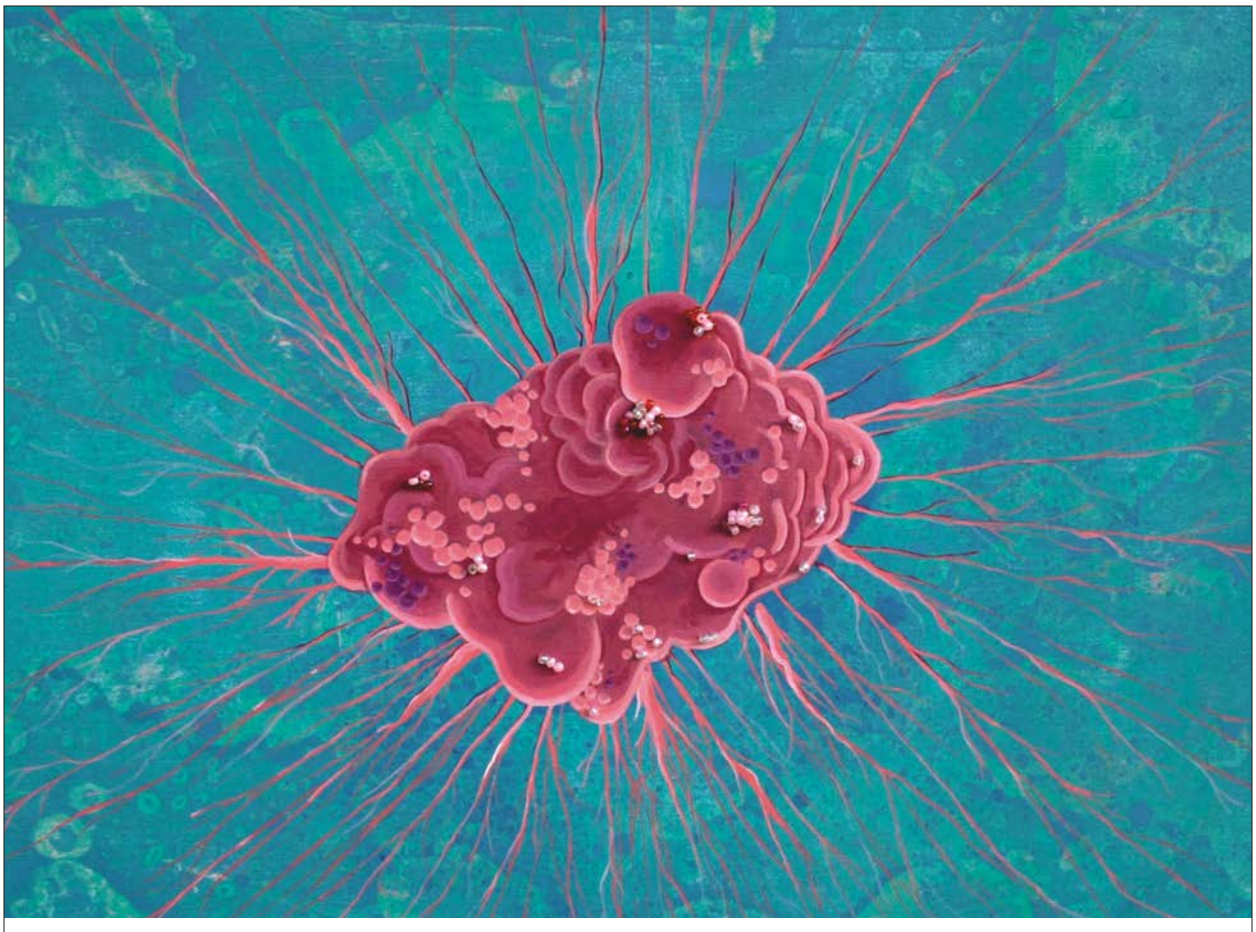

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Research Article
Immunohistochemical Analysis of Nf-κB Expression and its Relation to Apoptosis and Proliferation in Different Odontogenic Tumors

