EXPRESSION OF MATRIX METALLOPROTEINASE MMP-2 AND ITS TISSUE INHIBITOR TIMP-2 IN INTRAORAL PLEOMORPHIC ADENOMA AND ADENOID CYSTIC CARCINOMA

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Abstract

Matrix metalloproteinases (MMPs) are proteolytic enzymes that are capable of degrading different substrates within extracellular matrix (ECM), and are believed to be crucial for tumor invasion and metastasis. Tissue inhibitors of MMP (TIMPs) can inhibit the action of MMPs

The aim of this study was to analyze protein expression of MMP-2 and TIMP-2 in intraoral pleomorphic adenoma (PLA) and adenoid cystic carcinoma (ACC).

A total of 35 formalin-fixed paraffin-embedded specimens comprising 19 PLA and 16 ACC were utilized in this study. A standard immunohistochemical technique was used to determine the expression levels of MMP-2 and TIMP-2 proteins. Sections were assessed semi quantitatively .Staining was scored as 0 (< 1% positive tumor staining), 1+ (< 25% positive tumor cells), 2+ (20-50% positive tumor cells) and 3+ (> 50% positive tumor cells). For statistical analysis, tumors were divided into two groups, low expressors (0-1+) and high expressors (2-3+).

PLA showed higher TIMP-2 expression than ACC ($p<0.05$). No significant difference was observed between PLA and ACC regarding MMP-2 expression. MMP-2 and TIMP-2 expressed mainly in the cytoplasm of epithelial/ myoepithelial components of PLA and neoplastic epithelial cells of ACC.

Myoepithelial cells may be the primary source of gelatinases in PLA and the down regulation of TIMP-2 expression in ACC might be responsible for metastasis and recurrence. The ratio value of MMP-2/TIMP-2 is valuable parameter to demonstrate the ECM degradation/ deposition imbalance.

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Introduction

Intraoral minor salivary gland tumors (MSGTs) constitute a heterogeneous group of neoplasm with great histomorphologic variation[1].

In spite of their relative infrequency in term of total number of cases in oral or hospital surgical pathology services[2], minor salivary glands tumors continue to generate considerable research interest. Several studies[2,3,4] have documented the distribution of series of minor salivary gland tumors seen in some countries. Reports from various parts of the world indicate that there are differences in the frequency of particular histological types and in the frequency with which minor salivary glands are involved.

Benign and malignant tumors have almost equal frequency. However, some studies have previously reported that pleomorphic adenomas and adenoid cystic carcinoma seems to have a higher prevalence among Japanese [5]. The palate was the main site of occurrence in many studies followed by cheek, lip and gingiva [6]. Most studies in the literatures indicated that minor salivary gland tumors are somewhat more common in females than in males with Male to Female ratio varying from 1:1.2 to 1:1.5 [6,7].

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PLA cases	Age/ Years	Gender	Site	MMP-2	TIMP-2		ACC cases	Age/ Years	Gender	Site	MMP-2	TIMP-2
1	17	Female	Palate	**2+**	**1+**		**1**	64	Male	Palate	**0**	**3+**
2	25	Male	Palate	**0**	**3+**		**2**	60	Male	Palate	**3+**	**1+**
3	51	Male	Palate	**3+**	**0**		**3**	61	Male	Palate	**2+**	**3+**
4	14	Female	Palate	**3+**	**3+**		**4**	51	Male	Palate	**1+**	**0**
5	28	Female	Palate	**3+**	**3+**		**5**	55	Male	Palate	**3+**	**0**
6	45	Male	Palate	**3+**	**3+**		**6**	49	Male	Palate	**1+**	**1+**
7	42	Male	Palate	**2+**	**3+**		**7**	55	Female	Palate	**1+**	**1+**
8	15	Male	Palate	**0**	**3+**		**8**	59	Female	Palate	**0**	**1+**
9	45	Female	Palate	**1+**	**0**		**9**	60	Female	Palate	**2+**	**0**
10	50	Male	Palate	**0**	**0**		**10**	68	Male	Palate	**2+**	**0**
11	57	Male	Palate	**0**	**3+**		**11**	60	Male	Palate	**0**	**0**
12	61	Male	Palate	**0**	**1+**		**12**	71	Male	Palate	**1+**	**0**
13	18	Female	Palate	**0**	**0**		**13**	38	Female	Palate	**2+**	**0**
14	23	Female	Palate	**0**	**0**		**14**	44	Female	Palate	**0**	**0**
15	15	Male	Palate	**2+**	**1+**		**15**	55	Female	Palate	**0**	**0**
16	70	Female	Palate	**0**	**1+**		**16**	65	Female	Palate	**1+**	**0**
17	25	Female	Palate	**0**	**1+**							
18	73	Female	Palate	**0**	**0**							
19	60	Female	Palate	**0**	**0**							

