Vol. 112 No. 5 November 2011

Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology

ORAL AND MAXILLOFACIAL PATHOLOGY Editor: Paul C. Edwards

Immunohistochemical expression of matrix metalloproteinases 1, 2, 7, 9, and 26 in the calcifying cystic odontogenic tumor

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Objective. The aim was to evaluate immunoexpression of matrix metalloproteinases (MMPs) 1, 2, 7, 9, and 26 in calcifying cystic odontogenic tumor (CCOT).
Study design. Ten cases of CCOT were assessed by immunohistochemical expression of MMPs 1, 2, 7, 9, and 26 in the parenchyma and stroma. Metalloproteinase immunoexpressions and their distribution pattern were semiquantitatively scored.
Results. MMPs were expressed in the parenchyma and stroma in all cases of CCOT. Regarding the percentage of immunostained parenchymal cells, MMPs 1, 7, and 9 showed score 2 in 100% of cases. For MMP-2, there was a predominance of score 0 (90%), whereas for MMP-26 immunostaining was varied.
Conclusions. The staining of these metalloproteinases, with the exception of MMP-2, suggests their contribution to tumor growth and expansion. The presence of these metalloproteinases in stromal cells reveals the active participation of these cells in the degradation of the extracellular matrix, contributing to the growth of the tumor studied. **(Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2011;112:609-615)**

The calcifying odontogenic cyst was first described by Gorlin in 1962 as a distinct pathologic entity, named a non-neoplastic cystic lesion. However, Praetorius et al. in 1981 proposed a new classification and reviewed the neoplastic potential of this process. In the current 2005 World Health Organization (WHO) classification, the calcifying odontogenic cyst is defined as a benign cystic neoplasm derived from odontogenic epithelium, with the participation of ectomesenchyma that may or may not have hard tissue formation and is renamed calcifying cystic odontogenic tumor (CCOT).[1-4]

The CCOT constitutes 1% of odontogenic lesions and may be intra- or extraosseous. The maxilla and mandible are affected in the same proportion, more commonly in the anterior region.[1,5,6] Histopathologically, it is characterized by the proliferation of ameloblastomatous epithelium consisting of cubic or columnar cells in the basal layer similar to ameloblasts. In the shallower portions, cells are loosely arranged, remnants of the stellate reticulum of the enamel. Ghost cells are evident in varying amount, and some may be calcified. The presence of dysplastic dentin and proliferation of odontogenic epithelium may be observed adjacent to the tissue.[4,5]

Matrix metalloproteinases (MMPs) comprise a family of calcium- and zinc-dependent endopeptidases that are capable of degrading components of extracellular matrix (ECM) and basal layer, participating in physiologic events and pathologic processes and facilitating tumor growth, invasion, and metastasis.[7-9]

To date, 24 types of MMPs have been identified, and their classification is based on the specific substrate that they degrade and their molecular structure. MMPs are

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Received for publication Jun 14, 2010; returned for revision Jun 5, 2011; accepted for publication Jun 13, 2011.
1079-2104/$ - see front matter

doi:10.1016/j.tripleo.2011.06.009

Table I. Applied monoclonal antibodies and stained conditions

Clone	*Specification*	*Source*	*Dilution*	*Incubation time*	*Antigen retrieval*
41-1E5	MMP-1	Calbiochem	1:100	Overnight (18 h)	Citrate pH 6.0 Pascal
17B11	MMP-2	NovoCastra	1:50	60 min	EDTA pH 8.0 Pascal
2C3	MMP-9	Novocastra	1:20	Overnight (18 h)	Citrate pH 6.0 Pascal
Ab-1/ID2	MMP-7	Labvision/Neomarkers	1:250	Overnight (18 h)	Pepsin pH 1.8, oven 37°C, 60 min
AHP756	MMP-26	Serotec	1:250	Overnight (18 h)	Pepsin pH 1.8, oven 37°C, 60 min

divided into -soluble MMPs and membrane-associated MMPs. Among the soluble MMPs are the collagenases (MMP-1, -8, and -13), gelatinases (MMP-2 and -9), stromelysins (MMP-3 and -10), matrilysins (MMP-7 and -26) and a heterogeneous group of MMPs (MMP-12, -19, -20, -21, -23, -27, and -28). MMPs associated with the membrane are represented by the MMPs 14, 15, 16, 17, 24, and 25.[7,10,11]

MMP-1 is a type of collagenase that has the ability to degrade collagen types I, II, III, VII, VIII, and X and other molecules.[12,13] Degradation of fibrillar collagen leads to the formation of molecules that are thermally unstable and form gels that are subsequently degraded by gelatinases, represented by the MMP-2 and -9.[12] MMP-7 and -26, the matrilysins, are involved in cell proliferation, apoptosis, cell invasion, and metastasis.[14]

To better understand the interaction between tumor cells and extracellular matrix in CCOT, the present study aimed to evaluate and compare the immunohistochemical expression of MMPs 1, 2, 7, 9, and 26 in calcifying cystic odontogenic tumors.

