

Immunohistochemical Expression of Collagen IV and MMP9 in Odontogenic Cysts and Tumors

Homeyra Mardani, Nastaran Dinarvand*,
Gholamreza Jahanshahi and Elaheh Sofiyan

Department of Oral and Maxillofacial Pathology, School of Dentistry, University of Medical Sciences, Isfahan (Khorasgan) Branch, Islamic Azad University, Isfahan, Iran.

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The growth mechanism of odontogenic cysts and tumors along with their invasion and destructive ability can be influenced by the secretion of metalloproteinases from epithelial and mesenchymal cells. An important activity of matrix metalloproteinases is destruction of the components of basement membrane, including collagen IV. The present study was conducted to analyze the role of collagen IV and its association with matrix metalloproteinases9 in odontogenic cysts and tumors. In this study, 19 samples of keratocyst odontogenic tumor and 11 samples of dentigerous cyst in mesenchymal stroma and odontogenic epithelium, and in basement membrane of odontogenic epithelial neoplastic islands and basement membrane of cystic epithelium were analyzed by immunohistochemical method. Collagen IV expression in keratocyst odontogenic tumors and dentigerous cysts was >50%, which showed no statistically significant difference ($p=0.62$). The consistency and thickness of collagen IV in dentigerous cysts were significantly higher than those of keratocyst odontogenic tumors in terms of the staining pattern of collagen IV ($p=0.003$, $p=0.001$). Also, no significant difference was observed between the epithelium of keratocyst odontogenic tumors and dentigerous cysts with regard to the expression of matrix metalloproteinase-9 ($p=0.18$). Expression of metalloproteinases9 in the mesenchymal stroma of keratocyst odontogenic tumors was significantly higher than dentigerous cysts ($p=0.04$). Higher expression of matrix metalloproteinase-9 and inconsistency and low thickness of collagen IV in keratocyst odontogenic tumors may be associated with diverse biological behavior of keratocyst odontogenic tumors than dentigerous cysts.

Key words: Keratocystic odontogenic tumor, dentigerous cyst, matrix metalloproteinase9, collagen IV, immunohistochemical .

Odontogenic cysts and tumors constitute a heterogeneous group of jaw lesions with diverse histopathological features and clinical expression. Keratocystic odontogenic cyst has currently been classified as Keratocystic odontogenic tumor (KOT) in new classification owing to more invasive behavior and higher recurrence rate than odontogenic cysts¹⁻². In contrast, dentigerous cyst(DC) has a mild behavior and rarely recurs

after removal. Keratocystic odontogenic tumor (KOT) has a cystic structure similar to dentigerous cyst, but it has an aggressive development pattern similar to ameloblasts³.

Given the varied behavior of these lesions, a number of studies have been carried out to analyze the epithelial and mesenchymal factors that determine the different behaviors of these lesions. Some events, the same as histomorphological differentiation of tooth development in odontogenic lesions, occur during tumor development and mesenchymal-epithelial interaction.

* To whom all correspondence should be addressed.

Most of the studies have been conducted on the epithelial components of tumors and odontogenic cysts⁴. The development mechanism of odontogenic cysts and invasive and destructive capacity of some odontogenic tumors may be influenced by matrix metalloproteinases, secreted from epithelial and mesenchymal cells⁵⁻⁶.

Changes in the expression of specific proteins of extracellular matrix (ECM) with increasing presence of matrix metalloproteinases and lack of expression of metalloproteinase inhibitors (TIMPs) may influence the behavior of these lesions and consequently affect the development and aggressiveness of tumors⁷.

Matrix metalloproteinases are the most important enzymes, responsible for disintegration and destruction of extracellular matrix (ECM), whose major function is removal of the molecules in extracellular matrix.⁸ Matrix metalloproteinases are a family of calcium- and zinc-dependent endopeptidases that are classified based on the molecular structure and the substance they destroy. Matrix metalloproteinases are divided into two groups of soluble matrix metalloproteinases and membrane-related matrix metalloproteinase. The soluble types include collagenases, gelatinases, stromelysines, matrilizines and a heterogeneous group⁹.