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Abstract

Background and Objective: Odontogenic tumors comprise a complex group of lesions which can pretense diagnostic challenges because of overlapping and diverse histopathologic types and clinical behaviors. An understanding of the biological behavior of these lesions is of fundamental importance for the final diagnosis and treatment planning, as these have an influence on the prognosis. The goal of the present study was to analyze and correlate the immunohistochemical expression of NF-κB and its transcription targets Bcl-xL and COX-2 in different odontogenic tumors. **Materials and Methods:** Thirty paraffin blocks of different odontogenic tumors (six dentigerous cyst, fourteen simple ameloblastoma, five unicystic ameloblastoma and five ameloblastic carcinoma) were selected. Immunohistochemistry was performed using the standard method (Avidin biotin peroxidase) to detect the polyclonal anti-NF-κB, Bcl-xL and COX-2 antibodies. One-way ANOVA and Spearman correlation test was used for statistical analyses. **Results:** The overall expression of the three antibodies (NF-κB, Bcl-xL and COX-2) increased significantly (p-value was considered significant when $p \leq 0.05$) from dentigerous cyst to ameloblastoma and reached their highest values in the cases of ameloblastic carcinoma. **Conclusion:** The interaction between the three proteins in blocking apoptosis and increasing proliferation may constitute an important pathogenic mechanism of tumorigenesis and hence might play a role in the behavior of odontogenic tumors.

Key words: Odontogenic tumors, nuclear factor kappaB, apoptosis, proliferation, immunohistochemistry

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Odontogenic cysts and tumors are developed because of the abnormalities that occur during odontogenesis. Therefore, they are considered as developmental disorders of odontogenic tissues. Odontogenic cysts are more frequent in the oral cavity but odontogenic tumors are less possible to be seen[1]. Dentigerous cyst is the most common developmental odontogenic cyst with an sluggish behavior and a low recurrence rate[2], whereas ameloblastoma is a benign odontogenic tumor with an aggressive behavior and anobvious invasive potential that leads to multiple recurrences after enucleation and curettage[3]. Unicystic ameloblastoma is a prognostically diverse entity[4]. It represents 5-22% of all ameloblastomas and it can occur inside dentigerous cysts[5,6].

However, Ameloblastic carcinoma is one of the uncommon odontogenic malignancies which merge the histological features of ameloblastoma with cytological atypia, even in the absence of metastasis[7].

Nuclear factor KappaB (NF-κB) is a ubiquitous transcription factor which commonly exists as a p65/p50 heterodimer, which was retained in the cytoplasm in inactive state bound to an inhibitor IκBα[8]. The stimulation of cells with various inflammatory and oxidative stresses activates IκBα kinase, leading to the sequential phosphorylation and degradation of IκBα and liberation of the p50/p65 heterodimer[9]. NF-κB is then translocated to the nucleus, where it binds to multiple DNA sequences to begin the transcription of a broad assortment of genes with a whole variety of functions[10].

It activates the transcription of many genes able for suppressing cell death, among them are prosurvival members of the mammalian Bcl-2 gene family Bcl-xL and Bfl-1[11-13]. Chen *et al.*[14] institute that NF-κB differentially regulates the appearance of particular Bcl-2-related death inhibitors and directly activates the expression of Bcl-xL. Moreover, they concluded that stimuli that activate endogenous NF-κB factors also upregulated Bcl-x gene expression which was antagonized by an inhibitor of NF-κB activity and this raised the opportunity that some of these factors may add to oncogenesis associated with aberrant Rel/ NF-κB activity.

In addition, one of the downstream targets of NF-κB was cyclooxygenase-2 (COX-2), which was a proinflammatory enzyme concerned in the synthesis of prostaglandins from arachidonic acid[15]. COX-2 overexpression has been shown to encourage tumorigenesis by activation of procarcinogens, stimulating cell proliferation, inhibiting apoptosis, induction of neoangiogenesis and adjustment of cell adhesion molecules[16,17].

The objective of the present study was to analyze the immunoexpression of NF-κB and its transcription factors Bcl-xL and COX-2 in odontogenic tumors and to determine the correlation between the expressions of these proteins in these tumors.