MATERIALS AND METHODS

The research was approved by the Ethics Committee of the Federal University of Rio Grande do Norte. Ten cases of calcifying cystic odontogenic tumor were obtained from the files of the Pathology Laboratory of the Department of Oral Pathology, Federal University of Rio Grande do Norte. The diagnosis was confirmed by the authors through the review of slides stained with hematoxylin and eosin, following the WHO classification (2005). Of the 10 cases, 2 were associated with odontoma and 1 showed islands of odontogenic epithelium similar to ameloblastoma.

Immunohistochemical method

The material selected had previously been fixed in 10% formalin and embedded in paraffin; 3 μm thickness that were extended on glass slides containing the adhesive 3-amino-propiltrietoxi-silane (Sigma Chemical Co., St. Louis, MO, USA). Sections were subjected to deparaffinization in xylene through 2 baths, the first being 60°C for 30 minutes and the second at room temperature for 20 minutes. The sections were rehydrated in a sequence of alcohol to water and washed in 2 passages of distilled water for 5 minutes each chromogenic blocking of endogenous peroxidase was done using hydrogen peroxide (10 volumes). Subsequently, the sections were washed in water twice and immersed in a buffered solution of Tris (hydroxymethyl) aminomethane (Tris-HCl), pH 7.4, for 5 minutes each. The incubation of sections was performed with antibodies diluted in buffered Tris-HCl solution (Table I) with streptavidin-biotin complex (LSAB + System-HRP; Dakocytomation, Glostrup, Denmark) for 30 minutes at room temperature. Peroxidase activity was visualized by immersing tissue sections in diaminobenzidine (D5637; Sigma Chemical, St. Louis, MO), resulting in a brown reaction product. For counterstaining, Mayer hematoxylin was used for 10 minutes, washing with water after each step. To finish the process, dehydration in alcohol and clearing in xylene were applied and the coverslip mounted with Erv-mount.

Evaluation of immunohistochemical expression

The immunohistochemical analysis, verified by 4 examiners at different times was performed to identify presence or absence of immunohistochemical expression of MMPs 1, 2, 7, 9, and 26 and their distribution pattern (focal and diffuse). Semiquantitative analysis of immunostained cells was performed by using parenchymal scores (adapted from Nagel et al.[15]): 0 (<10% of tumor cells positive), 1 (11%-50% of tumor cells positive), and 2 (>50% of tumor cells positive). The stroma was evaluated for the presence or absence of immunoreactivity. After obtaining the data, a descriptive analysis of the results was performed.

RESULTS

MMPs 1, 2, 7, 9, and 26 were shown to be expressed in variable amounts in both the parenchyma and the stroma in all cases of CCOT with predominance of MMPs 1, 7, and 9. The neoplastic cells exhibited cytoplasmic immunoreactivity. Ghost cells, sometimes calcified, also exhibited immunopositivity for the MMPs studied.

Regarding the percentage of parenchymal cells immunostained, MMPs 1, 7, and 9 were scored as 2 in 100% of cases (Figs. 1-3). For MMP-2, there was a predominance of score 0 (90%), whereas MMP-26 immunostaining was varied (Table II; Figs. 4 and 5).

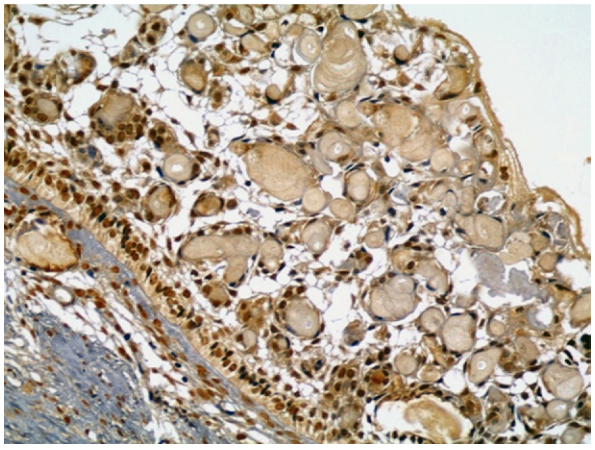

Fig. 1. Immunoexpression of matrix metalloproteinase 1 in calcifying cystic odontogenic tumor demonstrating cytoplasmic reactivity of neoplastic cells and ghost cells (×400).

