

Odontogenic epithelial proliferation is correlated with COX-2 expression in dentigerous cyst and ameloblastoma

MOHAMMED AMJED ALSAEGH¹⁻³, HITOSHI MIYASHITA^{1,4}, TAKAHIRO TANIGUCHI⁴ and SHENG RONG ZHU¹

¹Department of Oral and Maxillofacial Surgery, Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei 430030, P.R. China;

²Department of Oral and Maxillofacial Surgery, College of Dentistry, Ajman University, Al-Hulifat, Fujairah 2202, UAE;

³Department of Oral and Maxillofacial Surgery, College of Dentistry, University of Mosul, Mosul, Ninawah 41002, Iraq;

⁴Department of Oral Medicine and Surgery, Division of Oral and Maxillofacial Surgery, Graduate School of Dentistry, Tohoku University, Sendai 980-8575, Japan

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Abstract. Investigation of cyclooxygenase (COX)-2 in dentigerous cyst and ameloblastoma may help to improve understanding of the nature and behavior of odontogenic cysts and tumors, and in addition may eventually represent a definitive target for a pharmacological approach in the management of these lesions. The aim of this study was to evaluate COX-2 expression and its correlation with the proliferation of odontogenic epithelium in these lesions. Dentigerous cysts (n=16) and ameloblastomas (n=17) were evaluated. Detection of Ki-67 and COX-2 protein expression was conducted by immunohistochemistry. Data were statistically analyzed using Mann-Whitney U test and Spearman's rank correlation coefficient. No significant differences were found in the expression of Ki-67 and COX-2 between dentigerous cysts and ameloblastomas ($P>0.05$). A significant positive correlation ($P=0.018$) and highly significant positive correlation ($P=0.004$) were found between Ki-67 and COX-2 expression in the odontogenic epithelium of dentigerous cyst and ameloblastoma, respectively. COX-2 was expressed in the odontogenic epithelium of dentigerous cyst and ameloblastoma. It may contribute to local extension of these lesions by increasing the proliferation of their odontogenic epithelial cells.

Introduction

Dentigerous cyst is an odontogenic lesion that represents the second most common odontogenic cyst after radicular cyst, accounting for ~24% of all true cysts in the jaw (1). A typical dentigerous cyst presents clinically as an asymptomatic unilocular radiolucency enclosing the crown of an unerupted or impacted tooth (2). However, dentigerous cyst can cause local destruction, bony expansion, root resorption, or displacement of teeth, which occurs more commonly with long-standing lesions (3).

Ameloblastoma represents the second most common odontogenic tumor. It is slowly growing, locally invasive and has a high rate of recurrence if not treated adequately (4). It accounts for ~1% of all tumors and cysts of the jaws (5). There are three clinicoradiographic variants of this tumor, namely the solid or multi-cystic variant (86%), the unicystic variant (13%), and the peripheral variant (1%) (6).

The lining of a dentigerous cyst develops from reduced enamel epithelium that envelops the crown prior to eruption (7), whereas the tissues from which ameloblastoma may arise involve dental lamina rests, the developing enamel organ, the epithelial lining of an odontogenic cyst, or the basal cells of the oral mucosa (6). However, the etiology and the precise histogenesis of dentigerous cyst and ameloblastoma remain unclear (8,9).

The Ki-67 protein is a nuclear and nucleolar protein. It is tightly associated with somatic cell proliferation (10). Ki-67 is a widely used proliferating marker. It is expressed in all stages of the cell cycle, with the exception of the G₀ phase (11).

