

# Kallikrein 4 and matrix metalloproteinase-20 immunoexpression in malignant, benign and infiltrative odontogenic tumors

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## Abstract

**Context:** Matrix metalloproteinase-20 (MMP20) (enamelysin) and kallikrein 4 (KLK4) are enzymes secreted by ameloblasts that play an important role in enamel matrix degradation during amelogenesis. However, studies have shown that neoplastic cells can produce such enzymes, which may affect the tumor infiltrative and metastatic behaviors.

**Aims:** The aim of this study is to assess the biological role of MMP20 and KLK4 in odontogenic tumors.

**Materials and Methods:** The enzymes were analyzed immunohistochemically in ameloblastoma, adenomatoid odontogenic tumor (AOT), calcifying epithelial odontogenic tumor, keratocystic odontogenic tumor with or without recurrence and odontogenic carcinoma.

**Statistical Analysis Used:** Clinicopathological parameters were statistically correlated with protein expression using the Fisher's exact test. Kruskal–Wallis and Wilcoxon-independent methods were used to evaluate the differences in median values.

**Results:** Positive Immunoeexpression was detected in all benign lesions, with a prevalence of 75–100% immunolabeled cells. Patients were predominantly young, Caucasian, female, with slow-growing tumors located in the mandible causing asymptomatic swelling. No KLK4 expression was seen in carcinomas, and the amount of MMP20-positive cells varied between 20% and 80%. Rapid evolution, recurrence and age >60 years characterized the malignant nature of these lesions.

**Conclusions:** Data showed that KLK4 and MMP20 enzymes may not be crucial to tumoral infiltrative capacity, especially in malignant tumors, considering the diversity and peculiarity of these lesions. The significant immunoeexpression in benign lesions, remarkably in AOT, is likely associated with differentiated tumor cells that can produce and degrade enamel matrix-like substances. This would be expected since the histogenesis of odontogenic tumors commonly comes from epithelium that recently performed a secretory activity in tooth formation.

**Key Words:** KLK 4, MMP 20, odontogenic tumors

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## INTRODUCTION

Matrix metalloproteinase 20 (MMP20) and kallikrein 4 (KLK4) are enzymes secreted by ameloblasts that play an important role in enamel matrix degradation during amelogenesis. However, studies have shown that neoplastic cells are also able to produce such enzymes, which may affect the tumor infiltrative and metastatic behaviors.<sup>[1-6]</sup>

The replacement of enamel matrix proteins by hydroxyapatite crystals depends on the enzymatic activity of MMP20 (enamelysin), which plays an important role in the degradation of the newly deposited matrix. MMP20 is produced by ameloblasts during the secretory stage, and their cleavage products are absorbed and degraded by these same cells.<sup>[7,8]</sup> In odontogenic tumors, immunoeexpression of some MMP family members have been related to typical local infiltration of ameloblastomas (AMs) and Gorlin–Goltz’s keratocystic odontogenic tumors (KOTs)<sup>[2,9-14]</sup> or to the hamartomatous benign nature of adenomatoid odontogenic tumors (AOTs).<sup>[10,13]</sup> To the best of our knowledge, only Takata *et al.*<sup>[15]</sup> assessed MMP20 immunohistochemically in odontogenic tumors. The protein was expressed in the enameloid and amyloid products secreted by the epithelia of some tumors, suggesting tumoral cellular differentiation to secretory ameloblasts.

KLK4, a serine protease family enzyme, performs its biological role during the final stages of amelogenesis, especially in enamel maturation. It helps in complete decomposition of enamel proteins, allowing crystals to grow and contact each other, giving the final hardness to the enamel.<sup>[7,8]</sup> Prostate, kidney, liver, epithelium and benign tumors express this protease, and its overexpression has been associated with tumor invasion and metastasis in malignant neoplasms.<sup>[3-6,16]</sup>

To the best of our knowledge, no studies have assessed KLK4 protein expression in odontogenic tumors. Thus, the aim of this study was to detect and assess the biological role of MMP20 and KLK4 enzymes in odontogenic tumors.