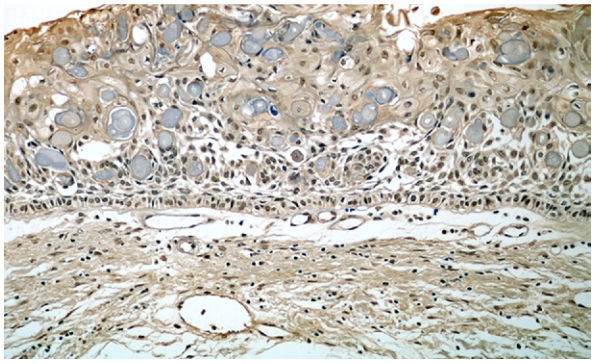

Fig. 2. Immunohistochemical staining for matrix metalloproteinase 7 in calcifying cystic odontogenic tumor (×200).

Considering the stroma, 100% of cases were positive for MMPs 1 (Fig. 6), 7, 9, and 26, whereas MMP −2 was expressed weakly in 80% of cases. It is noteworthy that there was an even staining pattern of these MMPs in the ghost cells that are part of the tumor parenchyma.

In analyzing the distribution pattern, a predominance of diffuse pattern for MMPs 1 (100%), 7 (100%), 9 (90%), and 26 (100%) was observed, while for MMP-2 only 60% of cases exhibited this pattern.

DISCUSSION

Since the first description of calcifying odontogenic cyst by Gorlin in 1962, different classifications have been proposed in an attempt to define the nature of this pathology. In the WHO classification of 1971, it was regarded to be a cystic lesion. In 1992, WHO defined it as a neoplasm, classified as an odontogenic tumor. According to this classification, all calcifying odontogenic cysts had a neoplastic nature. However, other proposed classifications are based on the dualistic concept of the existence of 2 separate entities, one cystic and the other neoplastic.[16-19] In 2005, WHO classified the calcifying odontogenic cyst it as a benign cystic neoplasm.[1]

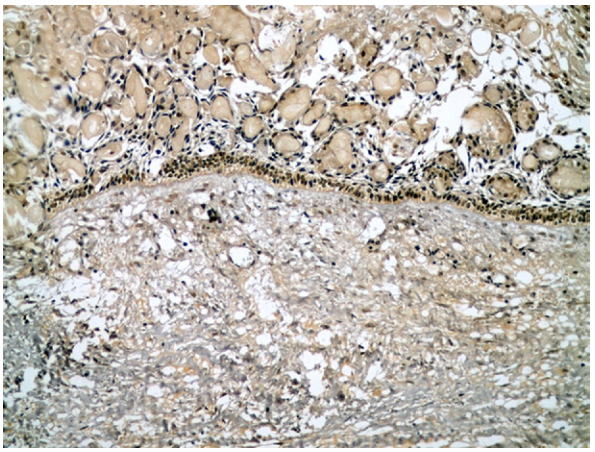

Fig. 3. Matrix metalloproteinase 9 immunoexpression in calcifying cystic odontogenic tumor (×200).

Table II. Immunoreactive score for MMPs 1, 2, 7, 9, and 26 in calcifying cystic odontogenic tumor, n (%)

	MMP-1	*MMP-2*	*MMP-7*	*MMP-9*	*MMP-26*
Score 2	10 (100%)	0 (0%)	10 (100%)	10 (100%)	4 (40%)
Score 1	0 (0%)	1 (10%)	0 (0%)	0 (0%)	2 (20%)
Score 0	0 (0%)	9 (90%)	0 (0%)	0 (0%)	4 (40%)
Total	10 (100%)	10 (100%)	10 (100%)	10 (100%)	10 (100%)

The participation of metalloproteinases in the progression of odontogenic lesions has been shown in various studies.[20-28] These proteases have the ability to modulate the ECM, modifying the structural and functional components. Several MMPs are present in the formation of dental tissues and may play an important role in the biomineralization of dentin and enamel,[29,30] but with low expression under physiological conditions. On the other hand, in pathologic processes, there is an overexpression of these proteins, due to the imbalance between the activity and their inhibitors.[7,31,32]

Considering the calcifying cystic odontogenic tumor, few studies have been conducted to evaluate the expression of metalloproteinases in these lesions. In the present work, in general, MMPs were expressed in both parenchymal and stromal cells but a immunoreactivity for MMPs 1, 7, and 9 was observed, which reinforces the idea of the involvement of stroma cells in the degradation of matrix components.