Cyclooxygenase (COX)-2 is a cytokine-inducible enzyme which is present in the nuclear membrane and luminal side of the endoplasmic reticulum (12,13). It converts free arachidonic acid into prostanoids, including prostaglandins (PGs) and thromboxanes (14). COX-2 is involved in multiple physiological functions and triggers key pathological processes, such as inflammation and tumorigenesis (14,15). Upregulation of COX-2 and an increased prostaglandin E₂ (PGE₂) level are frequently detected in premalignant and malignant tissues of epithelial origin (16). COX-2 may play a role in different

Correspondence to: Dr Mohammed Amjed Alsaegh, Department of Oral and Maxillofacial Surgery, College of Dentistry, University of Mosul, Al-Majmo'a Street, Mosul, Ninawah 41002, Iraq
E-mail: mo_am_77@yahoo.com

Professor Sheng Rong Zhu, Department of Oral and Maxillofacial Surgery, Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology, 1095 Jie Fang Avenue, Hankou, Wuhan, Hubei 430030, P.R. China
E-mail: tongji36@yahoo.com

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steps of tumor progression by increasing the proliferation of mutated cells (17). Previous studies have shown that COX-2 has a positive correlation with the expression of Ki-67 proteins in various types of tumor (12,18-22).

The expression and role of COX-2 in odontogenic lesions have not been thoroughly elucidated. Studies of COX-2 in dentigerous cyst and ameloblastoma may help to improve understanding of the nature and behavior of odontogenic cysts and tumors, and eventually may provide a definitive target for a pharmacological approach in the management of those lesions. The aim of the current study was to evaluate COX-2 expression and its correlation with the proliferation of odontogenic epithelium in dentigerous cyst and ameloblastoma.

Material and methods

Patient samples. A total of 33 samples were collected, 29 of which were retrieved from the archive of the Oral Pathology Laboratory, Tongji Hospital, Huazhong University of Science and Technology (Wuhan, China) over the 3 years from 2008 to 2010 as formalin-fixed paraffin-embedded (FFPE) samples. The remaining 4 samples were surgically removed from patients attending the Department of Maxillofacial Surgery of Tongji Hospital, Huazhong University of Science and Technology, and collected as frozen tissue samples. These samples were distributed into two categories which were: Dentigerous cyst (n=16; 13 FFPE and 3 frozen) and ameloblastomas (n=17; 16 FFPE and 1 frozen). Dentigerous cyst and ameloblastoma represent odontogenic lesions that encompass reactive tissues and tumors, respectively, which replace the healthy bone; there is no true tissue equivalent to serve as a negative control. The protocol followed the criteria established by the World Medical Association Declaration of Helsinki. This study was approved by the Institutional Review Board of Tongji Medical College, Huazhong University of Science and Technology.

Immunohistochemical analysis

Antibodies. Immunohistochemical analysis was performed using the following primary polyclonal antibodies: Rabbit anti-human Ki-67 antigen, corresponding to a sequence mapping at the C-terminal of Ki-67 (BA1508; Wuhan Boster Biological Technology, Ltd., Wuhan, China; dilution 1:100) and rabbit anti-human COX-2 antigen raised against a synthetic peptide corresponding to a sequence at the N-terminal of human COX-2 (BA0738; Wuhan Boster Biological Technology, Ltd.; dilution 1:100).

Immunostaining method. Immunostaining was performed using the standard streptavidin-biotin peroxidase complex assay method (Wuhan Boster Biological Technology, Ltd.). FFPE samples were cut into 5- μ m tissue sections and then were dewaxed and rehydrated. Endogenous peroxidase activity was quenched using 3% H₂O₂ solution before the antigen unmasking step in 0.01 M citrate buffer that had been heated to boiling point in a microwave. The frozen samples were cut into 5- μ m sections in a cryostat chamber (Leica Biosystems Nussloch GmbH, Nussloch, Germany), then dried at room temperature and fixed in 4% paraformaldehyde. Endogenous peroxidase activity was blocked using 0.03% H₂O₂ in absolute methanol. After that, both FFPE and

frozen samples were treated equally with normal goat serum (Wuhan Boster Biological Technology, Ltd.) for 50 min at room temperature and then incubated with the aforementioned primary antibodies at 4°C overnight. Following treatment with 10 μ g/ml biotinylated secondary antibody (goat anti rabbit IgG; BA1003; Wuhan Biological Technology, Ltd.) for 2 h at room temperature, the slides were stained with 20 μ g/ml streptavidin-biotin-peroxidase complex. Finally, the sections were developed with 3,3'-diaminobenzidine substrate and slightly counterstained with Mayer's hematoxylin. Negative controls were incubated with phosphate-buffered saline instead of the primary antibodies.