MATERIALS AND METHODS

Case selection: A total of 30 paraffin blocks (six dentigerous cyst, fourteen simple ameloblastoma, five unicystic ameloblastoma and five ameloblastic carcinoma) were retrieved from the archives of Oral Pathology Department, Faculty of Oral and Dental Medicine, Cairo University. A four micron section was cut from each block, stained with Hematoxylin and Eosin (H&E) staining and re-examined for formation of the diagnosis.

Immunohistochemical staining: Immunohistochemistry was performed using the standard method (avidin biotin peroxidase)[18]. Paraffin blocks were cut into 4 μm (micron) thick and mounted on positively charged glass slides (Optiplus, Biogenex, Milmont Drive, CA, USA) for immunostaining with anti-NF-κB, anti-COX2 and anti-Bcl-xL antibodies.

The paraffin embedded tissue sections on positively charged slides were deparaffinized in xylene, rehydrated through graded alcohols to water and treated with endogenous peroxidase in 0.3% H_2O_2 for 30 min to block the endogenous peroxidase activity. For antigen retrieval, the slides were boiled in 10 mM citrate buffer, pH 6.0 for 10-20 min followed by cooling at room temperature for 20 m. The sections were then incubated with the primary antibody rabbit polyclonal anti- NF-κB antibody (Cat #RB-9034-R7), the primary antibody rabbit polyclonal anti-COX2 antibody (Cat #RB-9072-R7) and the primary antibody rabbit polyclonal anti-Bcl-x Lantibody (Cat#PA5-32275) (Thermo Scientific, Lab vision, Kalamazoo, MI, USA) for 30 min at room temperature in a humified chamber. After washing with phosphate buffer solution (PBS), the slides were treated with the biotin labeled link antibody and then the streptavidin conjugated horse radish peroxidase was used.

The diaminobenzedine chromogen was applied to visualize the antigen antibody reaction. All these reagents belongs to the universal Labeled Streptavidin-Biotin 2 System, Horseradish Peroxidase (code no.K0673 DakoCytomation, Glostrup, Denmark). All the slides were immersed in Mayer's hematoxylin for counter staining. Finally, the sections were covered by cover slips using aqueous mounting medium.

Negative controls were performed, the sections were stained in the same technique but with omitting of the primary antibody and treating them with PBS instead.

Immunohistochemical evaluation: The ordinary light microscope was used to detect and localize the immunostaining of the three antibodies. For NF-κB, cells with nuclear staining were considered positive while cells with cytoplasmic staining were considered positive in case of COX-2 and Bcl-xL antibodies. Then, all the sections were examined by an image analyzer computer system using the software Leica Qwin 500 (Leica Microsystems Imaging Solutions Ltd, Cambridge, UK).

Five random fields in each specimen were captured using a magnification (x400) to determine the area percentage and immunostaining intensity of the positive tumor cells. The area percentage and immunostaining intensity were scored from 1-3 according to the difference between the largest and smallest mean value of each parameter in the studied cases. The scores of both area percentage and immunostaining intensity were then summed to obtain a single total score. The overall reaction was considered mild (Score 1 and 2), moderate (Score 3 and 4), or strong (Score 5 and 6) according to the single total score[19].

Statistical analyses: The total scores of immunoexpression of NF-κB, COX2 and Bcl-xL in the studied groups of specimens were represented as mean values and standard deviations (SD) for statistical evaluation. The one-way ANOVA test was used to compare the mean values among dentigerous cyst, simple and unicystic ameloblastoma and ameloblastic carcinoma. Spearman correlation test was used to evaluate the correlation between total scores of expression of the three antibodies. At 95% confidence interval, p-value was considered significant when $p \leq 0.05$.

RESULTS

Immunohistochemical detection of NF kappaB: Nuclear and cytoplasmic NF-κB immunostaining both were observed. Most of the cases of dentigerous cyst and unicystic ameloblastoma showed positive NF-κB immunostaining in the epithelial lining (Fig. 1a-b). However, the studied cases of simple ameloblastoma and ameloblastic carcinoma showed strongest NF-κB immunoexpression (Fig. 2a-b).

