# Expression of matrix metalloproteinases in human amelo-blastoma

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**Purpose**: To investigate the expression of matrix metalloproteinase-2 (MMP-2), matrix metalloproteinase-9 (MMP-9) and tissue inhibitor of metalloproteinase-1 (TIMP-1) in human ameloblastoma (AB), and to explore the relationship of the three factors with AB's biological behavior in combination with clinicopathological characteristics.
**Materials and Methods**: Specimens of 43 cases of AB, 10 cases of odontogenic keratocyst (OKC), and 16 cases of normal oral mucosa were examined immunohistochemically using the streptavidin-biotin method to determine the expression of MMP-2, MMP-9 and TIMP-1.
**Results**: Of the 16 cases of normal oral epithelia, MMP-2 was positively expressed in six cases. In the 10 OKC cases, MMP-2 expression was strongly positive in stratum spinosum in two cases and weak or lost in stratum basale. MMP-2 was strongly expressed in the central and peripheral cells of the tumor islands in 28 cases of AB. There was a significant difference among these three groups ($p<0.01$). MMP-9 expression was negative in all cases of normal oral mucosa, and extensively positive in the entire strata of peritumor epithelia in five cases of AB. MMP-9 was strongly expressed in two of nine cases of OKC. MMP-9 was strongly expressed in the central and peripheral cells of the tumor islands in 30 cases of AB. There was no difference between normal epithelia and OKC ($p>0.05$). There was a great difference between AB and normal oral mucosa ($p<0.01$). The positive rate and intensity increased as AB recurred and transformed malignantly, but the increases were not associated with age, sex, pathological type, or disease location. MMP-2 and MMP-9 were positively expressed in fibroblasts, endothelial cells, and inflammatory cells including monocytes, neutrophils, plasmacytes and lymphocytes. TIMP-1 was weakly or not expressed in normal oral mucosa, OKC and AB.
**Conclusion**: The high expression of MMP-2 and MMP-9 is related to the biological behavior of AB. Imbalance in the expression of MMP-2 and MMP-9 proteins is one mechanism for the invasiveness of AB. The MMPs activation produced by stromal cells is also related to the invasiveness of AB.
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**Clinical Significance**: The study provided that activity of MMP-2 and MMP-9 was closely related to the clinico-biological behavior of AB, which provided a new target to therapy for AB by gene.
**Key Words**: ameloblastoma, immunohistochemistry, matrix metalloproteinase, odontogenic keratocyst.

## Introduction

Ameloblastoma (AB) and odontogenic keratocyst (OKC) of oral jaw are diseases that have a negative impact on families and society in general. Currently, the most common treatment option for these diseases is surgery, which does not improve quality of life and causes great mental anguish to, and places an economic burden on, the patients. Furthermore, the prominent biological features of AB are aggressive growth and have the possibility that cells will transform malignantly and metastasize with a relatively high postoperative recurrence rate. However, the pathogenesis of AB and mechanisms for its aggressive growth have not been fully clarified. Previous studies have demonstrated that the development and progression of numerous carcinomas are not only associated with epithelial adhesive molecules,[1,2] but are also related to the degradation and synthesis imbalance of extracellular matrix (ECM). Generally speaking, six types of enzyme are involved in ECM degradation by tumor cells, including the important matrix metalloproteinase

(MMP). In this study, we measured MMP-2, MMP-9 and TIMP-1 expressions in AB immuno-histochemically by the standard streptavidin-biotin method, with the aim of exploring the relationship of the three factors to the biological behavior of AB.