Immunohistochemical scoring. Standard light microscopy was used to semiquantitatively score the staining by counting the percentage of positive cells and scoring the intensities in ≥ 10 continuous and representative high power (x400) fields. Ki-67 expression was identified as a yellowish-brown nuclear stain that was scored as follows: i) Absent, when there was no identified staining of the odontogenic epithelium or when the staining was questionable; ii) weak for $\leq 20\%$; iii) mild for 21-40% and iv) strong for $>40\%$ positivity rate of the odontogenic epithelium. COX-2 expression was recognized as granular yellowish-brown staining of different intensities that was mainly located in the nuclear membrane and the cytoplasm of the positive cells. For COX-2 scoring, firstly the average COX-2 staining intensity was rated on a scale from 0 to 3 as follows: 0, no staining at all; 1, weak staining; 2, moderate staining; and 3, strong staining. Then, the percentage of positively stained cells in ≥ 10 continuous and representative high power (x400) fields was determined and scored as follows: 0, no identified staining of the odontogenic epithelium or unnoticeable staining; 1, $\leq 20\%$ staining; 2, 21-40% staining; and 3, $>40\%$ staining. The final score was calculated by adding the score obtained from the staining intensity to that derived from the percentage of positive cells, with a maximum score of 6. A final score of 0 was regarded as negative, 2 as weak, 3 or 4 as mild, and 5 or 6 was considered as strong immunoreactivity.

Statistical analysis. The Statistical Package for the Social Sciences (SPSS) 19.0 software (IBM SPSS, Armonk, NY, USA) was used for analyzing the results. Comparisons and correlations between the immunohistochemical results for protein expression were statistically analyzed using Mann-Whitney U test and Spearman's rank correlation coefficient, respectively. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Clinical features. The most relevant clinical features of the patients in this study are summarized in Table I. A total of 11 cases were females (33.3%) and 22 cases were males (66.7%). The patients' ages ranged from 12 to 74 years, with a mean of 36.2 years. The mandible was the most common site of involvement (20 cases; 60.6%), whereas the maxilla was involved in the remainder 13 cases (39.4%).

Ki-67 protein expression. Ki-67 protein expression was detected as yellowish-brown nuclear staining in the

Table I. Clinicopathological characteristics of the involved patients (n=33).

Variable	Total	Dentigerous cyst	Ameloblastoma
Patients (n)	33	16	17
Age (years)			
Mean	36.2	36.7	32.8
Range	12-74	12-74	12-70
Gender, n (%) ^a			
Male	22 (66.7)	13 (39.4)	9 (27.3)
Female	11 (33.3)	3 (9.1)	8 (24.2)
Location, n (%) ^a			
Maxilla	13 (39.4)	12 (36.4)	1 (3.0)
Mandible	20 (60.6)	4 (12.1)	16 (48.5)

^aCalculated as a percentage of the total study population.

odontogenic epithelial cells of dentigerous cysts and ameloblastomas (Fig. 1). In the dentigerous cysts, positive cells were distributed throughout the cystic wall. Ki-67 expression was absent in 25%, weak in 50%, mild in 12.5%, and strong in 12.5% of odontogenic epithelial cells in dentigerous cyst samples (Table II). The distribution of positive Ki-67 stained cells in ameloblastomas was variable; some samples showed an equal distribution of Ki-67 staining in the central stellate reticulum-like cells and the peripheral columnar cells; other samples showed predominant staining of central cells, while the remaining samples expressed a predominant staining of peripheral cells. Ki-67 expression was absent in 23.52%, weak in 41.17%, mild in 17.64%, and strong in 17.64% of odontogenic epithelial cells in ameloblastoma samples (Table II).

