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Evaluation of Neoplastic Nature of Keratocystic Odontogenic Tumor Versus Ameloblastoma

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ABSTRACT

Objective: Although most of odontogenic tumors are benign, some of them will show locally destructive behavior, as keratocystic odontogenic tumor (KCOT) is now known as a benign but aggressive odontogenic neoplasm. The neoplastic characteristics in KCOT have been suggested from clinical as well as pathologic aspects. Matrix metalloproteinase-2 (MMP-2) is a gelatinase form of the MMPs family, which is a group of proteolytic enzymes that degrade many types of collagen. Cysteine aspartic acid-specific protease-3 (caspase-3) is the most downstream enzyme in the apoptosis-inducing protease pathway and is probably the most clearly associated with cell death. The aim of this study is to evaluate and compare the extracellular degradation potentiality (MMP-2) and apoptosis (caspase-3) of the epithelial lining in KCOT versus radicular cysts and ameloblastoma, in order to reinforce its classification as an odontogenic tumor.

Material and Methods: Twenty-six surgical specimens including keratocyst odontogenic tumor (KCOT; n=11), ameloblastoma (AB; n=8) and radicular cysts (RC; n=7) were examined for expression of MMP-2 and caspase-3 using the immunohistochemical method.

Results: For MMP-2 immunoexpression, AB showed the statistically significant highest mean area percentage, followed by KCOT, while RC showed the statistically significant lowest mean area percentage. As for caspase-3, there was no statistically significant difference between KCOT and AB, while RC showed the statistically significantly lowest mean area percentage.

Conclusion: Overexpression of MMP-2 protein related to growth and progression of lesions analyzed and may be one of the factors enhancing the recurrence of KCOT and invasion of AB. In addition, the epithelial lining of KCOT showed a high cell turnover reinforcing its classification as an odontogenic tumor.

Key Words: *Keratocystic odontogenic tumor (KCOT) – Ameloblastoma (AB) – Radicular cyst (RC) – Matrix metalloproteinase-2 (MMP-2) – Caspase-3.*

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INTRODUCTION

Odontogenic keratocysts (OKCs), originally defined by Philipsen more than 50 years ago **[1]**, have been re-classified in the 2005 edition of the WHO Classification of Head and Neck Tumors, from cystic to neoplastic lesions, and have been recently named "keratocystic odontogenic tumors" **[2]**. The WHO working group believed that the new term better reflects the neoplastic nature of the lesion.

Keratocystic odontogenic tumors (KCOTs), previously called odontogenic keratocysts, are lesions affecting the jaw bones especially in young adults. Their biological behavior differs from that of other odontogenic cysts. Classically, KCOTs are considered to be developmental cysts arising from remnants of the dental lamina **[3]**. However, their aggressive clinical behavior, associated with a high rate of recurrence, infiltrative growth and association with Nevoid Basal Cell Carcinoma Syndrome (NBCCS) suggests a neoplastic origin of these lesions **[3-6]**. KCOTs are frequently found in the posterior region of the mandible **[7-9]**. Moreover, recent molecular studies have implicated genetic alterations in odontogenic keratocysts, which supports the clonal/neoplastic nature of the cysts **[10]**. Odontogenic keratocysts are thus now categorized as odontogenic tumors **[2]**.

Ameloblastomas are the most frequently encountered tumors arising from odontogenic epithelium **[11,12]**. Although characterized as benign neoplasms, ameloblastomas are locally invasive with frequent recurrence and destruction tumor of the jaws, even following radical surgery **[12]**. Numerous studies have identified both genetic and molecular alterations in these

odontogenic tumors of the epithelium **[13,14]**, but the mechanisms underlying the local invasiveness of these neoplasms have yet to be clarified.

In multi-cellular organisms, the ability to regulate cell death to the same extent to cell growth and differentiation is vital. Programmed cell death mainly proceeds by apoptosis, a tightly controlled process to efficiently remove unwanted cells with characteristic cytoplasmic and nuclear condensation and DNA fragmentation. Apoptosis is also a fundamental process in tumor cell kinetics. It is believed that tumor cell growth results from a disturbance in the balance between the rate of proliferation and cell death, and therefore mutations affecting genes that are either inducers or repressors of apoptosis may be common occurrences during the development of neoplasms **[15,16]**.

Degradation of the extracellular matrix (ECM) is a prerequisite for cell migration into the matrix and represents a key element in the multistage processes of tumor invasion and metastasis. Matrix metalloproteinases (MMPs) are a family of zinc and calcium-dependent proteolytic enzymes that degrade extracellular matrix (ECM) macromolecules, such as collagens, gelatins, fibronectin, tenascin and laminin, at physiological pH **[14,17,18]**. These enzymes play central roles in the regulation of the extracellular matrix during embryonic development and tissue remodeling. MMPs also participate in ECM destruction associated with tumor invasion and metastasis **[19-21]**. Aberrant MMP activity in tumor cells and the surrounding stromal tissues has been implicated in tumor invasion and metastasis **[22,23]**. Therapeutic interventions that inhibit MMP activity appear to be promising based on a number of in vitro and in vivo tumor invasiveness studies **[24,25]**.