## MATERIALS AND METHODS

The tumors used were AMs ( $n = 5$ ), AOTs ( $n = 5$ ), calcifying epithelial odontogenic tumors (CEOTs,  $n = 5$ ), KOTs with or without recurrence (KOTr,  $n = 5$  and KOT,  $n = 2$ ) and odontogenic carcinomas (OC,  $n = 5$ , being 1 intraosseous carcinoma, 3 clear cell OCs and 1 ameloblastic carcinoma). Table 1 summarizes the immunohistochemical technique used to assess protein expression. Data were scored by observing the presence of a brown end-product at the site of the target antigen under a light microscope. The scores for immunoeexpression were “0” = 0% positive cells, “1” = 1–25% positive cells, “2” = 26–50% positive cells, “3” = 51–75% positive cells

**Table 1: Monoclonal antibodies used in paraffin sections of formalin-fixed tissues<sup>†</sup>**

Antibody	Clone	Concentration	Incubation time at room temperature (min)
MMP20 <sup>‡</sup>	Orb101641	1:200	120
KLK4 <sup>§</sup>	aa242-254	1:2500	30

<sup>†</sup>Antigen retrieval: citric acid (10 mM, pH 6.0) at 95°C steamer for 30 min; Detection system: REVEAL Polyvalent HRP-DAB, Spring BioScience, Catalog#SPD-060, and DAB, Counterstain: Harris hematoxylin, Positive control: Breast adenocarcinoma and oral squamous carcinoma immunostains, Negative control: Immunostain replacing primary antibody with TRIS buffer pH 7.4. <sup>‡</sup>Rabbit polyclonal antibody, Unconjugated from Biorbyt Ltd., <sup>§</sup>Rabbit polyclonal antibody, Unconjugated from LifeSpan BioSciences. DAB: 3,3-diaminobenzidine

and “4” = 75–100% positive cells, considering that 500 tumor cells were randomly counted.

Clinicopathological parameters were statistically correlated with protein expression using the Fisher’s exact test. Kruskal–Wallis and Wilcoxon-independent methods evaluated the differences in median values. Results were considered statistically significant if  $P < 0.05$ .

## RESULTS

Table 2 shows the number ( $n$ ) of tumors assessed and their respective MMP20/KLK4 immunoeexpression scores. Scores 3 and 4 were prevalent in all benign lesions [Figure 1a–e]. Overall, the immunolabeling pattern for both antibodies was similar. Positive AM cells were scattered or grouped in the center and periphery of tumor islands [Figure 1a]. More than 90% of AOT epithelial cells were positive, especially in solid nodules forming sheets, ductal-like and adenomatoid structures. Fusiform and stellate cells interspersed among solid nodules were sometimes immunonegative (data not shown). Positive CEOT cells were dispersed and did not correlate with any histopathological feature [Figure 1c and d]. In both KOTs and KOTr, all cystic epithelium cells were immunopositive, except for the parakeratin layer [Figure 1e]. Regarding the carcinomas, scores ranged between 1 and 2, with positive cells randomly dispersed in the tumor islands. One carcinoma was negative for both antibodies [Figure 1g] and one was positive in squamous cells only [Figure 1h].