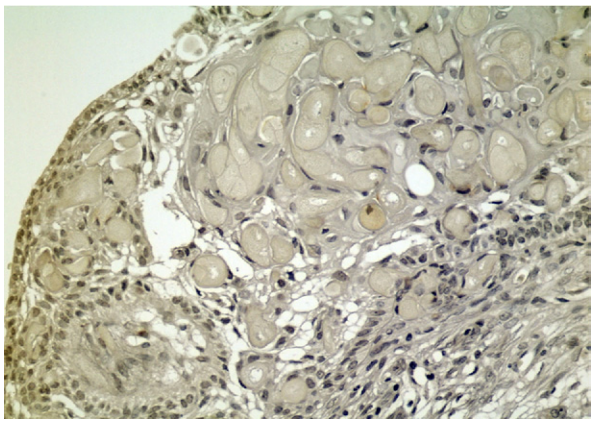

Fig. 4. Immunoexpression of matrix metalloproteinase 2 in a few tumor cells of calcifying cystic odontogenic tumor (×200).

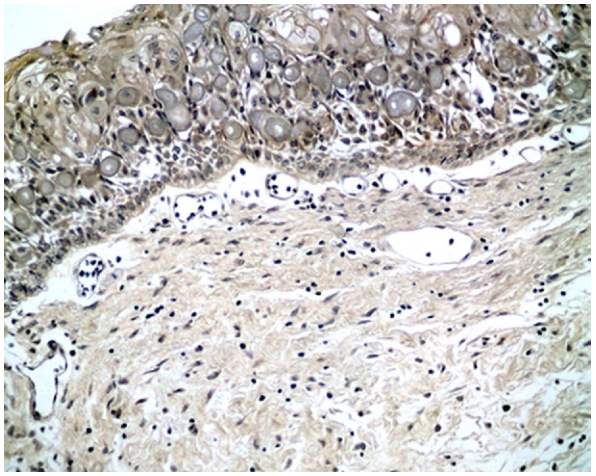

Fig. 5. Matrix metalloproteinase 26 immunoexpression in calcifying cystic odontogenic tumor (×400).

There are several substrates of MMPs 1, 2, 7, 9, and 26. MMP-1 degrades mainly collagens I, II, and III. Gelatinases (MMPs 2 and 9) degrade mainly denatured collagen (gelatin) and collagen type IV, and the matrilysins MMP-7 and -26 digest various components of the matrix, which include fibronectin and collagen type IV.[31]

Score 2 was observed in 100% of cases for MMPs 1, 7, and 9. The positivity displayed by MMP-1 demonstrates the importance of this protease for the degradation of ECM constituents, mainly collagen I, promoting tumor growth and expansion. Similar results in relation to the expression of MMP-1 have been demonstrated in other studies of odontogenic tumors, such as ameloblastoma,[22,24,27] odontogenic tumor keratocystic,[25] myxoma,[33] and adenomatoid odontogenic tumor.[27]

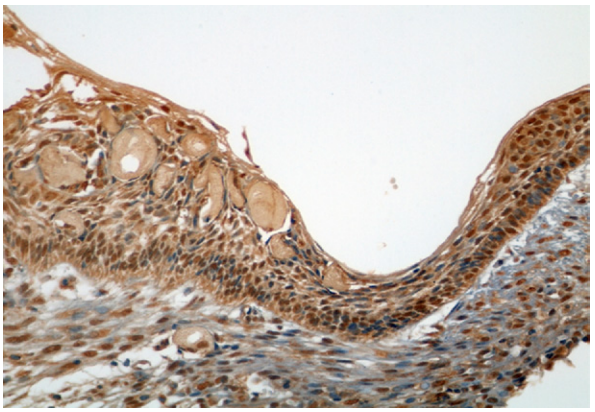

Fig. 6. Matrix metalloproteinase 1 immunopositivity in parenchymal and stroma cells of calcifying cystic odontogenic tumor (×400).