Matrix metalloproteinase-2 (MMP)-2 is a gelatinase of the MMP family, and degrades many types of collagens such as native types IV, V, and X collagen, and denatured fibrillar types I, II, and III collagen **[26]**. The role of MMPs in cell migration was studied by Mäkelä et al. **[27]** by measuring cell growth, migration, and production of MMP-2 and -9 in oral mucosal and skin keratinocytes cultured in the presence of synthetic MMP inhibitors. MMP-2 is the major gelatinolytic MMP produced by these cells. These results suggest that MMP-2 plays a specific role in epithelial migration, possibly by detaching the advancing cells from the pericellular matrix or by activating other MMPs [27]. Previous studies have shown that ameloblastomas have an elevated expression of MMP-2 **[28-31]**.

Caspases are cysteine proteases of the interleukin-1*ß* converting enzyme (ICE), which are required for programmed cell death, and 14 mammalian homologues have been identified so far **[32,33]**. Among these proteases, cysteine aspartic acid-specific protease-3 (caspase-3) is the most downstream enzyme in the apoptosis-inducing protease pathway and is probably the most clearly associated with cell death **[34,35]**. Caspase-3 has been found not only in the immune system but also in diverse normal tissues **[36]**. Recently, expression of caspase-3 has been proven to be correlated with the outcome of several neoplasms **[37,38]**.

The aim of the present study is to compare immunohistochemical expression of MMP2 (as an indicator of ECM degradation) and caspase-3 (as an indicator of apoptosis) in KCOT to ameloblastoma to clarify the neoplastic nature of KCOT.

MATERIAL AND METHODS

A- *Material:*

Twenty-six surgical specimens of KCOT were collected throughout a 2-year period (2007-2009), from the Department of Oral and Maxillofacial Surgery, Faculty of Dental Medicine for Girls, Al-Azhar University. All patients were surgically treated under general anaesthesia. Patients who had previously undergone medical therapy were excluded from the study. In addition, this study included only those cases who had normal levels of serum calcium, phosphorus and alkaline phosphatase to exclude hyperparathyroidism. Criteria for diagnosis included clinical examination, radiographic and histological features. Also, patients' data were analyzed in terms of age, gender, anatomic location, clinical and radiographic features, and type of surgery as well as the follow-up period.

B- *Radiographic examination:*

Digital orthopane tomograms were performed for all patients to evaluate mesiodistal

extension of the lesion in addition to its relation to teeth roots as well as the surrounding vital structures (Fig. 1). In addition, a multi-slice helical CT unit (Somatom plus S®, Siemens co, Germany) was used to determine buccolingual expansion of the lesions, presence or absence of cortical perforations and to evaluate lesions in all dimensions. The CT scan was set at 120KV and 200mA and a slice of 0.5mm thickness was obtained. The CT dataset presented in true sagittal, coronal, axial and three dimension views. The digital data from the CT scan were transformed to a computer for processing using the vision software (Figs. 2,3).

C- *Surgical procedures:*

An incisional biopsy was taken from intrabony lesions under local anaesthesia (zylocain 2%) for histological examination. The results of the biopsies revealed that 11 cases were keratocystic odonontogenic tumors, 7 radicular cysts and 8 ameloblastomas. A second surgical intervention was done under general anaesthesia for the definitive treatment of all cases. In mandibular cases (20 samples), 8 were ameloblastoma where the tumor mass was removed by an intraoral approach and marginal resection with at least a 5mm safety margin. In these cases mandibular continuity was conserved using reconstruction bone plates and the periosteum was minimally sacrificed (Fig. 4). The rest of the cases were 9 keratocystic odonontogenic tumors and 3 radicular cysts. Both types were treated by enculeation and root canal treatment to preserve the associated teeth. The maxillary cases (6 lesions, 2 keratocystic odonontogenic tumors and 4 were radicular cysts), were all treated by enucleation (Fig. 5). Carnoy's solution was used for chemical cauterization in cases of keratocystic odonontogenic tumors to avoid recurrence.

D- *Immunohistochemical investigations:*

The samples were fixed in 10% formalin for 24 hours and automatically processed and routinely embedded in paraffin blocks. Serial tissue sections cut at 4μm from paraffin blocks were stained with hematoxylin and eosin, as well as immunohistochemical staining.

For immunohistochemistry (IHC), serial sections 4-mm thick were cut and mounted on electrically positively charged slides and dried. Immunocytochemical staining was performed using a standard streptavidin-biotin-peroxidase complex method. The slides were first deparaffinized, dehydrated in graded ethanol concentrations, and incubated with 0.6% hydrogen peroxide in methanol for 10 minutes to block endogenous peroxidase activity. After rinsing with water, the slides were placed in a glass dish filled with 10mmol/L sodium citrate buffer, pH 6.0. Tissue sections were boiled in a microwave oven twice, 5 minutes each, to enhance immunoreactivity. The slides were allowed to cool and were rinsed with phosphate-buffered saline (PBS), pH7.2. The immunohistochemical staining was done according to the manufacturer's instructions using polyclonal antibodies against MMP-2 (1:800) (DAKO, Denmark) and caspase-3 (1:200) (DAKO, Denmark). Detection was carried out using the universal kit (DAKO, Denmark). Slides were washed in PBS for 5 minutes and incubated with secondary antiserum that was biotinylated goat serum conjugated to rabbit and mouse sera for 30 minutes. Sections were then washed for 5 minutes in PBS followed by development of antigen-antibody visualization by diaminobenzidine [DAB] in PBS containing 40% hydrogen peroxide. Sections were washed under running tap water for 10 minutes, then lightly counterstained with Mayer's haematoxylin and mounted. Negative controls were used on consecutive sections, using either an isotype antibody or omission of the primary anti-body resulting in no detectable staining.