COX-2 expression. COX-2 expression was demonstrated as a yellowish-brown granular stain of different intensities that was expressed in the cytoplasm and nuclear membrane of the positive odontogenic epithelial cells in dentigerous cysts and ameloblastomas (Fig. 1). In the dentigerous cysts, positive cells were observed throughout the cystic wall. COX-2 was absent in 18.75%, weak in 18.75%, mild in 43.75%, and strong in 18.75% of odontogenic epithelial cells in dentigerous cyst samples (Table II). In ameloblastoma samples, a predominant and high intensity of COX-2 staining was identified in the central stellate reticulum-like cells compared with the peripheral columnar cells. COX-2 expression was absent in 5.89%, weak in 29.41%, mild in 52.94%, and strong in 11.76% of odontogenic epithelial cells in the ameloblastoma samples (Table II).

Correlation analysis. Although the numerical mean values of Ki-67 and COX-2 expression levels were higher in ameloblastomas than in dentigerous cysts, analysis using the Mann-Whitney U test showed that the differences in the expression of Ki-67 and COX-2 between dentigerous cysts and ameloblastomas were not significant ($P>0.05$).

By contrast, analysis using Spearman's rank correlation coefficient showed that there was a positive correlation between

the expression of Ki-67 and COX-2 in the odontogenic epithelium of dentigerous cysts ($\sigma=0.582$; $P=0.018$; Table III) and a strong positive correlation between the expression of Ki-67 and COX-2 in the odontogenic epithelium of ameloblastomas ($\sigma=0.656$; $P=0.004$; Table III).

Discussion

COX-2 is a cytokine-inducible enzyme that converts free arachidonic acid into prostanoids including PGs and thromboxanes (12,14). It is involved in multiple physiological functions and triggers key pathological processes such as inflammation and tumorigenesis (14,15). The current study demonstrated the expression of COX-2 in the odontogenic epithelium of dentigerous cyst and ameloblastoma. COX-2 is upregulated in a wide variety of human tumors including oral cancer (12,23-25). The precise contribution of COX-2 to neoplastic growth has not been elucidated (26). However, the induction of COX-2 has been shown to inhibit apoptosis, promote cell growth, and enhance cell motility and adhesion (27). In addition, epidemiological studies indicate that the use of nonsteroidal anti-inflammatory drugs (NSAIDs) is associated with a reduced risk of malignancies, particularly in the digestive tract (28). Studies have indicated an association of COX-2 expression with the initiation of tumorigenesis (29), malignant transformation (19), local invasion (30), lymph node metastasis (23), and recurrence (28,31). The expression of COX-2 in precancerous lesions has been reported to be higher than that in malignancies in various human organs, including bronchial (32), colonic (33), esophageal (34), as well as head and neck tumors (25,35,36). These findings suggest that COX-2 may be involved in the early stages of carcinogenesis (34). The COX-2 gene is most commonly elevated by growth factors and mediators of inflammation such as lipopolysaccharide, tumor necrosis factor α , interleukin-1, and 12-O-tetradecanoylphorbol 13-acetate, whilst anti-inflammatory cytokines and glucocorticoids suppress COX-2 expression (13). The mechanism of COX-2 upregulation in tumors is unknown. One possibility is that the cancer cells become intrinsically more active in expressing COX-2 than do the non-neoplastic cells. Furthermore, the activation of oncogenes such as HER-2/neu, and inactivation of tumor suppressor genes such as p53, have been implicated in the induction of COX-2 expression (37).

Little is known about the expression and role of COX-2 in odontogenic cysts and tumors. Mendes *et al* showed a distinct overexpression of COX-2 in keratocystic odontogenic tumor, and they hypothesized that they would be likely to observe a certain degree of COX-2 expression in developmental cysts such as dentigerous cysts (38,39). In addition, a previous study of 30 radicular cyst specimens demonstrated the expression of COX-2 in the lining epithelium of all 30 specimens, and reached the conclusion that COX-2 may be involved in a possible mechanism for radicular cyst pathogenesis and expansion through its effect on the production of PG and matrix metalloproteinase (40).