From among matrix metalloproteinases, matrix metalloproteinase⁹ is the most important proteinase associated with bone resorption, whose increase in tissues causes the destruction and breakage of extracellular matrix molecules and molecules on the cell surface, thereby changing the cell-cell and cell-extracellular matrix interaction¹⁰. Matrix metalloproteinases can also aim to destroy molecules other than extracellular matrix components¹¹⁻¹². However, recent studies have shown that matrix metalloproteinases cannot explain the aggressive behaviors of the mentioned lesions alone, and their interaction with other factors such as collagen IV can affect their biological behaviors.

Collagen IV is one of the components of basement membrane (BM) that is involved in all stages of tooth development. Further, changes and remodeling of basement membrane collagen IV occur in most of the odontogenic neoplasms¹³. Collagen IV along the basement membrane also plays a pivotal role in cell attachment, migration,

differentiation and development, and its reduced production during angiogenesis processes also plays an important role in tissue remodeling and tumor growth¹⁴.

Few studies have investigated the role of above factors in odontogenic cysts and tumors as well as their biologic behaviors. Hence, for better understanding of biologic behaviors of odontogenic lesions, extracellular matrix assessment is required in order to determine different expression levels of proteins among indolent and invasive lesions¹⁵. Therefore, the present study was performed to investigate the immunohistochemical expression of matrix metalloproteinase⁹ and collagen IV in keratocystic odontogenic tumors and dentigerous cysts to better understand the association of matrix metalloproteinase⁹ with destruction of basement membrane and biologic behavior of these lesions.

MATERIALS AND METHODS

In this retrospective descriptive-analytical research, the odontogenic cysts including keratocystic odontogenic tumors and dentigerous cysts were studied. This study (research project number:2381020921038) was approved by the ethics committee and vice chancellor of research at Khorasegan branch, Islamic Azad University, Isfahan and conducted over one year, 2013-2014.

The blocks of 30 odontogenic cysts, 19 keratocystic odontogenic tumors and 11 dentigerous cysts were obtained from the pathology department of Isfahan school of dentistry. Clinical information, including age, gender and lesion site was analyzed. A 5 μ section was prepared for hematoxylin-eosin staining. Having observed hematoxylin-eosin slides, the slides with sufficient tissue and appropriate fixation were selected. The samples with improper fixation, extensive inflammation, extensive areas of bleeding and too much inflammation, Gorlin-syndrome-related odontogenic keratocysts and odontogenic lesions with inadequate tissue were excluded from the study. Diagnosis of odontogenic cysts and tumors was performed according to the special classification of World Health Organization (WHO) in 2005 and other studies¹⁶⁻¹⁸. The expression levels of collagen IV and matrix metalloproteinase-9 were

determined and compared in both groups.

Immunohistochemical methods

Immunohistochemical staining was performed by envision method using matrix metalloproteinase-9 monoclonal antibody (Biogenex co, USA) and collagen IV monoclonal antibody (Dako co, USA). 3 μ sections prepared from the blocks were attached on the glass slides. During deparaffinization and dehydration processes, the slides were put in thermal furnace (60 °C) for 30 minutes and then placed in two xylol containers for 5 minutes and three descending ethanol (100%, 80% and 65%, respectively) containers for 2 minutes in order to dehydrate. The slides were then placed in 3% oxygenated water and washed with distilled water after 5 minutes.

During antigen retrieval stage for matrix metalloproteinase-9 marker, slides were placed in Tris-EDTA buffer (pH=9) in microwave for 20 minutes. For collagen IV marker, slides were put in proteinase K for 20 minutes. Then, the primary antibodies of matrix metalloproteinase-9 and collagen IV were separately poured on the tissue. After 60 minutes incubation for collagen IV and 30 minutes incubation for matrix metalloproteinase-9 at room temperature, the slides were washed with phosphate buffered saline.