## Materials and Methods

### Tissue Samples

All specimens were selected from archival paraffin blocks collected between 1987 and 2001 at the Department of Pathology, College of Stomatology, China Medical University. Forty-three cases of AB were studied, consisting of 16 primary, 21 recurrent and six malignant ABs. There were 19 males and 24 females, with a mean age of 36.5 years (range 11-65). Furthermore, mean ages in the primary, recurrent and malignant AB cases were 31.8, 40.7 and 43.3 years, respectively. As for the disease sites, four cases of AB were at the gum, three at the left maxillary bone, four at the right maxillary bone, 18 at the left inferior maxilla, and 14 at the right inferior maxilla. For pathological patterns, the AB cases included 21 follicle-type, seven cluster-type, four granular cell, three monocyst, and eight solid ABs. Complete clinical records and follow-up data were available for all patients. The recurrent interval in the recurrent cases ranged from two to 19 months with one to six relapses. The majority of relapses were connected with surgical scaling and four cases relapsed after ilium transplantation. Of the malignant cases, two involved pulmonary metastases and one had regional lymphatic involvement. Ten cases of OKC and 16 cases of normal oral mucosa (at gum or cheeks) were picked as controls. All specimens were reviewed and diagnosed by two oral pathologists.

### Reagents

Monoclonal antibodies against MMP-2 (MAB-0244), MMP-9 (MAB-0245) and TIMP-1 (MAB-0282), an S-P ultrasensitive kit (kit-9703), and DAB (DAB-0031) were all purchased from Maxim Biotechnological Inc., Ltd, Fuzhou, Fujian, P. R. China.

### Immunohistochemistry

Further sections were cut from selected blocks at 4 μm thickness. Sections from each specimen were immunohistochemically examined separately by antibodies against MMP-2, MMP-9 and TIMP-1. However, because of detachments during staining procedures, there was a slight difference between numbers of cases stained by the three antibodies. Negative controls were set up by replacing the primary antibodies with 0.01 mol/L phosphate-buffered saline (PBS), and positive controls were prepared according to the manufacturer's instructions.

### Result Judgment

In general, the occurrence of yellow particles in cytoplasm was regarded as positive staining. Sections were evaluated by two scoring systems, A and B. For system A, scores were determined according to the color shade of cytoplasmic particles: no yellow particles was scored at 0, light yellow at 1, yellow brown at 2, and brown at 3 points. The scores for system B were based on the ratio of the number of tumor cells with positive particles to the total number of tumor cell in the sections. A ratio <25% was scored at 1,

25%-75% at 2, and >75% at 3 points. The final score of each case was expressed as AB, and stratified in accordance with the magnitude of the scores, where 0 points was classified as negative (-), 1-4 points as weakly positive (+), and >4 points as strongly positive (++). To perform a comparison between groups, we pooled the negative and weakly positive cases into one group, and the strongly positive cases were treated as one group.[3]

**Statistical Analysis**

Comparisons between groups were made by a $X^2$ test using SPSS 10.0 for Windows.

## Results

**Expressions of MMP-2, MMP-9 and TIMP-1 in Normal Oral Mucosa and Peritumor Epithelia**

The expression of MMP-2 in normal oral mucosa was negative in 10 and positive in six cases, being present mainly in stratum spinosum. There was no difference in expression between peritumor epithelia and normal epithelia. All cases (n=8) of normal mucosa had negative MMP-9 expression, but strong positive expression was found in the entire strata of peritumor epithelia (n=5) (Fig. 1). TIMP-1 was found to be weakly expressed in normal epithelia, vascular endothelial cells, fibroblasts, mononuclear cells, and plasmacytes.

**Expressions of MMP-2, MMP-9, and TIMP-1 in OKC and AB.**

In stratum spinosum of OKC, MMP-2 was negatively or weakly expressed in eight cases and strongly expressed in two weak cases, but in basal layer cells, there was weak or no expression (Fig. 2). MMP-2 was strongly expressed in stellate stratum and peripheral cells in 28 AB cases (Fig. 3), and weakly to moderately expressed in granular cells in four AB cases. In interstitial fibroblasts, MMP-2 was expressed positively in 33 and negatively in eight AB cases. Vascular endothelial and inflammatory cells including monocytes, neutrophils and plasmacytes etc. had positive expression of MMP-2 (Fig. 4).

