decreased in tumor cells of AM with more invasive growth in comparison with OKC and RC.

Bologna-Molina *et al.* (2009) also found greater loss of CD138 in ameloblastic carcinoma when its expression was compared in ameloblastic carcinomas, peripheral AM, and desmoplastic AM, indicating the relation of invasion and aggressiveness with loss of CD138. Similarly, weaker CD138 expression was found in well-differentiated oral squamous cell carcinoma than less-differentiated tumors (Muramatsu, 2012).

The distribution of CD138 expression was noticed in all epithelial layers of OKC and in superficial cells in RC with variable cytoplasmic stromal cell reaction. This finding was consistent with results of other authors (Nadalin *et al.*, 2011; Al-Otaibi *et al.*, 2013; Hammad *et al.*, 2020). However, Özcan *et al.* (2015) detected CD138 expression in basal and suprabasal layer only, which is unlike our study. This can be explained by that inflammation which is a key feature in RC and a secondary feature of inflamed OKC may alter the expression of CD138 (Nadalin *et al.*, 2011). Our studied OKC cases were without inflammatory reaction, together with the small number of the studied cases (only five) of Özcan *et al.* (2015) may contribute in this diversity.

Etemad-Moghadam and Alaeddini (2017) matches our study concerning the localization of CD138 in OKC and AM, but without significant difference between them, unlike the current work. This may be owing to that they considered the overall expression of CD138 without discriminating the epithelial from stromal expression despite stromal cell expression.

According to the current work, OKC has aggressive potential that is not equal the severity of AM but at the same time significantly different from nonneoplastic cystic lesions in relation to apoptotic protein (Bcl-2). This notation is against their classification as a developmental cyst in the newly WHO classification. Moreover, more pronounced epithelial CD138 expression of OKC and stromal shifting of CD138 expression in AM eliminate the more invasive ability of AM compared with OKC. Based on that, there is argument about the exact categorization of OKC. Further research studies considering more and

different biologic markers are recommended to clarify its classification.

Conclusion

The results of the current study registered (a) positive Bcl-2 expression in OKC and AM compared with negativity of RC; (b) increased CD138 epithelial expression in OKC and RC than in AM; and (c) increased stromal CD138 in both OKC and AM than RC. These results reflect the difference in their growth profile and invasive behavior. At the same time, it elucidates that molecular defect in Bcl-2 and CD138 protein is critical for biology of studied odontogenic lesions, with consequent effect on their diagnosis and treatment. However, the localization of Bcl-2 expression supports the classification of OKC as a neoplastic one rather a developmental cyst, in contrary to the new WHO classification. So, further studies with a panel of markers are recommended for more understanding the biological nature of OKC.

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Conflicts of interest
There are no conflicts of interest.

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