In chromogen stage, the slides were stained by diaminobenzidine chromogen for 5 minutes and then washed with distilled water. Omission of primary antibody was employed as negative control, while tissue of lung and skin were used as positive control for MMP9 and collagen IV. Brown cytoplasmic staining was considered as positive. The histological regions in which the given marker was expressed were observed as brown color under optic microscope. Hematoxylin staining was used for background staining. Finally, the slides were mounted with entelan glue and observed under optic microscope.

Having stained the slides, they were evaluated by two pathologists under optic microscope. According to the methods presented by Gomes Henriques *et al.* in 2011, for collagen IV staining, the basement membrane between epithelium and mesenchyme was analyzed. The percentage of collagen IV staining along the basement membrane was analyzed semi-quantitatively as >50% or \leq 50%. Also, the staining pattern of collagen was determined in terms of

consistency (continuous, discontinuous) and thickness (thin, thick and mixed). Expression of matrix metalloproteinase-9 was separately analyzed in epithelial and mesenchymal lesions. In mesenchyme, staining was observed in endothelial cells, fibroblasts and inflammatory cells. The staining percentage of matrix metalloproteinase-9 was determined semi-quantitatively using the method presented by Gong *et al.* in 2009. In this method, the staining percentage is determined by one of these three scores, 0 (<10% staining), 1 (10-50% staining) and 2 (>50% staining). Brown cytoplasm was considered as positive staining.

Statistical analysis

The obtained data were analyzed by SPSS-20 software using chi-square, Mann-Whitney and Fisher's exact tests.

RESULTS

A total of 19 keratocystic odontogenic tumors of the patients with age range of 15-70 years and mean age of 16.40 ± 33.63 and 11 dentigerous cysts of the patients with age range of 7-55 and mean age of 17.56 ± 29.81 were analyzed in this study. With regard to the lesion site, from the 19 keratocystic odontogenic tumors, 14 samples were related to posterior mandible and 5 samples were related to maxilla. Of 11 dentigerous cysts, 5 pericoronal samples were found in impacted mandibular wisdom tooth, 1 pericoronal sample in mandibular tooth 5, 1 pericoronal sample in maxillary canine and 4 samples in intraosseous area (3 samples in anterior maxilla and 1 sample in posterior mandible).

From the samples studied in OKC group, 64.3% were male and 31.6% were female. As for the dentigerous cyst group, 63.6% of samples were male and 36.4% were female. The distribution of gender was not significantly different between groups ($p=0.79$). The expression levels of matrix metalloproteinase-9 in the epithelial and mesenchymal components of keratocystic odontogenic tumors and dentigerous cysts are presented in Table 1 and Figure 4,5,6

The expression levels of collagen IV in the basement membrane and staining pattern of collagen in terms of consistency (continuous-discontinuous) in keratocystic odontogenic tumors and dentigerous cysts are presented in Table 2.

Table 1. Immunohistochemical expression of matrix metalloproteinase-9 in the epithelial and mesenchymal components of keratocystic odontogenic tumors and dentigerous cysts