Sonic hedgehog (SHH) signaling molecules have been demonstrated to be expressed in dentigerous cyst (41) and ameloblastoma (42). Concordance between the SHH pathway and the induction of COX-2 expression has been concluded

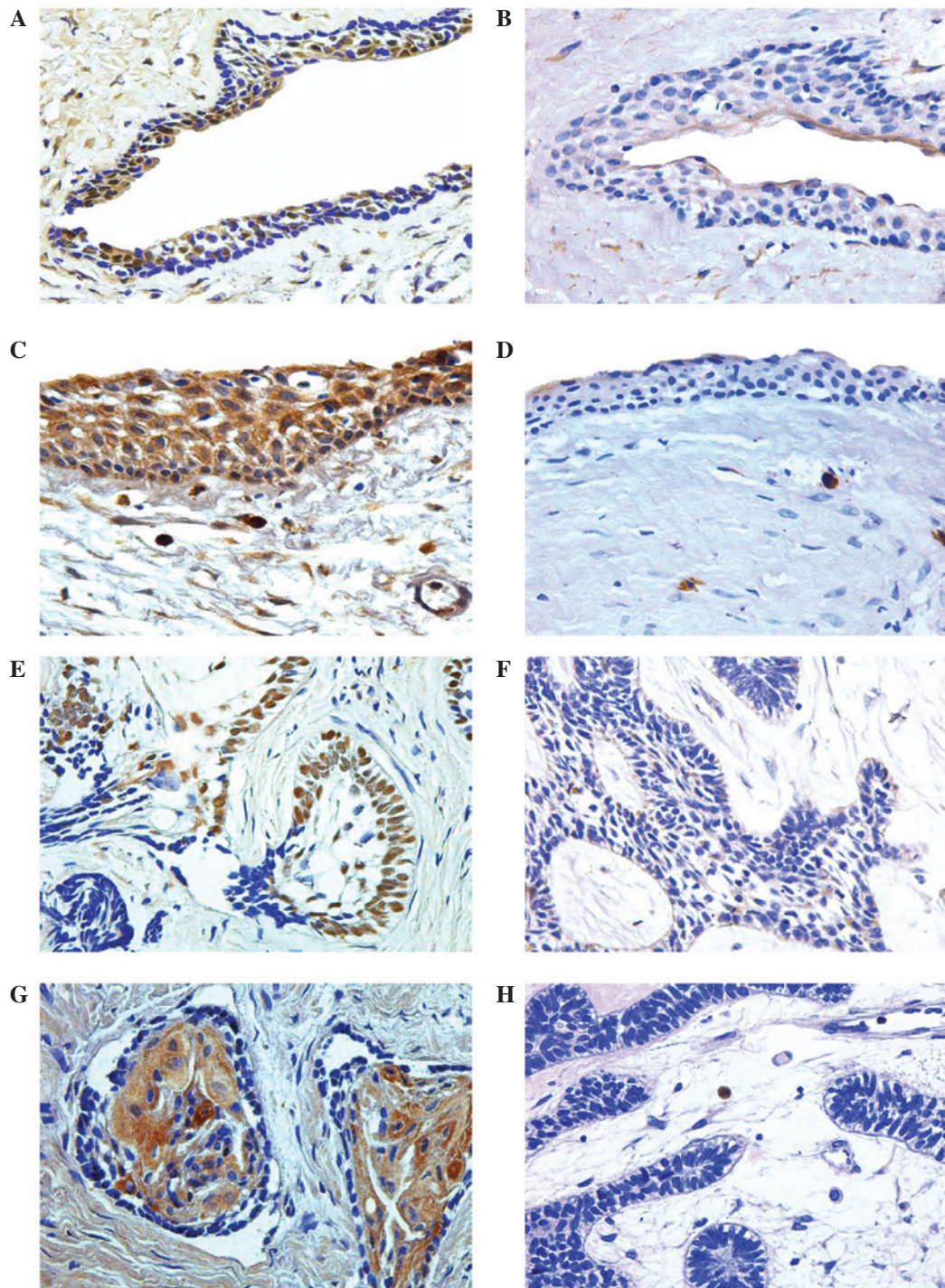


Figure 1. Immunohistochemical staining of Ki-67 and COX-2 proteins in dentigerous cyst and ameloblastoma (magnification, x400). (A) Dentigerous cyst shows yellowish-brown nuclear positive staining of Ki-67 protein distributed throughout the cystic wall. (B) Dentigerous cyst with negative Ki-67 immunostaining. (C) Dentigerous cyst shows yellowish-brown COX-2 staining, which appears mainly in the cytoplasm and nuclear membrane of the positive cells distributing throughout the cystic wall with some strongly positive subepithelial inflammatory cells. (D) Negative COX-2 results in dentigerous cyst; however, positively stained inflammatory cells are located in the subepithelial connective tissue. (E) Positive yellowish-brown Ki-67 nuclear staining is present in stellate reticulum-like cells and peripheral columnar cells of ameloblastoma. (F) Negative Ki-67 immunostaining of ameloblastoma. (G) Positive COX-2 staining in ameloblastoma is cytoplasmic and predominantly located in the central stellate reticulum-like cells. (H) Negative COX-2 staining of the odontogenic epithelium of ameloblastoma; however, a positive inflammatory cell is located in the connective tissue. COX, cyclooxygenase.