E- *Evaluation of immunostaining:*

Epithelial cells with unequivocal staining of the cytoplasm or the membrane were considered positive. In each section, at least 10 high-power fields (400X) for each slide were examined under the light microscope and the staining positivity for Perlecan was recorded using an image analyzer using the software Leica Qwin system, at the Department of Oral Pathology, Faculty of Oral and Dental Medicine for Girls, Al-Azhar University. The immunopositivity was measured in the form of an area and area percentage in a standard measuring frame per 10 fields.

F- *Statistical evaluation:*

Quantitative data were presented as means and standard deviation values. Analysis of Variance (ANOVA) was used to compare between means of the three groups. Duncan's post-hoc

test was used to determine significant differences between the means when the ANOVA test result was significant. Qualitative data were presented as frequencies and percentages. The Chi-square (X^2) test was used to compare between the groups. The significance level was set at $p \leq 0.05$. Statistical analysis was performed with SPSS 15.0® (Statistical Package for Scientific Studies) for Windows. Pearson's correlation coefficient was used to determine significant correlation between Caspase-3 and MMP-2.

RESULTS

Nineteen patients (73.1%) were males and seven (26.9%) were females. Patients' ages ranged from 7 to 45 years. In twenty patients (76.9%) the lesion was located in the mandible, while it was found in the maxilla in six patients (23.1%). In ameloblastic cases, the lesions were widespread and involved the mandible from the condyle to the premolar region. All patients showed expansion of the buccal plate in both the maxilla and mandible, resulting in partial obliteration of the buccal vestibule. Also, eight patients presented with pain, tooth mobility, and a rapidly enlarging facial swelling. Paraesthesia was present in three patients. However, the most common feature (15 patients) was an asymptomatic swelling of the face or in the oral cavity. When the lesion was confined within the cortical plates, the overlying mucosa was usually normal. On the other hand, if perforation of the cortical plates was present, the mucosal surface had an erythematous appearance.

The radiographic examination revealed that there were 16 cases (61.5%) who had teeth involved with the lesions. Scalloping of the lesion between teeth was found in 6 cases (23.1%), root resorption was seen in 5 cases (19.2%), and displacement of the teeth was observed in 12 cases (46.2%). Also, 13 cases (50%) were multilocular while the others were unilocular lesions (50%).

No complications were observed in terms of loss of teeth, wound dehiscence, infection of the surgical site, fracture or loss of plates and screws, and necrosis of bone segments. All patients were followed annually with clinical and radiographic examinations for periods ranging from 2 to 5 year with no evidence of recurrence.

Histopathology:

All cases of KCOTs showed a uniform, thin lining epithelium ranging from 8 to 10 cell layers with little or no evidence of rete ridge formation; a palisading cuboidal or columnar hyperchromatic basal cell layer; a predominantly parakeratotic keratinization, with a frequently "corrugated" inner cystic surface; Orthokeratinized foci, however, could also be found. Occasionally, budding of the basal cell layer into surrounding connective tissue and the formation of microcysts could be seen. Folding of the epithelial lining was a characteristic features. The fibrous cyst wall was relatively thin and usually lacking inflammatory cell infiltrate. Detachment of portions of the cyst-lining epithelium from the fibrous wall was commonly observed.

The epithelial lining of RCs consisted of stratified squamous epithelium of variable thickness with or without elongated rete ridge. Various degrees of inflammatory cell infiltration were also observed in the connective wall.

All cases of ameloblastoma showed follicular pattern with small areas of plexiform. Follicular ameloblastoma appeared as epithelial nests consisting of a core of loosely arranged stellate reticulum like cells. A single layer of tall columnar cells surrounded this central core. The nuclei of these cells were located at opposite pole to the basement membrane. In other areas, the peripheral cells may be more cuboidal. The plexiform type of ameloblastoma consisted of long, anastomosing cords or larger sheets of odontogenic epithelium. The cords or sheets of epithelium were bound by columnar or cuboidal cells surrounding more loosely arranged epithelial cells.

Immunohistochemical results:

MMP-2:

Immunoreactivity to MMP-2 was found in all KOCT samples analyzed. The MMP-2 expression was found in cytoplasm of suprabasal and superfacial epithelial cells of KCOT, clearly detectable also in the basal cells and basement membrane zone of the detached epithelium, (Fig. 7A). For RC, only five cases out of seven (71%) showed immunoreactivity to MMP-2. MMP-2 immunoexpression was shown in the cytoplasm of suprabasal and superficial epithe-

lial cells, (Fig. 7B). All ameloblastoma samples showed immunoexpression of MMP-2. Immunohistochemical reactivity was observed in the cytoplasm of the neoplastic cells. In tumor islands, expression of MMP-2 was mainly detected in the peripheral columnar cells, with few positive cells in the central island area, (Fig. 7C). For MMP-2, AB showed the statistically significant highest mean area percentage, followed by KCOT, while RC showed the statistically significant lowest mean area percentage (Table 1, Fig. 6).

