

MMP-1/2 and TIMP-1/2 expression levels, and the levels of collagenous and elastic fibers correlate with disease progression in a hamster model of tongue cancer

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Abstract. In the present study, the presence of extracellular matrix components, including collagenous and elastic fibers, and the expression of their key regulating enzymes, were investigated in different stages of hamster tongue carcinoma development. Immunohistochemical and computer-assisted morphological analyses were performed to quantify the staining intensity and area (integral optical density) of matrix metalloproteinase (MMP)-1 and -2, tissue inhibitors of metalloproteinase (TIMP)-1 and -2, and the extent of elastic and collagenous fibers in histological sections. MMP-1, MMP-2, TIMP-1 and TIMP-2 expression levels gradually increased with tongue cancer progression, and were associated with disease pathology staging ($r=0.705, 0.633, 0.759$ and 0.751 , respectively). By contrast, elastic fiber levels gradually decreased with cancer progression and were negatively correlated with disease staging ($r=-0.881$). The levels of collagenous fibers gradually increased with cancer progression and showed a positive correlation ($r=0.619$). In summary, the study demonstrated that MMP1/2 and TIMP1/2 expression levels, and collagenous and elastic fiber levels were significantly correlated with disease progression in a hamster model of tongue cancer.

Introduction

Collagenous and elastic fibers, the main components of the extracellular matrix (ECM), are distributed widely among the varying oral mucosa layers. While providing a protective screen for the cells, tissues and organs it supports, the ECM is also involved in cell proliferation, survival and apoptosis (1,2).

Clinically, a number of oral mucosal diseases are associated with the remodeling of collagenous and elastic fibers (3,4). Multiple studies have demonstrated that the fibrosis of the tongue mucosa can be considered as a precancerous lesion (5,6). For this reason, researchers are now focusing attention on the pathological changes in the structure of oral mucosal fibers.

Matrix metalloproteinases (MMPs) are degradative enzymes that exhibit a significant role in all aspects of tumor progression via the enhancement of tumor-induced angiogenesis and the destruction of the local tissue architecture, thus allowing tumor invasion and metastasis (7). Various MMPs are secreted during the growth, invasion, metastasis and angiogenesis of tumors (8). These MMPs affect the surrounding microenvironment, resulting in dynamic changes in the structure of the ECM (9). Indeed, MMPs can degrade almost all components of the ECM (8,10). MMP-1 (also known as collagenase-1) is the principal human enzyme that cleaves native fibrillar collagen, while MMP-2 (gelatinase) specifically degrades basement membrane (BM) type IV collagen, but can also cleave other ECM components. MMP-1 and MMP-2 have each been implicated in the degradation of the ECM in oral diseases (10), and through degradation of collagenous and elastic fibers, provide a path for tumor cells to invade and migrate (11-14). Studies have shown that during the invasion and metastasis period of numerous cancers, activated MMP-1 is located in the peripheral borders of tumor islands, where tumor cells possess invasion ability (15-17). MMP-1 has been shown to promote invasion and tumorigenesis through the degradation of the ECM as the main component of connective tissue in the oral mucosal, while MMP-2 has been associated with angiogenesis, tumor growth, progression, invasiveness and metastasis in numerous cancer types (18-22). Moreover, MMP-1 and -2 have been suggested to be the most important MMPs in the invasion and metastasis of oral mucosal cancer (23,24).

Recent studies have shown that MMPs and tissue inhibitors of matrix metalloproteinases (TIMPs), which strictly regulate MMP activity, are expressed in a relative equilibrium that facilitates the maintenance of the dynamic balance between the synthesis and degradation of the ECM (25,26). Currently, 4 TIMP enzymes have been identified, namely TIMP-1, -2, -3 and -4 (27). TIMP-1 and -2 have been the most well studied of these TIMP enzymes. TIMP-1 interacts with MMP-1 (28), while TIMP-2 selectively inhibits MMP-2 activity (29). To date, a number of studies have suggested a positive role for TIMP-1

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and -2 in the invasion and metastasis of oral cancer (30), head and neck squamous cell carcinoma (31), colon cancer (32) and breast cancer (22).