to proceed through the effect of SHH on the upregulation of proliferative markers such as p53 leading to the activation of the Ras/Raf/ERK cascade, which, in turn, induces the expression of COX-2 (38,43). The primary requirement for any lesion to expand within the bone is the ability to resorb the dense crystalline environment (44). COX-2 is upregulated during bone

repair and under pathological conditions such as inflammation and neoplasia. Thus, the skeleton is supplied with high levels of PGs, particularly PGE₂, which plays either a stimulatory or inhibitory role in bone metabolism. PGs, mainly PGE₂, are able to stimulate bone resorption by increasing the numbers and functional activity of osteoclasts (45).

Table II. Expression of Ki-67 and COX-2 proteins in dentigerous cyst and ameloblastoma.

Protein	N (%)	
	Dentigerous cyst (n=16)	Ameloblastoma (n=17)
Ki-67		
Positive	12 (75.0)	13 (76.5)
Weak	8 (50.0)	7 (41.2)
Mild	2 (12.5)	3 (17.6)
Strong	2 (12.5)	3 (17.6)
COX-2		
Positive	13 (81.3)	16 (94.1)
Weak	3 (18.8)	5 (29.4)
Mild	7 (43.7)	9 (52.9)
Strong	3 (18.8)	2 (11.8)

COX, cyclooxygenase.

Table III. Spearman's rank correlation analysis for odontogenic epithelial proliferation with Ki-67 and COX-2 in dentigerous cyst and ameloblastoma.

Sample type	Correlation of Ki-67 and COX-2	
	σ	P-value
Dentigerous cyst	0.582	0.018
Ameloblastoma	0.656	0.004

COX, cyclooxygenase.