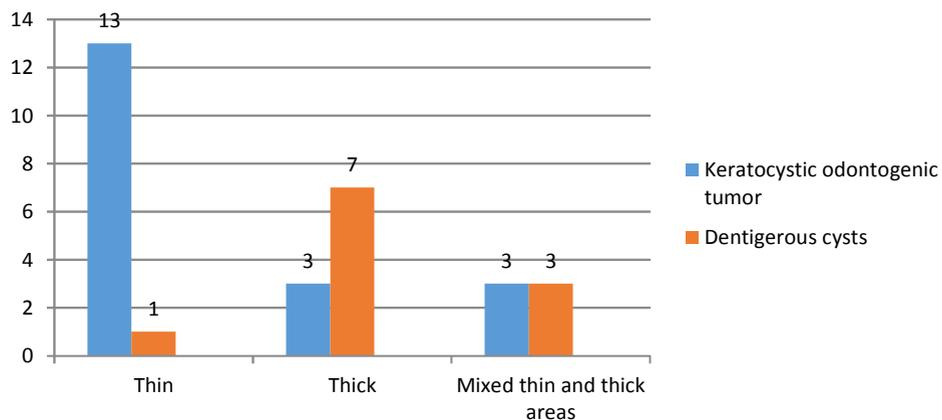
Immunohistochemical expression of MMP9	Keratocystic odontogenic tumor n=19	Dentigerous cysts n=11	P value
Epithelium[n(%)]			
-Score0	7(36.8%)	5(45.5%)	0.18
-Score1	12(63.2%)	6(54.5%)	
-Score2	0(0%)	0(0%)	
Mesenchyme [n(%)]			
-Score0	3(15.8%)	3(27.3%)	0.04
-Score1	12(63.2%)	8(72.7%)	
-Score2	4(21.2%)	0(0%)	

Table 2. Expression of collagen IV and its patterns (continues or discontinues) at the basement membrane of keratocystic odontogenic tumors and dentigerous cysts

	Keratocystic odontogenic tumor n=19	Dentigerous cysts n=11	P value
Expression of collagen IV			
-< 50% staining	4(21.1%)	2(18.2%)	0.62
->50% staining	15(78.9%)	9(81.8%)	
Staining distribution patterns			
- Continuous	5(26.3%)	9(81.8%)	0.003
- Discontinuous	14(73.7%)	2(18.2%)	

The staining patterns of collagen with regard to thickness (thin, thick and mixed) in keratocystic odontogenic tumors and dentigerous cysts are presented in Figure 1,2, 3. The thickness of collagen

IV in the basement membrane of dentigerous cyst was significantly higher than that of keratocystic odontogenic tumor ($p=0.001$).

**Fig. 1.** The staining patterns of collagen IV expression according to thickness (thin, thick and mixed thin and thick areas) at the basement membrane of keratocystic odontogenic tumors and dentigerous cysts

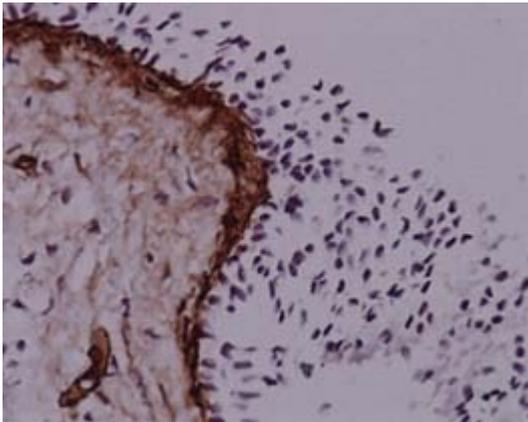


Fig. 2. Thick basement membrane in dentigerous cyst ($\times 400$)

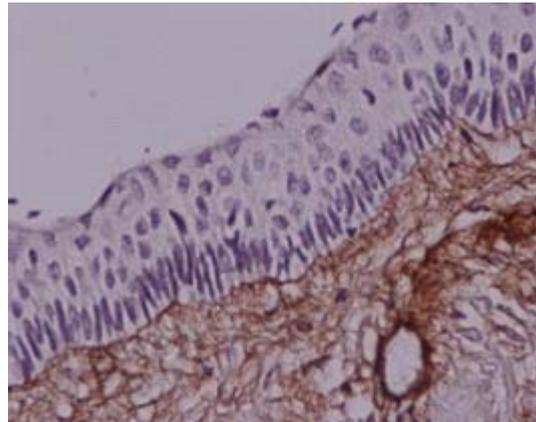


Fig. 3. Thin basement membrane in keratocystic odontogenic tumor ($\times 400$)