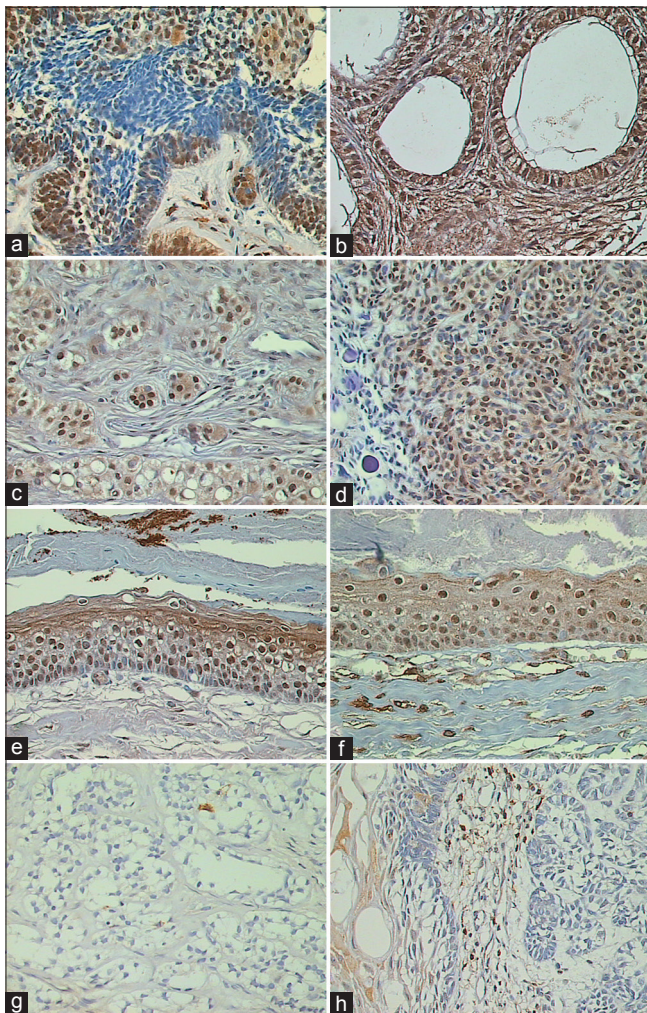
All tumors expressed MMP20 and KLK4 enzymes in inflammatory cells, endothelium, smooth muscle cells, remnants of odontogenic epithelium and keratinocytes. Few fibroblasts showed immunoeexpression, especially those among neoplastic tissues. Nevertheless, all KOT and KOTr cystic capsule fibroblasts were KLK4 positive [Figure 1f].

Patients with benign tumors were predominantly young, Caucasian, female, with slow-growing tumors located in the mandible causing asymptomatic swelling. Conversely, rapid evolution, recurrence and age >60 years were associated with malignant lesions [Tables 3 and 4].

**Table 2: Kallikrein 4 and matrix metalloproteinase 20 immunoections in ameloblastoma, adenomatoid odontogenic tumor, calcifying epithelial odontogenic tumor, keratocystic odontogenic tumor, recurrent keratocystic odontogenic tumor, odontogenic carcinoma†**

Odontogenic tumors	KLK4 epithelial cells scores - n (%)‡					MMP20 epithelial cells scores - n (%)‡				
	0	1	2	3	4	0	1	2	3	4
Am (n=5)	-	-	-	1 (20)	4 (80)	-	-	4 (80)	-	1 (20)
AOT (n=5)	-	-	-	1 (20)	4 (80)	-	-	-	1 (20)	4 (80)
CEOT (n=5)	-	-	1 (20)	2 (40)	2 (40)	-	-	-	1 (20)	4 (80)
KOT (n=5)	-	-	-	1 (20)	4 (80)	-	-	-	1 (20)	4 (80)
KOTr (n=2)	-	-	-	-	2 (100)	-	-	-	2 (100)	-
OC (n=5)	1 (20)	2 (40)	1 (20)	1 (20)	-	1 (20)	1 (20)	2 (40)	-	1 (20)

†All tumors showed epithelial cells positive to KLK4 and MMP20 immunostaining, ‡Scores - 0: 0% positive cells, 1: 1-25% positive cells, 2: 26-50% positive cells, 3: 51-75% positive cells, 4: >75% positive cells. AM: Ameloblastoma, AOT: Adenomatoid odontogenic tumor, CEOT: Calcifying epithelial odontogenic tumor, KOT: Keratocystic odontogenic tumor, KOTr: Recurrent keratocystic odontogenic tumor, OC: Odontogenic carcinoma, KLK4: Kallikrein 4, MMP20: Matrix metalloproteinase 20



**Figure 1:** Kallikrein 4 (KLK 4) and matrix metalloproteinase 20 (MMP 20) immunoections in odontogenic tumors. (a) Ameloblastoma: KLK4 expression, score 2 (IHC stain, ×200). (b) Adenomatoid odontogenic tumor: KLK4 expression, score 4 (IHC stain, ×200). (c) Calcifying epithelial odontogenic tumor: MMP 20 expression, score 4 (IHC stain, ×200). (d) Calcifying epithelial odontogenic tumor: KLK4 expression, score 4 (IHC stain, ×200). (e) Keratocystic odontogenic tumor: KLK4 expression, score 4 (IHC stain, ×100). (f) Keratocystic odontogenic tumor: Capsular fibroblasts positive to KLK 4 (IHC stain, ×200). (g) Odontogenic carcinoma: Negative to MMP 20 (IHC stain, ×200). (h) Odontogenic carcinoma: Malignant “squamous metaplastic” cells were KLK4-positive (left) (IHC stain, ×200)