Amorim et al. (2004)[34] analyzed the immunohistochemical expression of tenascin, fibronectin, and collagen IV in syndromic (SKOTs) and nonsyndromic (NSKOTs) keratocystic odontogenic tumors and observed that there were differences in the expression of these proteins between the lesions. Tenascin was present along the basal membrane in all cases of SKOT, whereas in 5 cases of NSKOT this protein was negative in certain areas. The distribution of tenascin was focal on the SKOT wall and diffuse in NSKOT. Fibronectin was detected with a discontinuous band in SKOT and discontinuous in NSKOT. Collagen IV was not present in most cases of SKOT.

MMPs 2 and 9 are gelatinases, their main difference being that MMP-2 can degrade collagen type I,[35,36] both are involved in angiogenesis and in tumor growth.[28]

Vincent et al. (2005)[37] argue that these gelatinases are important in the process of tumor invasion because of the ability to degrade collagen type IV, the main constituent of the basal membrane, which is the first barrier to be breached in the process. Gong et al. (2009)[38] evaluated the immunohistochemical expression of MMP-9 in CCOT and concluded that the positivity of this enzyme in the stroma is associated with the ability to promote tumor invasion. Our results demonstrate focal immunostaining for MMP-2, whereas for MMP-9 a score of 2 was observed in 100% of the cases and a diffuse distribution pattern in parenchymal cells, corroborating the studies of Ribeiro et al. (2009)[27] that, using the same pattern of immunostaining for these MMPs in ameloblastomas and adenomatoid odontogenic tumor, found a prevalence of 0 scores for MMP-2 compared with marked expression of MMP-9. The same was found by Kumamoto et al. (2003)[22] and Pinheiro et al. (2004)[24] in studies with ameloblastomas and by Silveira et al. (2007)[28] with odontogenic cysts.

MMP-2 degrades mainly collagen IV, the main constituent of the basement membrane (BM) and other ECM components. We believe that the low expression of MMP is due to the need to maintain a minimum of BM constituents, which are crucial in the process of cell differentiation.

Silveira et al. (2007)[28] evaluated the role of MMPs 1, 2, and 9 in radicular cysts (RCs), residual radicular cysts (RRCs) and keratocystic odontogenic tumors (KOTs). The expression of MMP-1 was predominantly diffuse in the parenchyma of these lesions. Immunoexpression of MMP-2 ranged from focal (RC 60% and KOT 100%) to diffuse (RRC 60%), and for MMP-9 immunoreactivity was predominantly focal, in contrast to the expression found in CCOT, where in 90% of the parenchyma immunostaining for MMP-2 was absent whereas for MMP-9 the score 2 was predominant. Considering the mesenchyme, there was a higher expression of these MMPs in KOT, as well as in CCOT in our study, where there was 100% staining for MMPs 1 and 9 and absence of staining for MMP-2 was observed in 80%, whereas that MMP was focal in 100% of KOT. Compared with the cystic lesions, it appears that most have not shown staining of MMPs, thus confirming the presence of these MMPs in the mesenchyme participating in the active growth of the lesion.

The etiology of radicular cysts has been investigated as correlated with MMPs. Soares et al. (2007)[39] studied the expression of MMPs 1, 2, and 9 in radicular cysts with and without endodontic treatment: In the cystic epithelium a strong expression of MMP-1 was noted regardless of the type of treatment and of MMP-2 and MMP-9 in lesions treated endodontically, but with no statistical difference. Comparing these with the inflammatory markers, there was no direct relationship between the marking of MMP-2 and inflammatory infiltrate, and this was also observed in the work of de Paula-Silva et al. (2009).[40] These data may explain the weak or the absence of marking of MMP-2 in CCOT, which is a neoplastic lesion, and in those cases studied did not observe any reaction of this nature.

Among the various MMPs, the matrilysins, MMP-7 and MMP-26, are involved in diverse processes, such as cell proliferation, apoptosis, invasion, and metastasis. Researchers have demonstrated their expression in malignant epithelial neoplasms[41-43] and KOTs.[25] However, until now, no study has shown expression in calcifying cystic odontogenic tumors.

MMP-7 is synthesized by epithelial cells and has the ability to trigger a cascade of activity of MMPs and degrade a variety of ECM substrates, including elastin, laminin, collagen type IV, and others.[44] MMP-7 also acts on other substrates, such as tumor necrosis factor alpha, myelin basic protein, Fas-ligand, E-cadherin, osteopontin, and tissue growth factor. These substrates can modulate cell behavior,[45] which suggests that matrilysin may have a central role in the process of invasion and tumor metastasis.[46]

MMP-26 is frequently expressed in both normal cells and endometrium, placenta, and kidney, as well as in epithelial neoplasms from various anatomic sites. It shows proteolytic activity on various ECM components, including fibronectin, collagen IV, gelatin, and fibrinogen.[7,47]

Cavalcante et al. (2008)[25] evaluated the expression of MMP-7 and MMP-26 in syndromic and nonsyndromic keratocystic odontogenic tumors, and observed a strong epithelial expression in cases associated with Gorlin syndrome compared to non-syndromic cases, which may explain the more aggressive behavior of syndrome-associated KOTs.