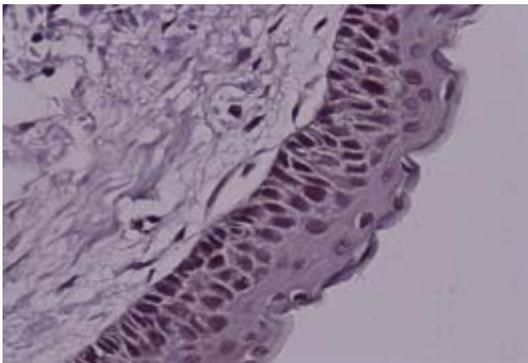


Fig. 4. Score 1 in the epithelial keratocystic odontogenic tumor ($\times 400$)

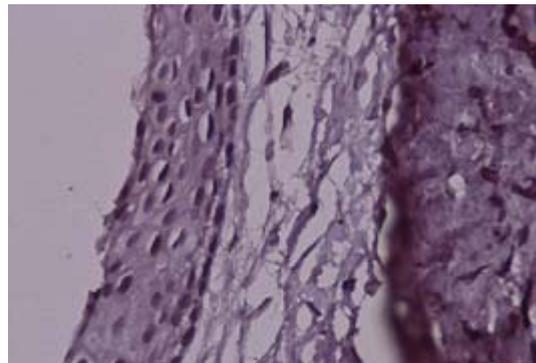


Fig. 5. Score 0 in Mesenchymal dentigerous cyst ($\times 400$)

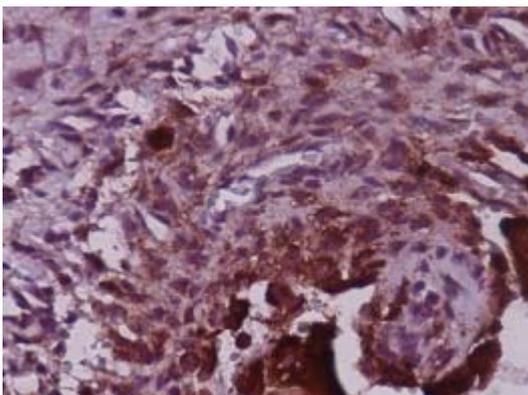


Fig. 6. Score 2 in the mesenchymal keratocystic odontogenic tumor ($\times 400$)

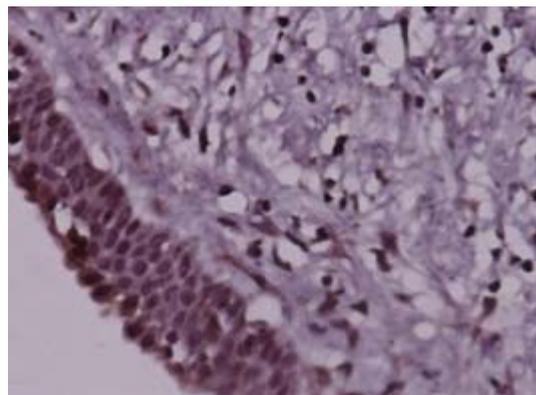


Fig. 7. Score 1 in the epithelial and mesenchymal keratocystic odontogenic tumor ($\times 400$)

DISCUSSION

In this study we investigated the immunohistochemical expression of matrix metalloproteinase-9 and collagen IV in keratocystic odontogenic tumors and dentigerous cysts. The results indicated that immunohistochemical expression of matrix metalloproteinase-9 in the epithelial component of odontogenic tumors and cysts was not significantly different, but its expression was significantly higher in keratocystic odontogenic tumors than in dentigerous cysts. Expression of collagen IV was similar in the two studied pathologies. Continuous staining patterns were more frequent in dentigerous cysts, whereas discontinuous patterns were more prevalent in odontogenic tumors. Expression of collagen IV was significantly thicker in dentigerous cysts.