Table 1. Clinico-pathologic data of studied sample.
(N.B: Histological subtype: PLA : Classic/ cellular ; ACC: Cribriform/ tubular)

One Iraqi study done on 140 minor salivary gland tumors revealed Male to Female ratio of about 1:1.1, the palate was the most commonly involved site and pleomorphic adenoma was the most common tumor type followed by mucoepidermoid carcinoma and adenoid cystic carcinoma[8]. In addition to traditional microscopical studies, other methods have become available to help define biologic behavior of these tumors.

The application of immunohistochemistry to salivary gland pathology has shown that matrix metalloproteinases (MMPs) and their inhibitors are useful markers to determine the biologic behavior of benign and malignant minor salivary gland tumors[9,10,11].

The matrix metalloproteinases (Matrixins) are a family of multi domain Zn-dependent proteolytic enzymes that are capable of degrading different substrates within extracellular matrix (ECM)[12,13] , both in physiological and pathological conditions such as embryogenesis, wound healing, angiogenesis, inflammation and tumor metastasis[14]. They are classified into four groups according to their substrate specificity: Collagenases (MMP-1,-8,-13 & -18), gelatinases (MMP-2 & -9), stromelysins (MMP-3, MMP-10 &MMP-11), membrane type MMP (MMP-14,-15,-16,-17,-24 & -25) and matrilysins (MMP-7 & -26)[15].

Tissue inhibitors of matrix metalloproteinases (TIMPs) are specific inhibitors of MMPs that participate in controlling the local activities of MMPs in tissues[16,17].

Four TIMPs (TIMP1-4) have been identified in vertebrates [18], and their expression is regulated during development and tissue remodeling. The expression of MMPs and TIMPs is still not well documented in intraoral minor salivary gland tumors. The aim of this study was to elucidate the expression and cellular localization of MMP-2 and TIMP-2 in intraoral minor salivary gland tumors by mean of immunohistochemistry.

Materials and Methods

Thirty five formalin-fixed paraffin embedded blocks were retrieved from Oral Pathology Laboratory specimen file of Dental School / University of Baghdad. The sample consisted of 19 specimens of pleomorphic adenoma of the palate and other 16 specimens of adenoid cystic carcinoma of the palate. The clinico-pathological characteristics of the patients from which the specimens were taken are summarized in table 1.

All the 35 blocks were cut at 3 µM thickness sections and mounted on poly-L-lysin-coated slides. The sections were deparaffinized

and stained by means of a standard immunohistochemical technique using a high – temperature water bath for antigen enhancement. US Biological (US Biological Co, Massachusetts) reagents was used in a peroxidase – based system to identify antigen-antibody conjugates. The sections were incubated with primary antibody against MMP-2 (mouse monoclonal anti-MMP-2, clone 0.N.430,USBM2420-51). Other sections were incubated with primary antibody against TIMP-2 (mouse monoclonal anti-TIMP-2, clone ZQ672, USB T5585-27). The incubation time for both antibodies was 30 minutes at room temperature. Negative control slides were incubated with normal phosphate buffer solution instead of primary monoclonal antibodies. The immunostaining procedure was performed using a labeled streptavidin-biotin system (LSAB system-HRP, Dakocytomation, Carpinteria, California, USA). The biotinylated secondary antibody was applied for 20 minutes. The presence of antigens was detected with streptavidin conjugated to horse radish peroxidase. DAB (3,3'-diaminobenzidine) (Dakocytomation) was used as a chromogen.

Mayer hemotoxylin counter stain was then applied to all sections for 2 minutes and washed with tab water for another 2 minutes, dehydrated in graduated alcohol concentrations, then mounted with DPX and slide cover. Ten more samples of non- neoplastic salivary gland tissue were included in this study and served as control group. All slide were examined and photographed with a light microscope (Olympus BX41) with digital camera at 100, 400 & 1000 magnification. All slides were scored by two investigators without knowledge of the clinical outcomes. Occasional disagreement was discussed later to reach a consensus. In case of persistent differences between them, the sections were studied by a third independent oral pathologist and the majority decision was then considered. Staining of MMP-2 and TIMP-2 was semiquantitatively assessed. Staining was graded as 0(negatively stained cells), 1+ (< 10% positive stained cells), 2+ (10-50% positive stained cells), 3+ (> 50% positive stained cells) (11). For statistical analysis of the immunoscores we used Fisher's exact test to test the statistical association between tumor types and expression levels for each marker.