The current study investigated the importance of MMP-1, MMP-2, TIMP-1 and TIMP-2 in the progression of tongue cancer in a hamster model. Additionally, the changes in the expression of MMP-1, MMP-2, TIMP-1 and TIMP-2, and the levels of collagenous and elastic fibers were analyzed during different pathological stages of tongue cancer, and computer-aided morphological analysis software (33) was applied to analyze the specific quantitative changes in the proteins during tongue cancer development in an attempt to reveal the intrinsic nature of the association between ECM metabolic disorders and tongue cancer development.

Materials and methods

Experimental animals, grouping and tissue preparation. All animal experiments were approved by the Institutional Animal Care Committee of the Laboratory Animal Committee of Harbin Medical University (Harbin, Heilongjiang, China; approval no. HMU-IACUC 2006107). The animal grouping and tongue cancer model were used as described in our previous study (33). Briefly, 4-week-old male hamsters (n=48; 35-55 g; SCXX2007-0005; Shanghai Laboratory Animal Center, Shanghai, China) were randomized into two groups: The control group (n=8), which was untreated, and the experimental group (n=40), in which 1.5% DMBA acetone solution (Sigma-Aldrich, St. Louis, MO, USA) was used to scratch the lingual mucosa 3 times a week, for up to 8 weeks. Each animal received an intraperitoneal injection of 40 mg/kg phenobarbital prior to sacrifice. Next, the tongues were excised, and tissue was fixed in formalin, processed in a standard manner by embedding in paraffin and sectioned into 4- μ m thick sections for immunohistochemical, Gomori's trichrome and Masson's trichrome staining. Histological sections were stained with hematoxylin and eosin for pathological grading purposes (33). Histological assessment was performed by 2 pathologists blinded to the imaging results and graded according to standards outlined by the World Health Organization (34).

Immunohistochemistry. Immunohistochemical staining for MMP-1, MMP-2, TIMP-1, TIMP-2 and type IV collagen was performed using 4- μ m thick, paraffin-embedded sections. Briefly, the sections were first deparaffinized with xylene and rehydrated in graded ethanol. Endogenous peroxidase activity was blocked by the immersion of the slides in 3% hydrogen peroxide. To block non-specific antigens, 1% bovine serum albumin was applied for 15 min. The sections were then incubated with the respective rabbit anti-hamster polyclonal primary antibodies [MMP-2 (cat no. BA0569; 1:300 dilution), MMP-9 (cat no. BA2202; 1:100 dilution), TIMP-1 (cat no. BA3727; 1:100 dilution), (TIMP-2 (cat no. BA0576; 1:50 dilution) and collagen, type IV (cat no. BA2174; 1:500 dilution); all purchased from Boster Bio-Engineering Co., Ltd., Wuhan, China] overnight in a humidified chamber maintained at 4°C. Subsequently, the sections were incubated with corresponding secondary antibodies (immediate-use goat anti-rabbit IgG horseradish-peroxidase polymers; cat no. PV-6001; Zhongshan Goldenbridge Biotechnology Co.,

Ltd., Beijing, China) for 30 min at 37°C. The antibody reaction was visualized using diaminobenzidine chromogen (Zhongshan Goldenbridge Biotechnology Co., Ltd.). Finally, all the slides were counterstained with hematoxylin. Sections incubated with immunoglobulins of the same species at the same final concentrations served as negative controls.

Gomori's aldehyde fuchsin (AF) staining. Formalin-fixed, paraffin-embedded tissues were sectioned, deparaffinized and rehydrated. AF-positive elastic fibers stain bluish violet and are continuous filaments that are widely distributed throughout the loose connective tissue. The nuclei stain blue and the cytoplasm stains pink. Gomori's AF staining (35) was used to observe the distribution of elastic fibers per unit area.