Several studies have investigated the role of matrix metalloproteinases in the growth of odontogenic tumors and cysts. Recently, Scariot *et al* 21 systematically reviewed the role of matrix metalloproteinases in this group of lesions and reported that the expression patterns of matrix metalloproteinases in odontogenic lesions were different from the expression patterns of other lesions. Accordingly, odontogenic lesions especially keratocystic odontogenic tumors showed a significantly positive immunoreactivity against matrix metalloproteinases. They suggested that matrix metalloproteinases could explain the aggressive biological behavior of these tumors. According to their findings, matrix metalloproteinases could degrade some types of collagen, such as collagen type IV and basement membrane laminin. They presented on the walls of odontogenic cysts and in the cystic fluid of these dental lesions. There is evidence regarding the involvement of matrix metalloproteinase-9 in the pathologic process of cyst expansion. Moreover, matrix metalloproteinase-9 is secreted in inactive form, and it should convert to its active form to represent its function.

Some previous studies have demonstrated a positive association between matrix metalloproteinase-9 activity and the type of odontogenic cysts²². Given the association between matrix metalloproteinase-9 and collagen IV as well as the recent reports regarding the interaction of matrix metalloproteinases with other

unknown factors for biological behavior of odontogenic tumors and cysts, we designed this study and compared the expression of both matrix metalloproteinase-9 and collagen IV in keratocystic odontogenic tumors and dentigerous cysts²³.

The structure of basement membrane, especially extracellular matrix plays an important role in regulating the cellular behavior. The role of basement membrane of vessels in regulation of angiogenesis is well-known and is used for the treatment of cancers²⁴. The origin of basement membrane collagen IV is still unknown, whether from epithelial or mesenchymal cells.

Several studies have indicated the immunohistochemical expression of $\alpha 3$, $\alpha 4$, $\alpha 5$ and $\alpha 6$ chains of collagen IV in normal tissues that are expressed based on the epithelial type of collagen IV.^{25,26,27} Many studies have shown that α chains of collagen IV are destroyed at the initial stages of invasion in many cancers^{27,29,30,31,32}.

The initial stages of invasion and metastasis of cancer cells occur as a result of destruction of basement membrane, which in turn happens through the effect of matrix metalloproteinases that are produced from cancer cells and reduction in inhibitors of matrix metalloproteinases. Apart from the aforementioned mechanism, some studies have shown that hypermethylation of collagen chains in COL4A6 and COL4A5 can account for the lost expression of $\alpha 5$ (IV)/ $\alpha 6$ (IV) chains and remodeling of basement membrane during the invasion of cancer cells³³.

Modification of basement membrane collagen IV has also been observed in odontogenic neoplastic lesions¹⁵. In the current study, the expression of collagen IV was not reported to be different between keratocystic odontogenic tumors and dentigerous cysts, but the staining pattern and collagen thickness were different in these lesions. Lack of difference between collagen IV in odontogenic tumors and cysts can be associated with hypermethylation of collagen chains. Since reduced expression of $\alpha 5$ (IV)/ $\alpha 6$ (IV) chains have been seen in many cancers, further studies are required to analyze odontogenic lesions in terms of hypermethylation and modification of basement membrane.

In normal and benign mucosa, collagen IV is expressed as a continuous and thick layer

with high staining capacity³⁴⁻³⁵. However, the results of studies by Gomes Henriques and Oliveira indicated continuous and thick expression of collagen IV in odontogenic lesions, and radicular and dentigerous cysts, and discontinuous and thin expression of collagen IV in keratocystic odontogenic tumors^{4,19}. The results for the staining pattern of collagen IV in dentigerous cysts and keratocystic odontogenic tumors in the present study were in line with those of the studies by Henriques, except that in the current study the staining intensity in dentigerous cysts and keratocystic odontogenic tumors was similar (both >50%-50%), with no significant difference between them.