Immunohistochemical detection of Bcl-xL: Bcl-xL positivity was observed as homogenous brown cytoplasmic immunostaining. Mild immunoexpression was observed in some of the cells of the epithelial lining of dentigerous cyst (Fig. 3a), whereas the epithelial lining of unicystic ameloblastoma showed more Bcl-xL immunostaining (Fig. 3b). On the other hand, a strong Bcl-xL immunoexpression was observed in ameloblastoma and ameloblastic carcinoma (Fig. 4a-b).

Immunohistochemical detection of COX-2: Positive cytoplasmic COX-2 immunostaining was observed in most of the dentigerous cyst cases (Fig. 5a) and unicystic ameloblastoma (Fig. 5b). Moreover, strongest COX-2 immunostaining was observed in ameloblastoma (Fig. 6a) and ameloblastic carcinoma (Fig. 6b).

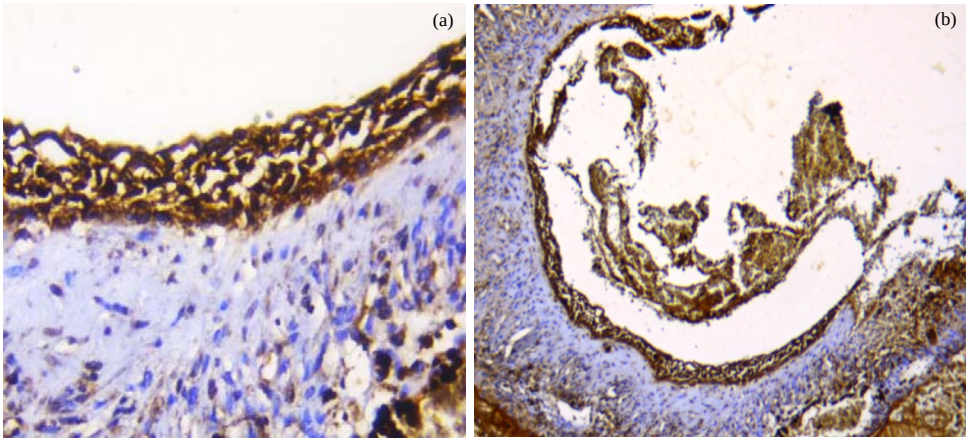

Fig. 1(a-b): (a) Photomicrographs showing NF-κB immunoexpression in the epithelial lining of both dentigerous cyst (X400) and (b) Unicystic ameloblastoma (X100)

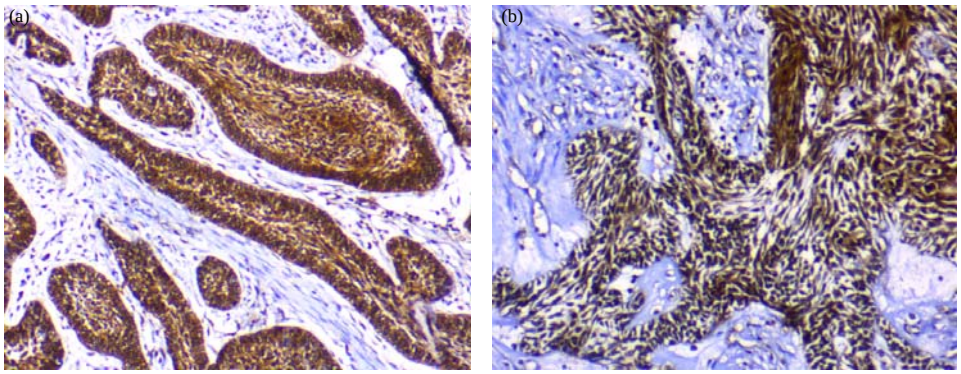

Fig. 2(a-b): (a) Photomicrographs showing strong NF-κB immunoexpression in simple ameloblastoma (X200) and (b) Ameloblastic carcinoma (X200)