Masson's trichrome staining. Formalin-fixed, paraffin-embedded tissues were sectioned, deparaffinized, and rehydrated. Masson's trichrome staining was used to observe deposited collagenous fiber (6). Collagen fibers stain blue and muscle fibers stain red. Furthermore, the nuclei exhibit black staining and the background exhibits red staining. The extent of collagenous fiber expression was assessed per unit area.

Computer-aided morphological analysis. Immunostaining reaction intensity and area [integral optical density (IOD)] for each protein, and staining per unit area in histological sections were separately assessed by 2 independent pathologists who were blinded to the animal grouping using computer-assisted morphological analysis. Slides that received different assessments from the 2 pathologists were re-analyzed with joint discussion. Image Pro Plus 6.0 (Media Cybernetics, Inc., Rockville, MD, USA) was used to calculate the intensity and extent of staining for the detected molecules, and the ratio of the Gomori's AF/Masson's trichrome staining area to the total area of the image in the different tissues. The per-area density of staining was calculated in this manner to reflect the percentage of the tissue that contained elastic and collagenous fibers, resulting in a semi-quantitative analysis. A total of 5 microscopic fields were randomly selected, and their images were cropped. The IOD levels of the stained cells and the per-area staining of the elastic and collagenous fibers in the tissue samples were then calculated by image analysis. Results were expressed as the mean \pm standard deviation (SD) per tissue examined.

Statistical analysis. SPSS 18 software (SPSS, Inc., Chicago, IL, USA) was used for all statistical analyses. All tests were 2-sided, and the significance level was set at $P \leq 0.05$. Data are expressed as the mean \pm SD. Differences between the mean values of 2 sets were assessed using the Student-Newman-Keuls q-test. Means, variances and interquartile ranges were examined, and Levene's test was used to test for homogeneity of variances, since the variances were found to be unequal. The Wilcoxon rank sum test was used for post hoc pair-wise analyses, and Spearman's rank correlation was used to analyze correlations between different biomarkers and pathological grades.

Results

Histological examination of hamster tongue tissues using hematoxylin and eosin staining. In our previous study (33),