Gomes Henriques *et al* conducted a study in 2011 and reported that the staining level in collagen IV was basically >50% in dentigerous cysts and <50% in keratocystic odontogenic tumors. They also showed that the staining pattern of collagen IV was continuous and thick in dentigerous cysts and discontinuous and thin in keratocystic odontogenic tumors¹⁹.

In the present study, the expression of collagen IV was >50% in 78.9% of keratocystic odontogenic tumors and 81.8% of dentigerous cysts. In most keratocystic odontogenic tumors, collagen IV showed a discontinuous staining pattern and was observed as a thin layer; whereas in most dentigerous cysts, collagen IV was observed as a continuous and thick layer all over the basement membrane. However, the findings of Gomes are different than our results in terms of the expression level of collagen IV, which can be associated with different immunohistochemical techniques used. The results of Gomes *et al*'s study are in agreement with the findings of the present research with regard to the thickness and consistency of collagen in keratocystic odontogenic tumors and dentigerous cysts.

Unlike the results of studies regarding the correlation of continuity and consistency of basement membrane with aggressive activity of lesions, Poomsawat *et al* reported no significant correlation in the expression levels of components of basement membrane (collagen IV, laminin 1 and 5, and fibronectin) among dentigerous cysts, keratocystic odontogenic cysts and radicular cysts. These results are in contrast with our findings, which can be due to the different immunohisto-

chemical techniques used in their study and ours³⁶.

As for the expression of matrix metalloproteinase-9, most of the studies have shown a significantly higher expression of matrix metalloproteinase-9 in the mesenchyme of keratocystic odontogenic cysts than dentigerous and radicular cysts. This can be a reason for more aggressive behavior and higher potential for development of keratocystic odontogenic tumors than other odontogenic cysts.

Silveira *et al* performed a study in 2007 and showed that the growth and development mechanism of keratocystic odontogenic tumors, and dentigerous and radicular cysts can be influenced by the development and secretion of matrix metalloproteinases⁵.

In addition, the findings of Gomes Henriques indicated that the expression of matrix metalloproteinase-9 in the epithelium of keratocystic odontogenic tumor, dentigerous cyst, radicular cyst and ameloblastoma showed no significant difference, but its expression in the mesenchyme of keratocystic odontogenic tumor and ameloblastoma was significantly higher than dentigerous and radicular cysts¹⁹.

Moreover, the results of De Andrade Santos *et al* showed no significant difference for the expression of matrix metalloproteinase-9 in the epithelium of keratocystic odontogenic tumors, and dentigerous and radicular cysts. However, the expression of matrix metalloproteinase-9 in keratocystic odontogenic epithelial cells was significantly higher than that of dentigerous cyst.³⁷ The findings of Gheisari *et al* showed that expression of matrix metalloproteinase-9 in the stroma of dentigerous cysts was significantly lower than keratocystic odontogenic tumors³⁸.

In the present study, the expression of matrix metalloproteinase-9 in the epithelium of 54.5% of dentigerous cysts and 63.2% of keratocystic odontogenic tumors showed the score 1. The expression of matrix metalloproteinase-9 in the mesenchyme of 72.7% of dentigerous cysts and 63.2% of keratocystic odontogenic tumors showed the score 1, but in 21.2% of keratocystic odontogenic tumors, score 2 was observed, whereas none of the dentigerous cysts showed score 2.

Since matrix metalloproteinase-9 plays a major role in the destruction of collagen IV and

basement membrane, it can be concluded that the increased expression of matrix metalloproteinase-9 in keratocystic odontogenic tumor brings about inconsistency and low thickness of collagen IV in this lesion. Inconsistency and low thickness of collagen IV in the basement membrane of keratocystic odontogenic tumor can result in an interaction in the performance of epithelium-mesenchyme, which can in turn be considered a factor for the increased development, invasive potential and higher recurrence (different biological behaviors) of keratocystic odontogenic tumors than dentigerous cysts.

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