For analysis we dichotomized results by comparing low expressors (0-1+) to high expressors (2-3+). All analysis was two tailed with a significant level set at (0.05).

Results

A benign intraoral salivary gland tumor was represented by nineteen cases of pleomorphic adenoma which were selected for this study. All were located in the palatal region as slowly-growing, painless mass. They presented histologically as epithelial cells arranged in strands, sheets and duct-like structure in mucoid or myxochondroid stroma background. Epithelial cells and modified myoepithelial cells are components of cell islands in pleomorphic adenoma. Immunoexpression of MMP-2 was detected in 8 out of 19 cases (42.1%). Four cases (50%) outof these 8 cases were strongly expressed MMP-2 (grade 3+), 3 (37.5%) showed immunostaining between 10% and 50% of tumor cells (grade 2+) and only one case (12.5%) expressed less than 10% of positive cells staining (grade 1+). MMP-2 staining intensity was most prominent in epithelial-myoepithelial components (figure1). The remaining 11 cases (57.9%) were negative for MMP-2.

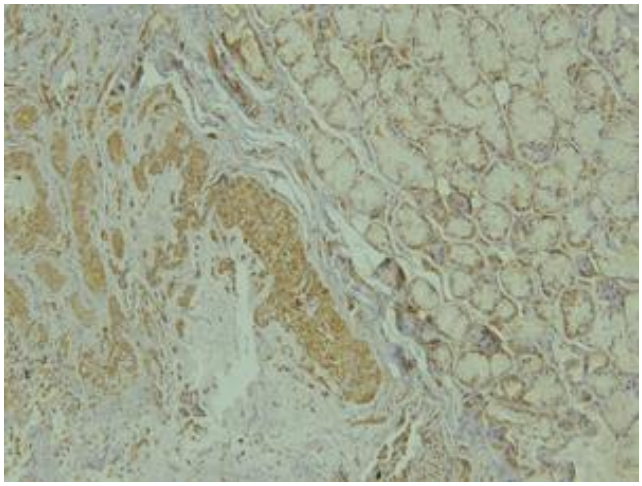

Figure 1. MMP-2 in PLA (grade 3).

TIMP-2 immunoexpression was detected in 12 pleomorphic adenoma (63.15%), seven cases (58.33%) were strongly positive (grade 3+), five case (41.66%) were with(grade 1+) .The cytoplasmic TIMP-2 staining was found in the majority of specimens and epithelial-myoepithelial cells were the most prominent cells that expressed TIMP-2 reactivity (Figure 2). The remaining seven cases (36.85%) were negative for TIMP-2 (table 2).

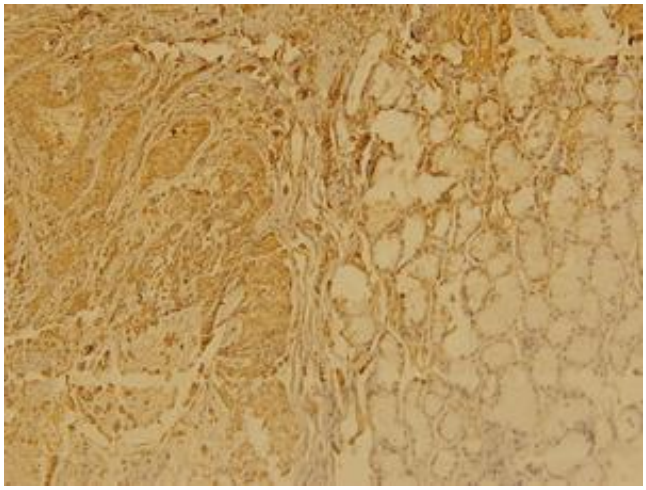
Figure 2. TIMP-2 in PLA (grade 3).