Caspase-3:

All cases of KCOT showed positive expression to caspase-3. Cytoplasmic and nuclear caspase-3 immunostaining was recognized from basal to suprabasal and superficial cells of the lining epithelium, (Fig. 8A). Six out of seven RC cases were immunoreactive to caspase-3 (85%). Caspase-3 was recognized in the cytoplasm of the superficial layer of the lining epithelium, nuclear staining became more evident upon moving to higher layers, (Fig. 8B). For AB, all cases positively stained for caspase-3. The immunoreactivity was detected in the cytoplasm of the tumor cells, mainly the stellate reticulum-like cells in the centre of tumor islands, with almost negative staining of the peripheral columnar or cuboidal cells, (Fig. 8C). For caspase-3, there was no statistically significant difference between KCOT and AB which showed the statistically significantly highest means. RC showed the statistically significantly lowest mean area percentage (Table 1, Fig. 6).

The correlation between caspase-3 and MMP-2, there was a statistically significant positive correlation between both parameters in each group (Table 2).

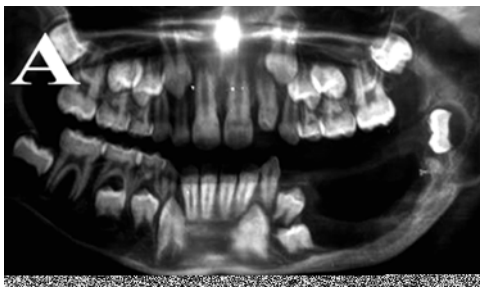

Fig. (1-A): Panoramic film showing a case of OKCT of the mandible.

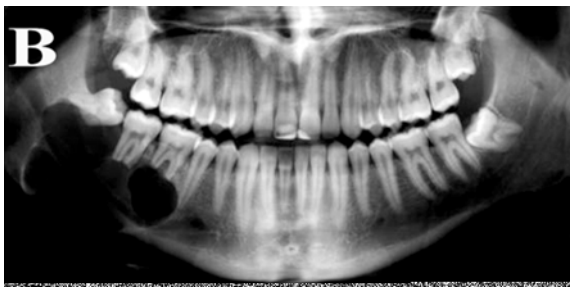

Fig. (1-B): Panoramic view demonstrating a case of ameloblastoma.

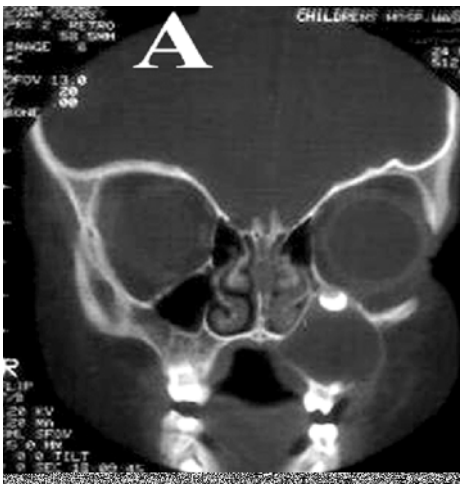

Fig. (2-A): Coronal CT view showing OKCT of the maxilla.

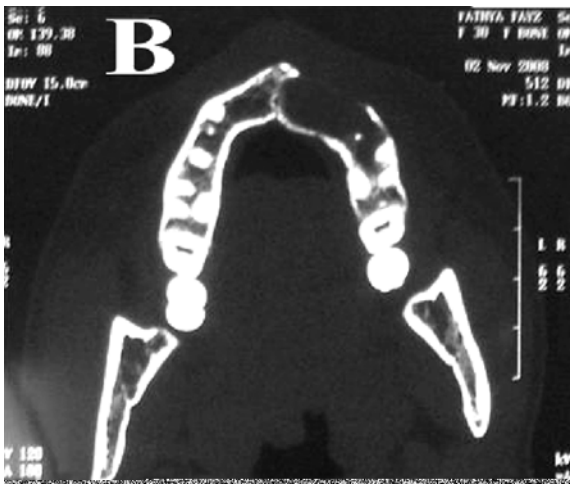

Fig. (2-B): Axial cut of the maxilla for the same case.

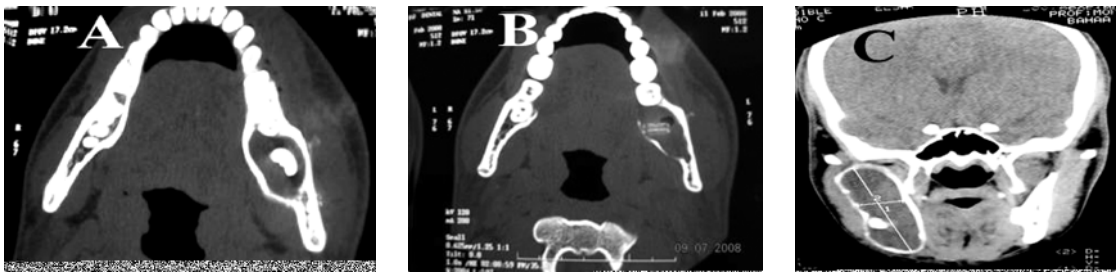

Fig. (3): A,B: Axial cuts for ameloblastoma of the mandible showing expansion of both buccal and lingual cortices and perforation of the lingual cortex. C: Coronal cut showing an ameloblastic lesion occupying the ramus of the mandible.