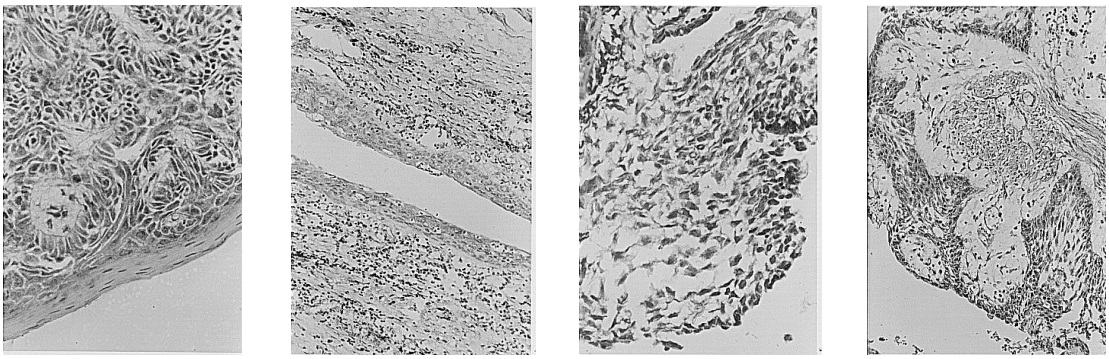

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**Fig. 1.** MMP-9 was strongly expressed in the epithelium surrounding AB (S-P method, x400).
**Fig. 2.** MMP-2 expression was weakly positive in stratum spinosum, and lost in stratum basale in OKC (S-P method, x200).
**Fig. 3.** MMP-2 was strongly expressed in stellate reticule stratum of AB (S-P method, x400).
**Fig. 4.** MMP-2 was strongly expressed in matrix fibroblasts and endothelial cells of AB (S-P method, x200).

**Table 1.** The expression of MMP-2 in normal oral mucosa, OKC, and AB.

| Groups | n | -~+ (%) | ++ (%) |
|---|---|---|---|
| Normal mucosa | 16 | 10 (62.5) | 6 (37.5) |
| OKC | 10 | 8 (80.0) | 2 (20.0) |
| AB | 41 | 13 (31.7) | 28 (68.3) |

Note: Among the three groups, $X^2$=9.769, p<0.01; between normal mucosa and OKC, p>0.05 between normal mucosa and AB, p<0.05; between OKC and AB, p<0.05.

**Table 2.** The expression of MMP-2 in primary, recurrent, and malignant AB.

| Groups | n | -~+ (%) | ++ (%) |
|---|---|---|---|
| Primary AB | 15 | 8 (53.3) | 7 (46.7) |
| Recurrent AB | 21 | 3 (14.3) | 18 (85.7) |
| Malignant AB | 5 | 2 (40.0) | 3 (60.0) |

Note: Between primary and recurrent AB, Fisher's p<0.05; between primary and malignant AB, and between recurrent and malignant AB, p<0.05.

**Table 3.** The expression of MMP-9 in normal oral mucosa, OKC, and AB.

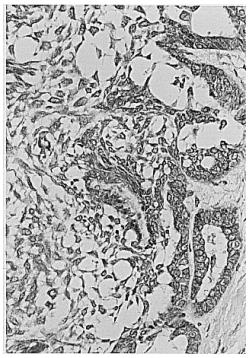
| Groups | n | -~+ (%) | ++ (%) |
|---|---|---|---|
| Normal mucosa | 8 | 8 (100.0) | 0 (00.0) |
| OKC | 9 | 7 (77.8) | 2 (22.2) |
| AB | 43 | 13 (30.2) | 30 (69.8) |

Note: Between normal mucosa and OKC, p>0.05; between normal mucosa and AB, p<0.01; between OKC and AB, p<0.05.

**Table 4.** The expression of MMP-9 in primary, recurrent, and malignant AB.

| Groups | n | -~+ (%) | ++ (%) |
|---|---|---|---|
| Primary AB | 16 | 10 (62.5) | 6 (37.5) |
| Recurrent AB | 21 | 3 (14.3) | 18 (85.7) |
| Malignant AB | 6 | 0 (00.0) | 6 (100.0) |

Note: Between primary and recurrent AB, p<0.01; between primary and malignant AB, p<0.05; between primary and malignant AB, p>0.05.