Studies were also performed on the immunohistochemical expression of these matrilysins in ameloblastomas and adenomatoid odontogenic tumors, trying to correlate with distinct tumor biologic behavior of these pathologies. However, Freitas et al. (2009)[26] found no statistically significant differences between the immunostaining of both lesions, but there was a significant staining for MMP-7 and MMP-26 in both the parenchyma and the stroma, suggesting a role in the process of remodeling and growth of these tumors.

In our results, the immunostaining of MMP-7 in the parenchyma scores were 2 in 100% of cases, whereas MMP-26 showed some variability. In the stroma, we observed 100% staining of the matrilysins, thereby demonstrating the involvement of these proteins in the interaction between epithelial cells and stroma in the process of tumor growth and expansion.[9,41] Besides degrading ECM components, MMP-7 and MMP-26 are also able to activate other metalloproteinases, such as MMP-9. MMP-7 activates MMPs 2 and 9.[48,49] MMPs 2 and 9 degrade collagen type IV, and these gelatinases are involved in processes of tumor invasion and metastasis,[50] as referenced above.

The positivity evidenced by metalloproteinases 1, 7, 9, and 26 in stromal cells demonstrates that these enzymes are also produced by fibroblasts, endothelial cells, inflammatory lymphocytes, plasma cells, and neutrophils, which are also involved in the degradation of ECM. Similar results were found in ameloblastomas,[22,24] adenomatoid odontogenic tumors (AOTs),[26,27] and odontogenic cysts.[28]

Ghost cells are necessary prerequisites for the diagnosis of CCOT, though not pathognomonic of these lesions.[19] There is still much controversy about the nature of these cells. Some researchers believe that they represent a normal or atypical keratinization,[51] simple cellular degeneration, or a product of the abortive enamel matrix,[52] or that they derive from apoptotic processes of odontogenic cells and originate from metaplastic transformation of odontogenic tumors.[51,53] In all of the cases studied, the ghost cells had the same staining pattern of MMPs in the pa-

renchyma with predominance of score 2 for MMPs 1, 7, and 9, variability for MMP-26, and weak labeling for MMP-2. Yoshida et al. (2001)[54] analyzed the presence of amelogenin protein in the ghost cells of CCOT, by immunohistochemistry study, and found that in 100% of cases there was positive staining for this protein.

In a study with confocal microscopy of 15 CCOTs, for analysis of the nature of these cells, an accumulation of high-molecular-weight keratin was observed.[51]The research of Kusama et al. (2005)[55] verified the presence of antibodies PA-HP1, PA-HP2, and MA-HP1 in 14 cases of CCOT.

Takata et al. (2000)[52] observed the presence of MMP-20 in some ghost cells of CCOT, and in late stages of odontogenesis within the immature enamel. Soares et al. (2004),[19] analyzing the presence of ECM proteins, found strong immunohistochemical reactivity for fibronectin followed in decreasing order by collagen I and tenascin C.

Watson et al. (1998)[56] demonstrated that the matrix produced by cells that are rapidly mineralizing contained an amount of collagen I and fibronectin 3 times higher than that secreted by clones of cells that were not mineralizing. Therefore, it is suggested that collagen I and fibronectin are critical in the formation of calcified structures, being the predominant components in the matrix produced by the mineralized cells. This evidence suggest that the staining for these components of the ECM in these cells is associated, probably, to the process of calcification of ghost cells, a widely observed phenomenon in CCOT.

CONCLUSION

MMPs 1, 2, 7, 9, and 26 are expressed in parenchymal and stromal cells of CCOTs, with the exception of MMP-2, suggesting their contribution to tumor growth and expansion. The presence of these metalloproteinases in stromal cells reveals the active participation of these cells, along with the parenchyma cells, in the degradation of ECM constituents, contributing to the tumor growth studied here. However, further studies investigating other MMPs as well as using other techniques, such as zymography and molecular biology, should be performed to better understand the role and influence of these enzymes in the behavior of the tumor studied here.

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