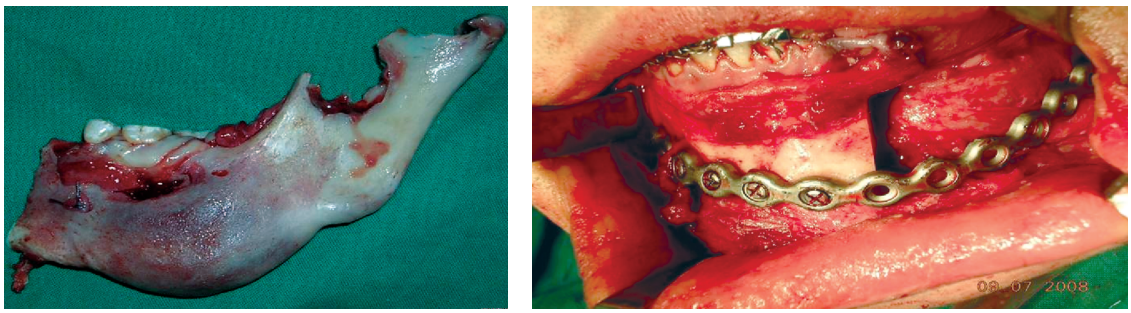

Fig. (4): Photographs demonstrating a resected mandible and reconstruction plate fixation.

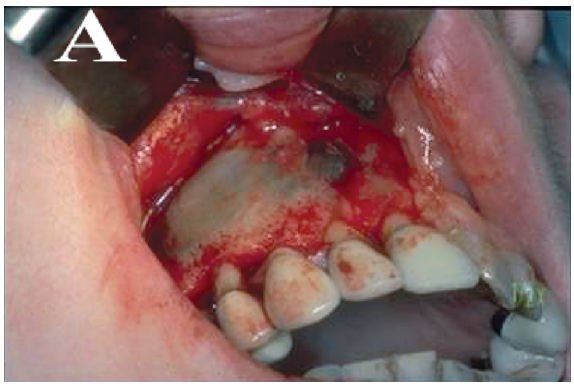

Fig. (5-A): A photograph showing OKCT of the maxilla.

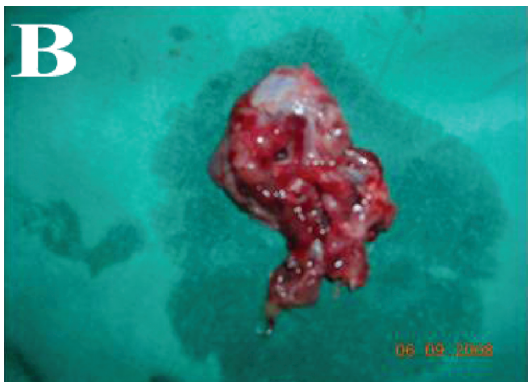

Fig. (5-B): A photograph illustrating the enucleated cyst.

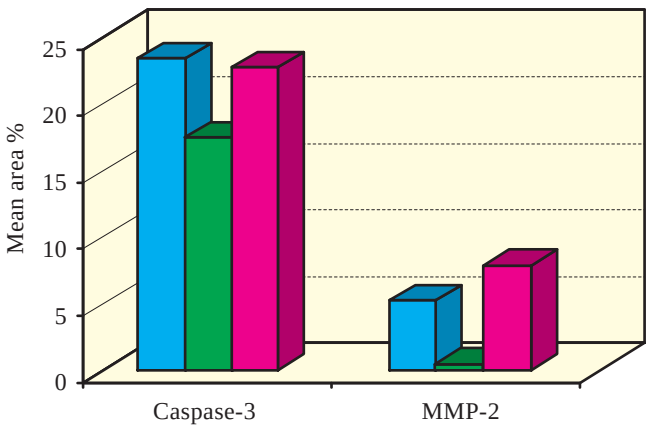

Fig. (6): Comparison between area percentage in the three studied groups.

KCOT : Keratocystic odontogenic tumor.
RC : Radicular cyst.
AB : Ameloblastoma.