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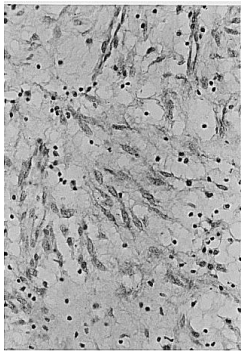

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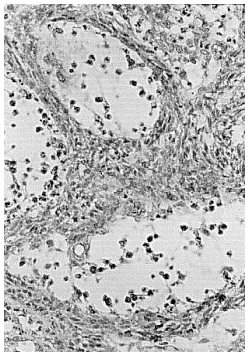

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**Fig. 5.** MMP-9 expression was intensely positive in malignant ABs with metastatic nodes (S-P method, x200).

**Fig. 6.** MMP-9 was strongly expressed in peripheral fibroblasts of AB (S-P method, x200).

**Fig. 7.** TIMP-1 expression was negative in AB, but positive in stroma, endothelia and inflammatory cells (S-P method, x200).

Table 1 shows the expression data of MMP-2 in normal oral mucosa, OKC and AB. As AB relapsed and transformed malignantly, the number of cases with strong positive expression of MMP-2 increased.

Table 2 shows the expression data of MMP-2 in primary, recurrent and malignant AB groups. For the

few malignant cases, no significant difference ($p>0.05$) was found between primary and malignant AB, and between recurrent and malignant AB. In OKC cases, only two had strong expression of MMP-9 and the other showed weak or negative expression. In AB cases, however, strong expression of MMP-9 was seen in 30 cases, being located in stellate and peripheral strata. The MMP-9 expression increased markedly as AB replased and transformed malignantly (Fig. 5). Granular cells had negative or weak MMP-9 expression. Monocyst AB displayed negative or weak MMP-9 expression. In addition, MMP-9 was strongly expressed in peritumor intertial fibroblasts (Fig. 6).

Table 3 shows the expression data of MMP-9 in normal oral mucosa, OKC and AB. As AB relapsed and become malignant, the intensity and positive ratio of MMP-9 expression rose significantly.

Table 4 shows the expression data of MMP-2 in primary, recurrent and malignant AB groups. TIMP-1 expression was negative or weak in four cases and strong in six cases of OKC, but the expression was negative or weak in the majority of AB cases (29/38). The intensity of TIMP-1 expression in AB was noticeably lower than that of MMPs (Fig. 7). However, TIMP-1 was positively expressed in vascular endothelia and inflammatory cells. No significant difference was found between normal oral mucosa, OKC and AB, or between primary, recurrent and malignant AB ($p>0.05$).

**Relationship between the Expression of MMP-2, MMP-9 and TIMP-1 Proteins and Clinico-pathological Characteristics of AB.**

Expressions of the three proteins were correlated with patients' age and sex, disease sites, X-ray phenotype and pathological classification, but no significant correlation was found ($p>0.05$) (Table. 5).

**Table 5.** The relationship of the expression MMP-2, MMP-9 and TIMP-1 proteins with clinico-pathological characteristics of AB.

| Parameters | Classification | No. | MMP-2 | | | MMP-9 | | | TIMP-1 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | - | + | ++ | - | + | ++ | - | + | ++ |
| Age | 30 or more | 25 | 3 | 10 | 12 | 6 | 5 | 14 | 7 | 10 | 4 |
| | <30 | 18 | 1 | 9 | 6 | 0 | 5 | 13 | 6 | 6 | 5 |
| Sex | Male | 19 | 2 | 7 | 7 | 3 | 3 | 13 | 6 | 9 | 4 |
| | Female | 24 | 2 | 11 | 12 | 3 | 7 | 14 | 7 | 7 | 5 |
| Site | Anterior region of lower jaw | 10 | 1 | 4 | 5 | 1 | 4 | 5 | 3 | 6 | 1 |
| | Molars and ascending ramus of mandible | 22 | 2 | 10 | 9 | 4 | 4 | 14 | 13 | 7 | 2 |
| | Maxilla | 7 | 0 | 4 | 2 | 0 | 1 | 6 | 2 | 0 | 2 |
| | Peripheral | 4 | 1 | 1 | 2 | 1 | 1 | 2 | 1 | 0 | 1 |
| X-ray | Mono-chamber | 6 | 1 | 3 | 2 | 1 | 2 | 3 | 3 | 2 | 1 |
| | Multi-chambers | 27 | 2 | 13 | 12 | 4 | 5 | 18 | 9 | 10 | 8 |
| Pathological patterns | Follicle | 21 | 1 | 11 | 7 | 4 | 5 | 12 | 6 | 7 | 6 |
| | Cluster | 7 | 1 | 1 | 5 | 0 | 2 | 5 | 1 | 3 | 3 |
| | Granular cell | 4 | 1 | 1 | 2 | 1 | 1 | 2 | 2 | 1 | 0 |
| | Monocyst | 3 | 0 | 2 | 1 | 1 | 0 | 2 | 2 | 1 | 0 |
| | Solid | 8 | 1 | 3 | 4 | 0 | 2 | 6 | 2 | 4 | 0 |