MMP-2	Score	Normal gland	PLA	ACC	Benign/Malignant tumor *(Fisher's Exact test)*
	0	5 (50%)	11 (57.9%)	5 (31.25%)	0.65
	1+	4 (40%)	1 (5.3%)	5 (31.25%)	
	2+	1 (10%)	3 (15.8%)	4 (25%)	
	3+	0	4 (21%)	2 (12.5%)	
TIMP-2					
	0	6 (60%)	7 (36.8%)	10 (62.5%)	0.04*
	1+	2 (20%)	5 (26.3%)	4 (25%)	
	2+	2 (20%)	0	0	
	3+	0	7 (36.8%)	2 (12.5%)	
MMP-2 / TIMP-2 ratio		1/2	1/1.6	2/1	

Table 2. Summary of results of Immunohistochemical staining.

A malignant intraoral salivary gland tumor was represented by sixteen cases of adenoid cystic carcinoma. All were located in the palate. They all presented histologically in a classical cribriform/ tubular pattern. Immunoexpression of MMP-2 was detected in 11cases out of 16 case (68.75%), of these 11 cases only 2 cases (18.18%) were strongly positive for MMP-2 (grade 3+) (figure 3), other 4 cases (36.36%) expressed immunreactivity between (10-50%) (Grade 2+), the remaining five cases (45.45%) expressed very mild immune reaction (grade 1+). The remaining 5 cases (31.25%) were negative for MMP-2. TIMP-2 immunostaining in adenoid cystic carcinoma was detected in 6 cases representing only (37.5%) of the total number, 2 cases (33.33%) expressed (grade 3+) and the other 4 cases (66.66%) expressed (grade 1+).

The remaining 10 cases (62.5%) were negative for TIMP-2 (figure 4) (table 2). TIMP-2 expression was significantly higher in pleomorphic adenoma compared with adenoid cystic carcinoma ($p<0.05$). The median values of MMP-2 and TIMP-2 immunoscores are summarized in table (2). The MMP-2/TIMP-2 ratio was calculated for both pleomorphic adenoma and adenoid cystic carcinoma which was 1:1.6 for pleomorphic adenoma and 2:1 for adenoid cystic carcinoma. Non-neoplastic minor salivary gland control group expressed mild-moderate MMP-2 and TIMP-2 immunostaining reaction in mucus acinar cells with MMP-2/TIMP-2 ratio of 1:2.

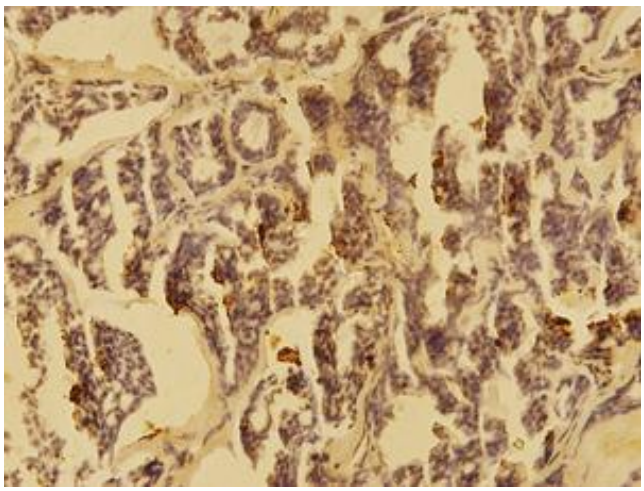
Figure 3. MMP-2 in ACC (grade 3).

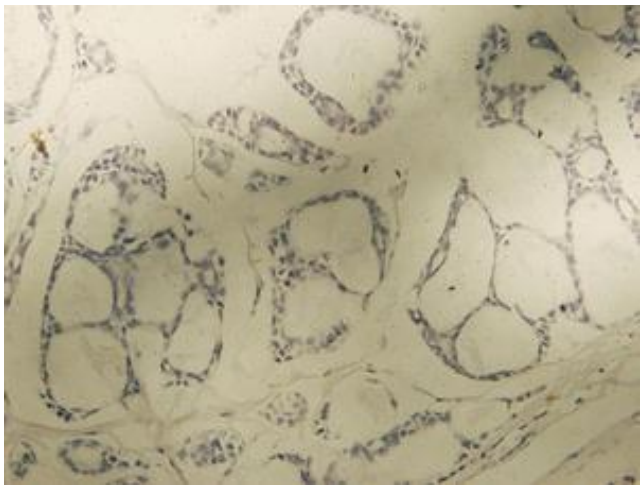
Figure 4. TIMP in ACC (grade 0).