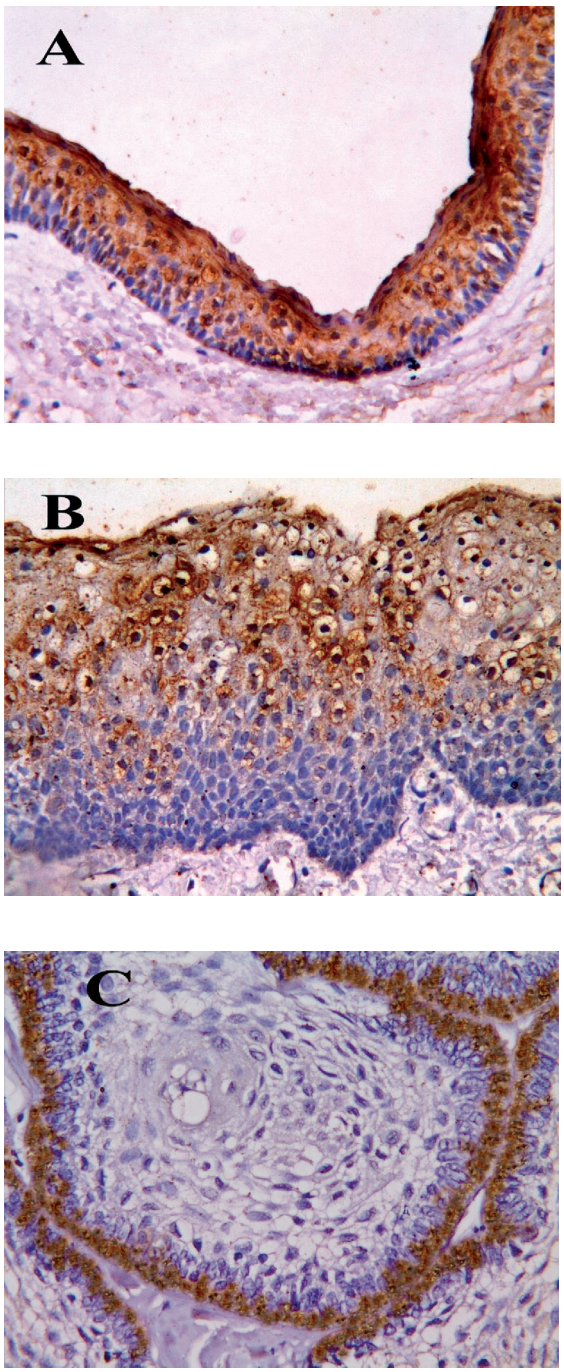

Fig. (7): Expression of MMP-2 (A) in KCOT, immunolocated in the cytoplasm of suprabasal and superfacial epithelial cells (original magnification X200), (B) in RC, immunolocated in the stratum spinosum but at higher layers than KCOT(original magnification X200), (C) in ameloblastoma, immunolocated in the cytoplasm of the neoplastic epithelial islands mainly detected in the peripheral columnar cells (original magnification X200).

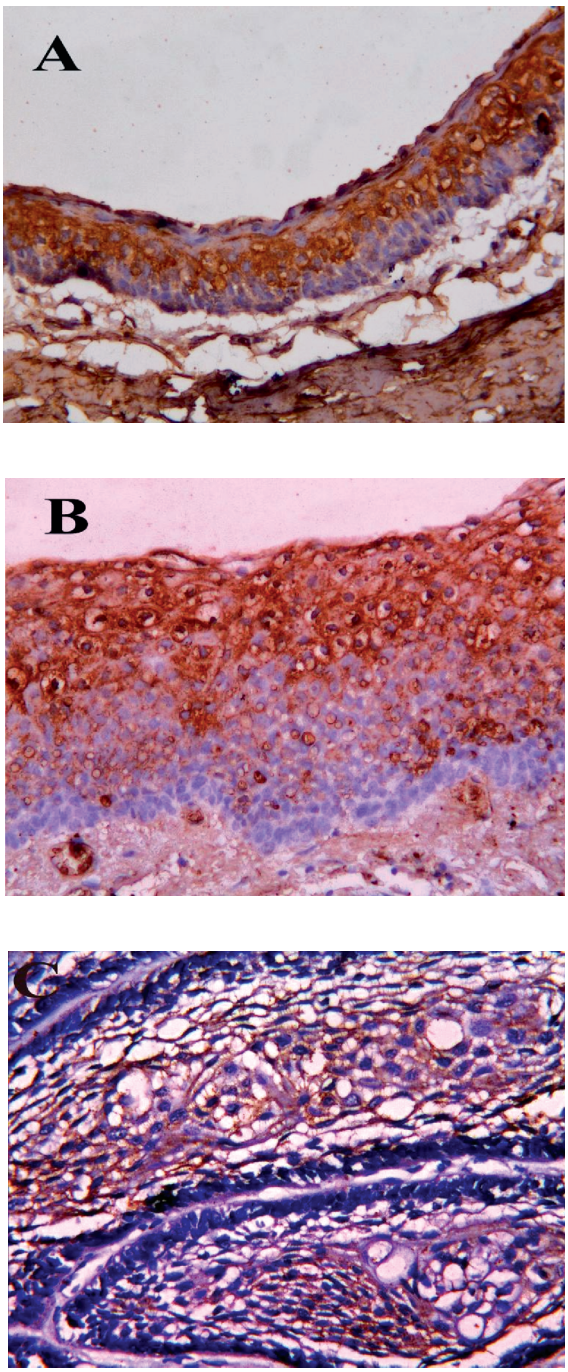

Fig. (8): Expression of caspase-3 (A) in KCOT, immunolocated in the cytoplasm of basal to suprabasal and superficial cells of the lining epithelium (original magnification X200), (B) in RC, immunolocated in the cytoplasm of suprabasal to superficial cells (original magnification X200), (C) in ameloblastoma, immunolocated in the cytoplasm of tumor cells, mainly stellate reticulum-like cells in the centre of tumor islands with almost negative staining at the peripheral columnar or cuboidal cells (original magnification X200).