Note: The expression of MMP-2 was measured in 41, MMP-9 in 43, and TIMP-1 in 38 cases of AB. X-ray results were not available for four of the 41 cases, six of the 43 cases with four peripheral ABs not included, and three of the 38 cases with two peripheral ABs not included.

## Discussion

ECM is constituted of proteoglycan and glycoprotein gel, in which various types of collagen and elastic fibers are embedded to form a tridimensional network structure. ECM plays an important role in the

maintenance of normal structure and function of tissues and in cell growth and differentiation. It is no longer thought to be static, but is believed to experience continuous metabolism and turnover, being in a dynamic balance of degradation and remolding. A large amount of research has indicated that loss of the balance is closely related to many pathological conditions, such as atherosclerosis, renal diseases and liver cirrhosis, and to tumor invasiveness and recurrence in particular. Since the discovery of specific proteolytic enzyme for collagen-collagenase, in 1962, many other enzymes acting on different components of ECM have been discovered.[3]

It is known that the acquisition of the ability to degrade ECM is necessary for in situ carcinomas to develop into invasive ones. The degradation of ECM relies primarily on various enzymes, of which MMPs are the most important. MMPs are capable of degrading almost all constituents of ECM. Therefore, MMPs have become the subject of much attention in the field of research into tumor invasiveness and metastasis.

MMPs are a family of zinc-dependent endopeptidase, comprising 18 principal to tumor members. Closely related aggression are MMP-2 (gelatinase A, 72 kDa) and MMP-9 (gelatinase B, 92 kDa), the principal action of which is to degrade ECM and basement membrane, promote tumor angiogenesis, and regulate cell adhesion to facilitate invasiveness and metastasis of carcinoma cells. Tissue inhibitor of metalloproteinases (TIMPs) are specific inhibitors of MMPs. TIMPs bind 1:1 with MMPs to inhibit the activity of MMPs,[4] leading to the suppression of tumor invasiveness and metastasis. Besides, TIMPs can repress tumor angiogenesis to inhibit tumor growth.

Our results indicated that a great difference was produced between MMPs and TIMP-1 expressions in AB, suggesting a loss of the balance between the two proteins. The expression of TIMP-1 in OKC was similar to that in normal mucosa, but was lost or weakened in 76.3% of AB cases (29/38). However, MMP-2 was strongly expressed in peripheral and stellate strata cells in 28 of 41 AB cases (68.3%), which was significantly different from that in normal oral mucosa ($X^2$=4.534, p=0.033<0.05) and OKC ($X^2$=5.875, p=0.015<0.05). Moreover, there was a significant difference in MMP-2 expression between primary and recurrent ABs (p=0.025<0.05), but no significant difference was found between primary and malignant, and between recurrent and malignant ABs, for a small number of malignant cases. In addition, our data demonstrated a stronger intensity of MMP-9 expression than that of MMP-2 in AB. MMP-9 expression was negative in normal mucosa, but strong in peritumor epithelia and in 30 of 43 cases of AB (69.8%), showing a significant difference between the two groups ($X^2$=10.827, p=0.001<0.01). MMP-9 expression was negative or weak in seven out of nine cases of OKC, having a significant difference from that in AB ($X^2$=5.241, p=0.022<0.05). As AB relapsed and transformed malignantly, the expression intensity and positive rate of MMP-9 increased. MMP-9 was intensely expressed in six out of 16 (37.5%) primary AB cases, but the percentage rose to 85.7% (18/21) in recurrent cases (p=0.005<0.01), and to 100% (6/6) in malignant cases (p=0.015<0.05). These results indicated a correlation between the strong expression of MMP-2 and -9 and AB's biological behavior, and in addition, that the imbalance of MMPs and TIMP-1 was also one of the principal mechanisms for the aggressive growth and high recurrence rate of AB. This has been documented in human carcinomas of breast, colon, stomach and bladder, and squamous carcinoma of head and neck.[3, 5]