**Table 3: Association between kallikrein 4 and matrix metalloproteinase 20 immunoection scores and clinico-radiographic characteristics: age, gender, race, pain and location**

Characteristics	KLK4 epithelial cells scores - n†					P‡	MMP20 epithelial cells scores - n†					P‡
	0	1	2	3	4		0	1	2	3	4	
Age (years)												
0-9	-	-	-	1	-	-	-	-	-	1	-	-
10-19	-	2	-	1	7	-	-	-	2	1	7	-
20-29	1	-	1	-	-	-	1	-	-	1	-	-
30-39	-	-	-	2	2	-	-	-	-	1	3	-
40-49	-	-	-	1	-	-	-	-	1	-	-	-
50-59	-	-	-	-	1	-	-	-	-	-	1	-
>60	-	1	2	-	2	-	-	1	2	2	-	-
NA	-	2	-	-	1	-	-	-	1	-	2	-
Gender												
Male	1	1	-	2	5	-	1	1	2	1	6	-
Female	-	4	3	3	8	-	-	-	4	3	9	-
Race												
Caucasian	1	3	2	4	10	0.0318	1	1	3	4	11	0.0049
Black	-	1	-	1	3	0.0083	-	-	2	-	3	0.0035
Japanese	-	-	1	-	-	0.0246	-	-	1	-	-	0.000
NA	-	1	-	-	-	-	-	-	-	-	1	-
Pain												
Yes	1	-	-	2	1	-	1	-	1	1	1	-
No	-	2	1	-	5	-	-	-	3	3	3	-
NA	-	5	2	3	5	-	-	1	2	-	11	-
Location												
Maxilla	-	-	1	2	3	-	-	-	-	-	6	-
Mandible	1	5	2	3	9	-	1	1	6	4	8	-
NA	-	-	-	-	1	-	-	-	-	-	1	-

†Scores - 1: 1-25% positive cells, 2: 26-50% positive cells, 3: 51-75% positive cells, 4: >76% positive cells. ‡Significant at P<0.05 (Chi-square test). NA: Not available, KLK4: Kallikrein 4, MMP20: Matrix metalloproteinase 20

Statistical tests were performed using GraphPad Prism software version 5.0 (GraphPad Software, Inc. La Jolla, CA 92037, USA). Regarding the correlation between immunohistochemical and clinico-radiographic data for both immunostainings, score 4 was prevalent in benign lesions and varied in carcinomas. A significant difference was detected between MMP20 expression, race and radiography, as well as lesion size (Fisher’s exact test, P<0.05). The comparison of means between groups showed statistical significance for localization and lesion size in MMP20 positive lesions (Kruskal–Wallis test, P<0.05, Table 5).

**DISCUSSION**

To discuss the pathogenesis and biological behavior of odontogenic tumors, several biomarkers have been immunohistochemically studied, including the MMP family of enzymes, especially MMPs-1, -2, -7, -9, -14, -20 and -26, as well as some proteins correlated with cell division or signaling pathways. Previous studies focusing on AMs have agreed with the typical aggressive and infiltrative tumoral properties.<sup>[9,10,12-14,17-19]</sup> This growth and infiltration potential would also be demonstrated by the positivity of AM stromal cells, and myxoma cells to MMPs-1, -2 and -9.<sup>[20,21]</sup> Our results showed the presence of MMP20 and KLK4 in these tumors, also indicating a probable participation in its aggressive potential. Only a part of the neoplastic epithelial cell population was labeled, with some areas being completely immunonegative. There was no preference for labeled cell types, i.e., for peripheral or central island cells. In contrast, one of the tumors showed over 75% immunopositive cells.