Table (1): Means, standard deviation (SD) and results of ANOVA test for the comparison between area percentage in the three groups.

Antibody	KCOT (n=11)		RC (n=7)		AB (n=8)		*p* value
	Mean	SD	Mean	SD	Mean	SD	
MMP-2	5.2^{b}	1.3	0.44^{c}	0.12	7.85^{a}	2.3	0.003*
Caspase-3	23.4^{a}	4.5	17.45^{b}	3.9	22.72^{a}	5.2	0.007*

*: Significant at $p \leq 0.05$, Means with different letters are statistically significantly different according to Duncan's test.

Table (2): Correlation between Caspase-3 and MMP-2 in the three groups.

KCOT (n=11)		RC (n=7)		AB (n=8)	
Correlation coefficient (*r*)	*p* value	Correlation coefficient (*r*)	*p* value	Correlation coefficient (*r*)	*p* value
0.576	0.002*	0.488	0.028*	0.423	0.033*

*: Significant at $p \leq 0.05$.

DISCUSSION

Development and diseases are consistent with a highly orchestrated process of apoptosis and ECM degradation. Resorption of angiogenic vessels following αvß3 integrin blockade is associated with apoptosis and ECM degradation **[39]**. In the formation of the preamniotic cavity, apoptosis of ectodermal cells is accompanied by ECM resorption **[40]**. MMP-2 is considered to play a major role in ECM remodeling because of its ability to initiate and continue degradation of fibrillar collagen **[41]**. Caspases could fulfill the role of ECM-degrading enzymes. They represent a large family of cysteine proteinases activated during the course of apoptosis **[42]**.

In accordance with Zhong et al. **[29]**, all our cases of KCOT showed intensively immunopositive MMP-2 in the stratum spinosum, and weekly immunopositive or negative MMP-2 in the stratum basale. This result is in contrast to Wahlgren et al. **[43]** who found that MMP-2 immunoexpression was located at the basal cells and basement membrane (BM) zone. Radicular cysts (RC) were used in this study for comparison due to their less aggressive clinical behavior and a growth mechanism related to fluid accumulation. It was found that the expression of MMP-2 in RC was statistically significantly lower than KCOT, the positive immunostaining was shown in the stratum spinosum but at higher layers than KCOT and increased in areas associated with inflammation. This is in accordance with Metwaly and El-Deeb **[44]**.

For ameloblastoma, immunoreactivity for MMP-2 was observed in the cytoplasm of the neoplastic epithelial islands mainly detected in the peripheral columnar cells, with few positive cells in the central cells. This is in line with previous reports by **[29,30,41,44-46]**. The immunoexpression of MMP-2 was higher in AB with a statistically significant difference ($p<0.05$) compared to KCOT, which is in line with Zhong et al. **[29]**.

The present results were not in accordance with Kumamoto et al. **[28]** who showed that stromal cells of ameloblastomas were strongly positive for MMP-2 and some tumor cells showed weak reactivity for MMP-2 in most ameloblastoma tissues. Expression of MMP-2 in stromal and epithelial components in ameloblastomas might be associated with regulation of epithelial-mesenchymal tissue interactions in neoplastic odontogenic tissues. The presence of MMPs in the stromal tissue is probably caused by tumor induction. It is known that neoplastic cells express a protein from the immunoglobulin super-family named extracellular matrix metalloproteinase inducer; this factor could induce secretion of MMPs by stromal cells **[47]**.

In the present study, caspase-3 was intensely expressed in the lining epithelium of KCOT from basal to suprabasal and superficial cells, with more intense staining in the suprabasal and superficial layers. In addition, both the cytoplasm and nuclei were stained but the nuclei were predominantly stained in the suprabasal and superficial cells. These results are in line with Kimi et al. **[48]** and Mitrou et al. **[49]** who found that caspase-3 was expressed in the full thickness of the epithelium of KCOT and suggested that caspase-3 was expressed during the transition from cell proliferation to cell death, and that caspase-3 might be transported into the nuclei of the suprabasal or superficial cells during apoptotic cell death in the lining epithelium of KCOT.

Kimi et al. **[50]** and Kichi et al. **[51]** revealed that apoptotic cells were found only in the surface layer **[51]** or suprabasal to superficial layers of KCOT epithelial lining **[50]**. However,

Kolář et al. **[3]** have shown that the immunoexpression of the anti-apoptotic protein bcl-2 was limited to the basal layer of KCOT. So, it is noteworthy that the intense expression of caspase-3 in the superficial cell layer of the epithelial lining of KCOT should explain why cystic lesions but not tumor masses are formed, despite the prominent proliferative activity **[51]**.