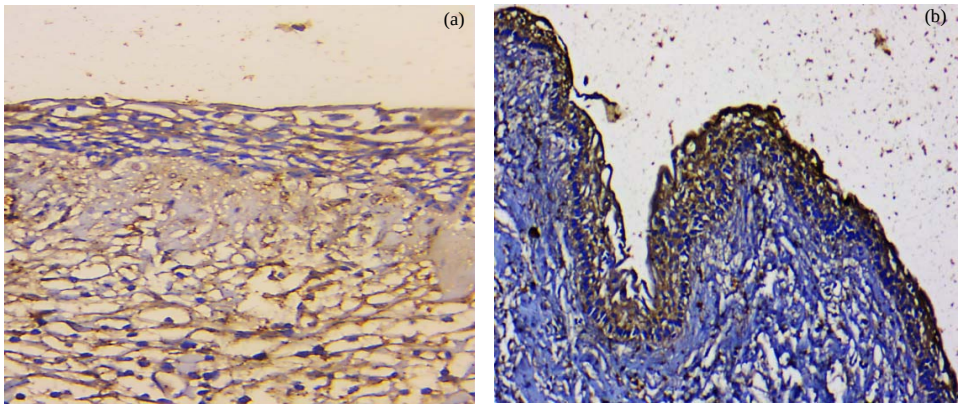

Fig. 3(a-b): (a) Photomicrographs showing Bcl-xL immunoexpression in the epithelial lining of dentigerous cyst (X400) and (b) Unicystic ameloblastoma (X200)

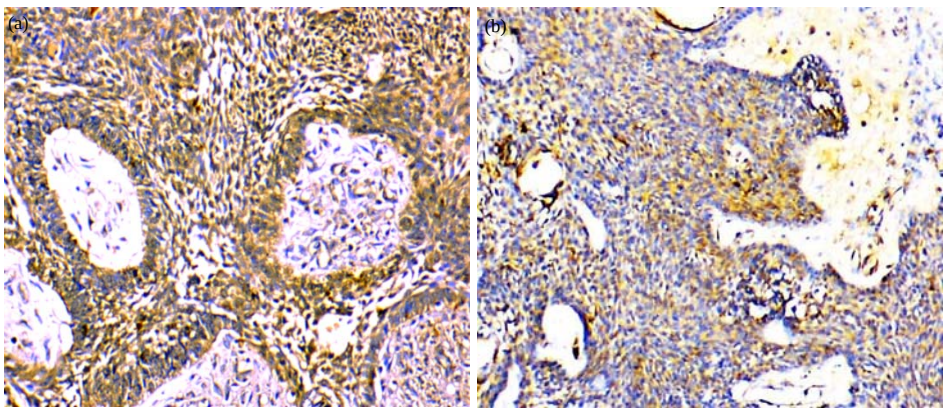

Fig. 4(a-b): (a) Photomicrographs showing Bcl-xL immunoexpression in ameloblastoma (X200) and (b) Ameloblastic carcinoma (X200)

Statistical results: A highly significant difference was obtained on comparing the total scores of NF-κB, COX-2 and Bcl-xL immunoexpression among the studied groups. The overall expression of the three antibodies increased

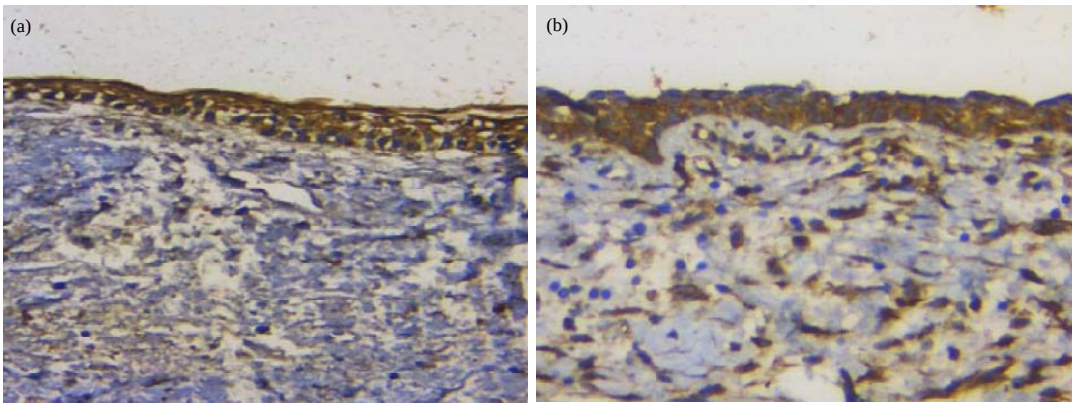

Fig. 5(a-b): (a) Photomicrographs showing cytoplasmic immunostaining of COX-2 in the epithelial lining of dentigerous cyst (X200) and (b) Unicystic ameloblastoma (X200)