According to the results of the current study, the proliferation rate of the odontogenic epithelium, which is indicated by the Ki-67 protein expression level, was not significantly different between dentigerous cysts and ameloblastomas. Ki-67 is a widely used proliferating marker. It is expressed in all stages of the cell cycle with the exception of the G₀ phase (11). Studies comparing the proliferation rate between dentigerous cysts and ameloblastomas have generated conflicted results, which may reflect differences in the markers and criteria used for the cellular proliferation assessment. Gadball *et al* found a significantly higher Ki-67 labeling index in ameloblastomas than in dentigerous cysts (46). Also, in another study, Ki-67 expression was observed to be higher in ameloblastomas that arise from dentigerous cysts than in the dentigerous cysts (47). Piattelli *et al* showed that ameloblastoma had higher proliferating cell nuclear antigen-positive cell counts than dentigerous cyst (48). Similarly, when IPO-38 was used as a proliferation marker, it was found that the labeling index of IPO-38 was higher in ameloblastomas than in dentigerous cysts (49). However, another study conducted by de Vicente *et al* demonstrated that Ki-67 expression was higher in dentigerous cysts than in ameloblastomas (50). Furthermore, dentigerous cyst

have been found to have significantly higher counts for the proliferative activity marker nucleolar organizer regions than unicystic ameloblastoma (51).

Bôas *et al* found that there was no correlation between the immunoeexpression of COX-2 and Ki-67 in oral squamous cell carcinoma (52). Similar results were observed in ovarian tumors (53). These studies concluded that COX-2 may be required for carcinogenesis in pathways other than those affecting the proliferation of cells in oral squamous cell carcinoma or ovarian tumor (52,53), stating that the interference of COX-2 with tumor growth and dissemination is not limited to the stimulation of mitogenesis (53). Another study did not find a correlation between COX-2 and Ki-67 expression in keratocystic odontogenic tumor (39). The current study results demonstrated that proliferation activity and COX-2 expression were positively correlated in the odontogenic epithelium of dentigerous cyst and ameloblastoma, respectively. These results are in accordance with other studies that revealed such a correlation in other different tumor types, for example, in mucosa-associated lymphoid tissue lymphoma (20), colorectal adenoma (19), cervical adenocarcinomas (12), renal cell carcinoma (18), esophageal adenocarcinoma (21), and esophageal squamous cell carcinoma (22). It has been shown that COX-2 may play a role in different steps of tumor progression by increasing the proliferation of mutated cells, thus, favoring tumor promotion (17). In addition, overexpression of COX-2 gene alters the response to growth regulatory signals and inhibits apoptosis (54). The inhibition of apoptosis by COX-2 includes effects on the intrinsic and extrinsic pathways of apoptosis (55). This action prolongs the survival of abnormal cells, which in turn favors the accumulation of sequential genetic changes that increase the risk of tumorigenesis (56). Furthermore, COX-2 induction or overexpression is associated with an increased production of PGE₂, which is known to modulate cell proliferation, cell death and tumor invasion in different cancer types (55). NSAIDs have been demonstrated to inhibit the proliferation of different cancer cell types expressing COX-2, supporting the evidence that PGs produced by COX-2 intervene in tumor cell proliferation (17).

Ki-67 and COX-2 exhibited higher scores and stronger correlations in their expression levels in ameloblastoma samples than in dentigerous cyst samples in the present study. However, the results of the current study reflect a similarity in the expression levels and correlation of Ki-67 and COX-2 in the odontogenic epithelium of the two odontogenic lesions dentigerous cyst and ameloblastoma. However, it has been demonstrated that the functions of COX-2 gene are complex and may involve different mechanisms depending on the cell types and the conditions studied (29). Therefore, it is necessary to further elucidate other effects of COX-2 in the epithelium and connective tissue of odontogenic lesions and to compare dentigerous cyst with ameloblastoma. Previous studies have shown that each of ameloblastoma and dentigerous cyst presents a distinct evolution and biological behavior, and in addition to their clinical and behavioral differences (57), they reflect diversities in various features such as gene expression (58), pattern of expression of cytokines (3), interaction of different proteins, and collagen components (57).

In conclusion, COX-2 was found to be expressed in the odontogenic epithelium of dentigerous cyst and ameloblas-

toma. Furthermore, it may contribute to local extension of dentigerous cyst and ameloblastoma by increasing the proliferation of their odontogenic epithelial cells. This study presents COX-2 as a possible target in the management of dentigerous cyst and ameloblastoma.

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