In RC, nuclear and cytoplasmic expression of caspase-3 in suprabasal to superficial cells was noted with increasing intensity toward the surface; this is in accordance with Suzuki et al. **[52]**. On the other hand, this was in contrary to other studies by Kimi et al. **[48]** and Mitrou et al. **[49]** where the caspase-3 expression of RCs was seen in all epithelial layers. In the present study, the expression of caspase-3 in RC showed statistically significant lower immunoexpression than KCOT, which is in contrary to the findings of Mitrou et al. **[49]**.

Loyola et al. **[53]** stated that apoptosis was always present in the epithelium of the RC, although it was more frequent in lesions with atrophic (quiescent) epithelium. In the endodontic literature, cavitation in epitheliated granulomas occurs when the inner cells of the proliferative epithelial islands become distant from the supportive connective tissue **[54]**. The resulting ischemia decreases the ATP levels in the cell, breaking down electrolytic pumps, and allowing cytoplasmic Ca^{2+} influx. It activates enzymes that cause morphologic cellular alterations characteristic of necrosis and the consequent formation of a cystic cavity **[55,56]**. This notwithstanding, mild ischemia has been shown to be able to induce apoptosis rather than necrosis **[55,57]**, and an abrupt impairment of oxygen supply does not occur in the periapical inflammatory lesion **[53]**.

In the present study, expression of the pro-apoptotic proteins, caspase-3, was mainly detected in the center of tumor islands of AB. These results are similar to those reported by Luo et al. **[58]**, but are not consistent with results described by Kumamoto et al. **[59]** who found that follicular and plexiform ameloblastomas showed strong caspase-3 reactivity in peripheral columnar or cuboidal cells and weak to moderate reactivity in central polyhedral cells. Although a higher mean of caspase-3 immunoexpression was percent in KCOT, yet there was no statistically significant difference when compared to AB.

Sandra et al. **[60]** suggested that ameloblastoma had two relatively distinct patterns, an anti-apoptotic proliferating site in the peripheral layer and a pro-apoptotic site in the central layer of the tumor islands. Where expression of pro-apoptotic proteins, such as Fas, FasL and caspase-3, was mainly detected in the foci of squamous and granular cells in the centre of tumor islands, the anti-apoptotic protein, Bcl-2, and the proliferating cell marker Ki67 were principally expressed in the peripheral basal cells. These results suggested that caspase-3-induced apoptotic cell death may function in the disposal of terminally differentiated (squamous metaplasia) or degenerative tumor cells (granular transformation and cystic changes) in ameloblastomas.

In oral carcinomas, the proportion of active caspase-3 positive cells with apoptotic morphology was markedly higher than in normal oral epithelium. Moreover, within the oral squamous cell carcinoma series, there was significantly more intense nuclear and cytoplasmic staining with increasing STNMP stage (p=0.017 and 0.03, respectively). Both caspase-3 staining intensity and the percentage of cells positive for caspase-3 were inversely associated with differentiation **[61]**. Hague et al. **[61]**, confirmed the findings of Macluskey et al. **[62]** that the apoptotic index is increased during oral carcinogenesis. Only tumor differentiation highlighted differences between nuclear and cytoplasmic staining intensity scores, in that cytoplasmic staining was higher in less differentiated tumors, whereas nuclear staining showed no association with differentiation. Hence, cytoplasmic staining may be the more valuable marker relating to prognosis **[61]**.

In the present study, there was a statistically significant positive correlation between caspase-3 and MMP-2 in each group. MMPs are implicated in the induction as well as inhibition of apoptosis in various cell types. MMP-2 is activated early during hepatic stellate cell (HSC) apoptosis and directly cleaves N-cadherin in vitro **[63]**. Also, upregulation of MMP-2 is associated with increased apoptosis in human umbilical vein endothelial cells (HUVECs) and vascular smooth muscle cells **[64,65]**. MMP-2 siRNA treatment significantly decreased cultured keratinocyte growth and migration, and stimulated apoptosis fourfold. MMP-2 appears

to act through caspase-3 signaling pathways as evidenced by stronger staining for active caspase-3 in response to MMP-2 siRNA **[66]**. In another study, MMP-2 inhibition reduced Bax expression and caspase-3 activity, as well as increasing Bcl-2 expression in cultured rat cardiac myocytes **[67]**. Lai et al. **[68]** found that matrix metalloproteinase family members (MMP)-1, -2 -3, -9 were involved not only in invasion and metastasis but, also in apoptosis of cancer cells characterized by the activation of caspase-3.

KCOTs have a tendency to form satellite cysts, and the epithelial lining locally detaches from the connective tissue wall. The mitotic activity of the KCOT epithelial cells is greater than any other odontogenic cysts **[69,70]**. Wahlgren et al. **[43]** proved that MMP-2 can induce epithelial migration by fragmenting the Ln-5 γ2 chain; this may explain the induced migration, tendency to detach from the connective tissue capsule and the growth potential of KCOT. These characteristic factors have been thought to be behind the frequent recurrence of KCOT after surgical enculeation.

In conclusion, the present study demonstrates that the overexpression of caspase-3 and MMP-2 proteins related to growth and progression of lesions analyzed and may be one of the factors enhancing the recurrence of KCOT and invasion of AB. Also, epithelial lining of KCOTs showed a high cell turnover reinforcing its classification as an odontogenic tumor.

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