The development of oral cavity and maxillofacial region and dental germs involves the induction of epithelia by

ECM. This induction is also seen in odontogenic tumors and cysts. ECM acts strongly on epithelia and can join the epithelia in invasive growth. Previous research found that interstitial tissues actively proliferated and telomerase had a high activity in AB.[6] Results in this study found MMP-2 to be positively expressed in matrix fibroblasts in 33 of 41 AB cases. Similarly, matrix fibroblasts had relatively strong expression of MMP-9 in 34 of 43 AB cases, but the expression of TIMP-1 was weak. It is now clear that tumor invasiveness depends on there being enough enzymes to degrade ECM, which enzymes are not only produced by tumor cells themselves, but also by tumor matrix and inflammatory cells. There is also evidence from previous studies that the large quantities of MMPs in tumor tissues can be produced by matrix cells.[7, 8] This demonstrates that tumor cells can avail themselves of MMPs generated by matrix cells in tumor invasive growth.[9] However, the action of the matrix did not receive sufficient attention in past studies. Kubota et al.[10] measured the levels of IL-1α and -1β, IL-6 and TNF-α, and gelatinase species in intracystic fluids of ABs and OKCs, and demonstrated significant differences in IL-1α and IL-6 between the two diseases. Our data indicated that only two cases of OKC had strong expression of MMP-2 and -9 in the epithelial cells. Therefore, OKC might depend to some degree upon the MMPs derived from the fluids or matrix to spread invasively. It is generally believed that MMPs levels are upregulated and TIMPs levels decreased in carcinomas, which is supported by our results. However, other research found that with the enhancement of MMPs levels, the expression of TIMPs also increased.[11] These results suggest that TIMPs are bifunctional cytokines that are differentially expressed in different tumors. Research by Kumamoto[12] showed that both MMP-2 and -9 and TIMP-1 were weakly expressed in AB, but that TIMP-2 was strongly expressed in matrix.

Like other carcinomas, AB is a typical disorder of vascular dependence.[13] In certain conditions, the impact of TIMPs upon tumor growth might be related to their action on tumor angiogenesis, because research has demonstrated that TIMPs can block angiogenesis.[14] The mechanisms of tumor inhibition by TIMPs might indicate an indirect effect of their influence on angiogenesis and a direct action on tumor cell growth. Therefore, the strong expression of MMPs and loss or weakness of TIMPs expression in AB promote tumor angiogenesis, allowing AB to proliferate and differentiate, which reflects the biological features of AB, i.e., aggressive growth and high potential to recur. Currently, some researchers believe that MMPs are a kind of angiogenesis associated protein.[15] Basic fibroblast growth factor (bFGF) can stimulate the production of MMPs by endothelial cells. MMPs degrade the collagen in ECM surrounding vessels, unmasking the hidden RGD-binding sites in the helical structure of collagens. Integrin αVβ3 binds to these sites, causing the invasion of vascular endothelial cells into ECM.[16] Other researchers believe that MMPs may produce bioavailable VEGF, which is an important pro-angiogenic factor and prognostic indicator of tumors with great promise.[17] Our results revealed positive expressions of MMPs and VEGF in endothelial cells.

In general, in-depth research into MMPs will deepen our understanding of the biological behavior of AB and OKC. Meanwhile, further studies of TIMPs will give rise to advances in the field of tumor biology and treatment.

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