The prevalence of the low immunolabeling percentage only in the epithelium suggested that only epithelial compartments would be infiltrative, whereas other populations would have a less invasive behavior. However, the production of enzymes may be variable between tumors, with higher enzyme production being associated with more aggressive AM lesions.

The literature has agreed with the hamartomatous nature of the AOTs, whose cells would be in active proliferation.<sup>[10,13]</sup> We found high immunoexpression of both MMP20 and KLK4 in the tumor tissue. The only unlabeled elements were fusiform and stellate cells scattered in woven structures. Since AOTs are typically benign, slow-growing and never permeate adjacent tissues, our results initially contradict the facilitating role of tumor invasion due to enzymatic activity. High immunoexpression of MMP20 and KLK4 enzymes probably results from the fact that AOT cells are analogous to those of the reduced enamel epithelium, which likely drives histogenesis. The reduced enamel epithelium is adherent to the recently

**Table 4: Association between kallikrein 4 and matrix metalloproteinase 20 immunoexpression scores<sup>‡</sup> and clinico-radiographic characteristics: facial asymmetry, radiography, lesion size, time evolution, recurrence**

Characteristics	KLK4 epithelial cells scores - n <sup>†</sup>						MMP20 epithelial cells scores - n <sup>†</sup>					
	0	1	2	3	4	P <sup>‡</sup>	0	1	2	3	4	P <sup>‡</sup>
Facial asymmetry	-	-	-	-	-	-	-	-	-	-	-	-
Yes	-	1	2	1	4	-	-	1	2	2	3	-
No	-	-	-	-	1	-	-	-	-	-	-	-
NA	1	4	1	4	8	-	1	-	4	2	12	-
Radiography												
Peripheral	-	-	-	-	-	-	-	-	-	-	-	-
Intra-osseous radiolucent	1	5	5	2	11	0.0028*	1	-1	5	4	10	0.0135*
Intra-osseous radiopaque	-	-	-	-	-	-	-	-	-	-	-	-
Intra-osseous radiolucent/radiopaque	-	-	-	-	1	-	-	-	-	-	1	-
NA	-	-	-	1	1	-	-	-	1	-	4	-
Lesion size (mm)												
0-10	-	-	-	1	-	-	-	-	-	-	2	0.0263*
11-20	-	-	1	1	5	-	-	-	-	1	5	0.0380*
21-30	-	-	-	-	4	-	-	-	-	-	4	0.0263*
31-40	-	-	-	-	-	-	-	-	-	-	-	-
>40	1	1	1	2	2	-	2	1	1	3	-	-
NA	-	4	1	1	2	-	-	-	4	-	4	-
Time evolution (months)												
1-3	-	-	-	-	-	-	-	-	-	-	-	-
4-6	-	-	1	-	1	-	-	-1	-	-	1	-
7-9	-	-	-	1	-	-	-	-	1	-	-	-
10-12	1	-	-	1	-	-	1	-	-	1	2	-
>12	-	2	1	-	1	-	-	-	1	1	-3	-
NA	-	3	1	3	11	-	-	-	-4	-1	10	-
Recurrence												
Yes	-	1	-	-	3	0.6554	-	-	-	2	2	-
No	-	-	-	-	-	-	-	-	-	-	-	-
NA	1	4	3	5	10	-	1	1	6	2	13	-

<sup>†</sup>Scores - 1: 1-25% positive cells, 2: 26-50% positive cells, 3: 51-75% positive cells, 4: >76% positive cells, <sup>‡</sup>Significant at P<0.05 (Chi-square test). NA: Not available, KLK4: Kallikrein 4, MMP20: Matrix metalloproteinase 20

**Table 5: Difference between maxilla and mandible lesions in matrix metalloproteinase 20 immunoexpression**

Variable	Groups	n	q	Effect	Upper limit	P <sup>†</sup>
Localization	Maxilla	6	3.113209417	4	4.886790583	0.0451
	Mandible	20	2.4307524	2.904761905	3.37877141	
	Not available	1	1.827815563	4	6.172184437	

<sup>†</sup>Significant at P<0.05 (Chi-square test)

formed enamel of an unerupted tooth, composing the dental follicle. Reduced enamel epithelial cells play a secretory role in enamel formation, exactly in the phase in which production of MMP20 and KLK4 enzymes for prism formation and maturation occurs. Therefore, AOT cells would come from postsecretory ameloblast lineage cells, and they would also produce these enzymes, besides the residual capacity to produce enamel matrix. This characteristic would be represented by dark eosinophil accumulations and concentrations of typical mineralization seen in histopathology, which produces the nearly pathognomonic radiographic image of “snowflakes.”