Discussion

Intraoral minor salivary gland tumors are relatively uncommon lesions in daily practice. The balance between MMPs and their tissue inhibitors (TIMPs) is involved in the morphogenesis of normal salivary glands as well as in the mechanism of tumor invasion and

metastasis[10]. The role of MMPs and TIMPs in pleomorphic adenoma and adenoid cystic carcinoma has not been well defined yet.

Imunohistochemistry allows the study of the presence of MMPs & TIMPs in tumors and surrounding tissues. In this study MMP-2 and TIMP-2 expression was compared in pleomrphic adenoma and adenoid cystic carcinoma. The immunoscores of MMP-2 detected in tumor cells were significantly increased in malignant tumors compared with benign tumors. In this study, MMP-2 and TIMP-2 were mainly expressed in epithelial/ myoepithelial cells of pleomorphic adenoma. Previous studies have demonstrated that although tumors cells may be the source of gelatinases in some patients, in the majority of patients only stroma cells express gelatinase[19].

Tumor stroma is one of the critical elements that promote the transition from carcinoma in situ to invasive cancer[20]. However, the present study , however, strongly suggest the possibility that myoepithelial cells may be the primary source of gelatinases and perhaps play a critical role in development and/or progression of pleomorphic adenoma. This finding is in accordance of other studies[10].

The increased expression of MMPs was followed by increased expression of TIMPs. This suggested a positive correlation between MMP-2 and TIMP-2. Zhang et al[10] suggested that the increase in both MMP and TIMP levels represented a physiological attempt of cells to control MMP activity and maintain a balanced ratio between the two proteins. It is well known that the gelatinolytic activity of MMPs is antagonized by TIMPs in a stoichiometric manner [21,] maintenance of this equilibrium is essential and any disturbance of this balance will probably result in tissue damage due to increased proteolysis. Hence, the high expression of MMP-2 (without TIMP-2 expression) in epithelial/ myoepithelial components of pleomorphic adenoma may facilitate the local invasiveness. Careful follow up is of great importance to early detect and prevent invasion or malignant transformation. The metastatic ability of human salivary gland cancer cells was closely associated with decreased or altered TIMP-2 expression. The down regulation of TIMP-2 in malignant epithelia of adenoid cystic carcinoma might be capable of concerning acquisition of abilities of recurrence and metastasis by the effects of TIMP-2 regarding the inhibition of MMPs, tumor growth and anti angiogenic activity (10). The expression of MMP-2 in tumor stromal interface of Adenoid Cystic Carcinoma in this study suggest that stroma may play some critical role than epithelia in progression of this tumor. It has also been reported that some TIMPs can directly affect cell growth and/ or cell survival independent of their actions of MMPs[22].

The expression of TIMP-2 has been correlated with the metastatic ability and poor prognosis of squamous cell carcinoma of tongue[14] and some studies reported the positive role of TIMP-2 in tumor metastasis and decreased survival[23]. The finding of the current study does not support this concept since most of ACC cases (14 out of 16 case) were low TIMP-2 expressors (0 -1+). The ratio of MMP-2/TIMP-2 takes into account variations in the expression of enzymes and its inhibitor and is an indicator of an imbalance between MMP (degradation of ECM) and TIMP (deposition of ECM). Because the balance of these enzymes is critical to matrix destruction, this ratio may adequately reflect the net proteolytic capacity which was significantly higher in carcinoma than in adenoma in this study. Other researchers have found a significant positive correlation between lymph node metastasis and MMP-2/TIMP-2 value[24], and the evaluation of MMP-2/TIMP-2 ratio has a higher prognostic value than the evaluation of MMP-2 and TIMP-2 expression alone[23]. Therefore we evaluated the ratio of MMP-2/TIMP-2 for all studied sample and non-neoplastic minor salivary glands. We found that the ratio in ACC were higher than that of pleomorphic adenoma and non-neoplastic minor salivary glands. The increased MMP-2/TIMP-2 ratio was ascribed to overproduction of MMP and MMP/TIMP balance apparently favors gelatinolytic activity.

Conclusions

In summary, the results presented here provide supporting evidence that MMP-2 expressed in epithelial/myoepithelia cells of pleomorphic adenoma may be important in the development and progression of this tumor and the high TIMP-2 expression may prevent invasion and metastasis. In Adenoid Cystic Carcinoma, the lack or low expression of TIMP-2 in malignant epithelial cells may favor ECM degradation produced by various MMPs and the ratio value of MMP-2/TIMP-2 is valuable parameter to demonstrate this imbalance.

Declaration of Interest

The authors report no conflict of interest and the article is not funded or supported by any research grant.

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