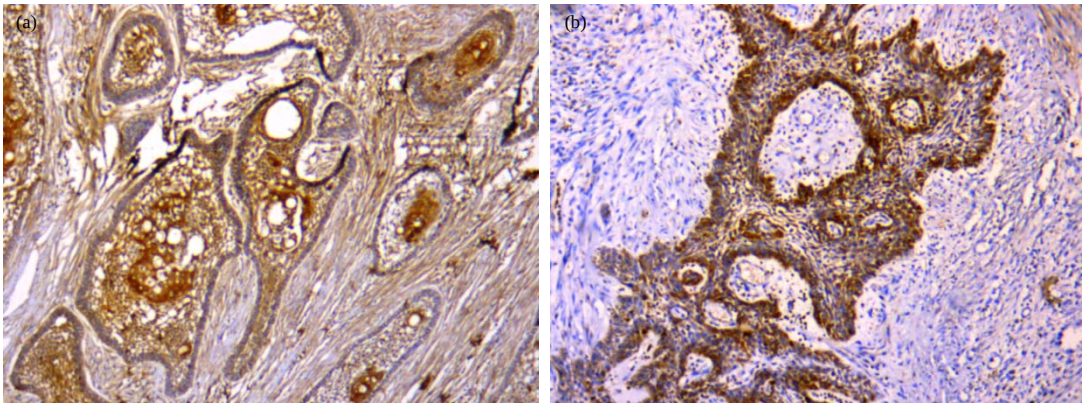

Fig. 6(a-b): (a) Photomicrographs showing strong cytoplasmic COX-2immunoexpression in simple ameloblastoma (X200) and (b) Ameloblastic carcinoma (X200)

Table 1: Total expression of NF-κB, COX-2 and Bcl-xL among studied groups

	Cases				
Markers	Dentigerous cyst Mean±SD	Simple and unicystic ameloblastoma Mean±SD	Ameloblastic carcinoma Mean±SD	p-value	**r
NF-κB	2.8±0.66	4.57±0.75	5.5±0.5	0.001*	0.00615
COX-2	2.6±0.4	3.5±0.5	4.9±0.9	0.001*	
Bcl-xL	2.2±0.4	3.3±1	4.7±1	0.001*	

*p≤0.001 = significant, **r = 0.00615 = direct correlation, SD = Standard deviation

significantly from dentigerous cyst to ameloblastoma and reached their highest values in the cases of ameloblastic carcinoma (Table 1). Direct correlation was detected between total scores of the immunoexpression of NF-κB, COX2 and Bcl-xL in studied cases.

DISCUSSION

NF-κB belongs to a family of transcription factors[20] that regulates the expression of several genes including COX-2 and Bcl-xL whose products are implicated in tumorigenesis[14,15]. However, little information is known about the role of NF-κB in odontogenic lesions[21]. This study was performed in an attempt to explore its immunoexpression in dentigerous cyst, ameloblastoma and ameloblastic carcinoma with correlation to its outcome on apoptosis, cell proliferation and tumorigenesis by transcription of certain proteins as Bcl-xL and COX-2.

In normal tissues NF-κB exists in the cytoplasm and remains inactive, once triggered by factors, it releases IκBα

and translocates to the nucleus and affect the expression of its target genes[1]. In this study, both nuclear and cytoplasmic immunostaining of NF-κB were detected in the epithelial lining of both dentigerous and unicystic ameloblastoma. It was supposed that the epithelial lining of the dentigerous cysts has an elevated proliferative potential than the epithelial lining of inflammatory cysts[22]. In addition, a high proliferative activity was established in unicystic ameloblastoma than dentigerous cyst linings[23].