The CEOTs typically produce amyloid-type material, considered at times abortive enamel matrix, besides mineralized structures that can predominate in the lesion. These tumors showed a high percentage of MMP20 and KLK4 immunolabeled cells, scattered around the tissue. We believe that this result also correlates with an ameloblastic profile of cells that would be able to produce and degrade enameloid matrix, with the participation of these enzymes. Since CEOTs are not rarely presented as clinically aggressive, their nature would probably be benign neoplastic, containing cells with lower differentiation and higher infiltration power compared with AOTs.

MMPs-1, -7 and -26 have been demonstrated in KOTs, being more expressed in lesions associated with the Gorlin–Goltz’s syndrome as a consequence of higher aggressiveness.<sup>[11]</sup> At present, KOT is considered a benign cystic neoplasia, and not a real cyst, as previously denominated keratocyst. In our results, both KOT and KOTr presented the same immunohistochemical profile of the evaluated enzymes, showing no difference between primary or recurrent lesions. The significant results corroborated the infiltrative behavior; however, with distinction for KLK4 expression in stromal cells near and distant from the cystic epithelium. In other odontogenic tumors, this stromal immunolabeling selectively included inflammatory, endothelial and smooth muscle cells, eventual fibroblasts, besides keratinocytes and odontogenic epithelium remnants. In the capsular region, adjacent to the KOT and KOTr epithelium, all stromal cells were immunolabeled; whereas in distant areas, the selective positivity pattern of other tumors was followed. This data reinforced the opinion that the stroma of KOTs and KOTrs would play an important infiltrative role, and not only is the epithelium involved. We could, therefore, consider KOTs as mixed tumors composed of epithelium and active odontogenic ectomesenchyme. As for the positive epithelial cystic cells, they could collaborate with cyst infiltration, or only be a reflex of the squamous cell nature.

Mutations of p16, p53 under-expression and immunoexpression of Ki67 have been suggested as important events in malignant transformation of odontogenic tumors,<sup>[22,23]</sup> besides

abnormalities in the WNT5A signaling pathway<sup>[24]</sup> and altered expression of Msx and Dlx homeoproteins.<sup>[25]</sup> In our results, the immunopositivity of MMP20 and KLK4 enzymes in malignant cells varied widely, being one case immunonegative and another case KLK4-positive only in squamous cells similar to keratinocytes in the basal layer. The lack of immunolabeling consistency suggested a less important role of the enzymes in the infiltrative ability of malignant odontogenic tumors, or perhaps no participation in this process, depending on the case.

There was no statistically significant correlation between immunoexpression of KLK4/MMP20 and clinical and radiographic data. In benign lesions, the clinical profile of patients was young, female, Caucasians/blacks with slow-growing mandibular tumors, and significant prevalence of radiolucent intraosseous lesions. MMP20 reached significantly higher scores in mandibular lesions measuring up to 30 mm. In carcinomas, the characteristics were typical of malignancy, however with a wide cell positivity and variation, not establishing an obvious correlation.

## CONCLUSION

Considering the diversity and peculiarity of the tumors assessed herein, we suggest that MMP20 and KLK4 enzymes may not play a significant role in tumor infiltration and malignancy. Expression of these enzymes may reflect the functionality of cells in producing and degrading enameloid material or in cell differentiation. AOT, CEOTs and KOTs and KOTrs showed MMP20 and KLK4 enzymes immunoexpression in a high percentage of tumor cells. All mesenchymal cells of the KOT and KOTr capsule were KLK4-positive, suggesting a cooperative role of KLK4 in their infiltrative ability. As for the correlation between the immunohistochemical findings and clinical and radiographic data, there was a significant association between Caucasian patients and radiographic radiolucent intraosseous pattern.

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## Conflicts of interest

There are no conflicts of interest.

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