These study results showed a strong immunostaining of NF-κB in ameloblastoma and ameloblastic carcinoma. This indicated that NF-κB plays an important role in the development of such odontogenic lesions and might influence their progression. This finding was in contrast to Kumamoto and Ooya[24] and Gong *el al.*[21], who detected a scarce nuclear NF-κB immunostaining in classic ameloblastoma and normal odontogenic epithelium. On the other hand, it has been reported that there was a direct relationship between NF-κB immunoexpression and aggressiveness of malignant tumors[25-27], which explains the higher statistical significance of NF-κB immunostaining among the studied lesions in this study with highest value in ameloblastic carcinoma.

Consistent with the notion that NF-κB antagonizes cell death, we investigated the expression of Bcl-xL in the present study as one of the members of the Bcl-2 family that was directly regulated by NF-κB[14]. Bcl-xL showed mild to moderate expression in the studied odontogenic lesions of the current study with highest expression in ameloblastic carcinoma. This was supported by Gonzalez-Gonzalez *et al.*[28] and Tosios *el al.*[29], who detected a higher immunoexpression of Bcl-2 in multicystic ameloblastoma than of NF-κB, its unicystic variant and a focal expression in dentigerous cyst.

The present study results showed a direct correlation between the expression and Bcl-xL among the studied lesions, which might point to that NF-κB, is an inducer of Bcl-xL. This finding agrees with several reports which found that NF-κB dependent up regulation of Bcl-xL is important for Bcl-xL expression[30-32]. In addition, Khoshnan *et al.*[33] concluded that the NF-κB cascade is important for Bcl-xL expression and induction of antiapoptotic effects of primary human $CD4^+$ lymphocytes.

It has been reported that increased expression of COX-2 by interfering in biological processes such as cell proliferation, cell adhesion, cellular immunity, apoptosis and angiogenesis, plays asignificant role in growth and development of tumor[34]. Moreover, quite a lot of studies showed that COX-2 levels increase in various tumors[35-37]. These findings revealed positive cytoplasmic immunostaining of COX-2 in the epithelial lining of both dentigerous cyst and unicystic ameloblastoma, whereas ameloblastoma and ameloblastic carcinoma showed strongest immunostaining. This result might confirm that COX-2 has a role in the invasive behavior of odontogenic lesions as ameloblastoma and ameloblastic carcinoma when compared to lesions with less aggressive behavior as dentigerous cyst. This is coincident with Seyedmajidi *et al.*[38], who found higher expression of COX-2 in ameloblastoma in comparison to dentigerous cyst. Moreover, it has been concluded that COX-2 may have an effect on the local invasion of ameloblastoma[39].

A highly statistical difference was observed in the current study on comparing the immunoexpression of NF-κB, Bcl-xL and COX-2 among the studied odontogenic lesions ($p \leq 0.001$ = significant). The overall expression of the three antibodies increased significantly from dentigerous cyst to ameloblastoma and reached their highest values in the cases of ameloblastic carcinoma. It has been reported that NF-κB affect the upstream of COX-2 through IκBα kinase activation[40]. NF-κB interacts with COX-2 directly as COX-2 promoter contains a binding site for NF-κB[41,42]. In addition, NF-kB is a global regulator of death antagonists in the Bcl-2 family as it differentially regulates the expression of particular Bcl-2-related death inhibitors and directly activates the expression of Bcl-xL[14].

Therefore, the interaction between three proteins in blocking apoptosis and increasing proliferation may comprise an important pathogenic mechanism in the behavior of odontogenic tumors.

CONCLUSION

Nuclear factor KappaB and its transcription targets Bcl-xL and COX-2 may represent an important pathogenic system of tumorigenesis indentigerous cyst and ameloblastoma as well asthe highest expression of these proteins in ameloblastic carcinoma suggests that they play a central role in malignant transformation.

SIGNIFICANCE STATEMENTS

This study discovered that the immunohistochemical expression of nuclear factor KappaB and its transcription targets Bcl-xL and COX-2 correlated significantly with dentigerous cyst, ameloblastoma and reached their highest values in the cases of ameloblastic carcinoma that can be beneficial for detection of pathologic behavior of these odontogenic lesions. This study help the researcher to cover

the area of pathogenic mechanism of tumorgenesis regarding diagnosis and prognosis in odontogenic tumors that many researchers were not able to explore. Thus, a new theory on these immunohistochemical markers and transcriptional targets and possibly other combination, may be arrived at.

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