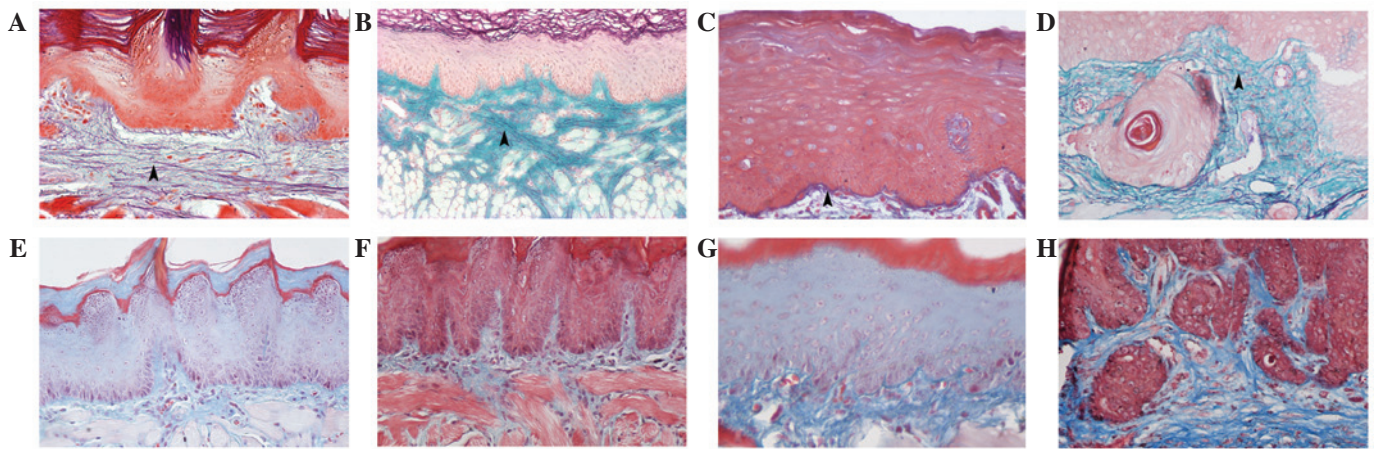


Figure 1. Staining of elastic and collagenous fibers during each progression stage of tongue squamous cell carcinoma and their correlations among (A and E) normal tongue mucosa (magnification, x200), (B and F) atypical hyperplastic tissues (magnification, x200), (C and G) carcinoma *in situ* (magnification, x400) and (D and H) invasive carcinoma tissues (magnification, x400). (A-D) Gomori's aldehyde fuchsin staining for elastic fibers. Arrowheads indicate a fracture in the elastic fiber. (E-H) Masson's trichrome staining for collagenous fibers.

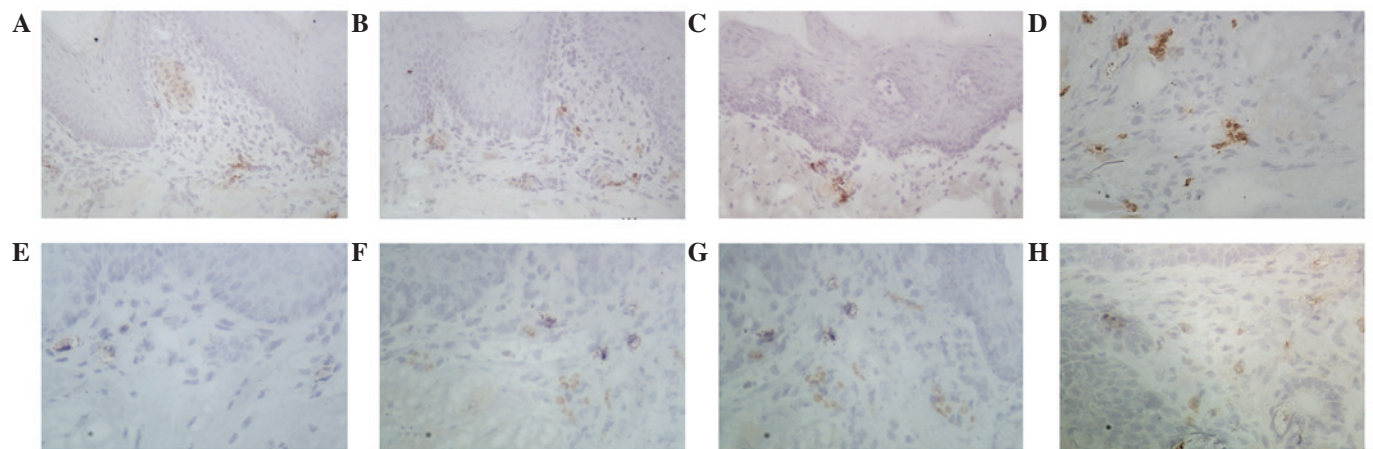


Figure 2. Immunohistochemical staining of (A-D) matrix metalloproteinase-1 (A-C: Magnification, x200; D: Magnification, x400) and (E-H) tissue inhibitors of metalloproteinase-1 (x400 magnification) during different stages of tongue squamous cell carcinoma progression. (A and E) Normal tongue mucosa, (B and F) atypical hyperplastic tissues, (C and G) carcinoma *in situ* and (D and H) invasive carcinoma.

lymphatic vessel changes were morphometrically analyzed in hamsters using 5'-nucleotide enzymes-alkaline phosphatase staining. The DMBA-induced lingual mucosa tissue was harvested and divided into two blocks for frozen and paraffin embedding in the previous and present study, respectively. The lingual mucosa of 40 hamsters were treated with 1.5% DMBA. Ten hamsters were sacrificed every 2 weeks. Using hematoxylin and eosin staining, it was observed that 16 hamsters had atypical hyperplasia, 13 exhibited tongue squamous cell carcinoma *in situ* and 11 had early tongue squamous cell carcinoma. In addition, 8 hamsters were left untreated, with 2 hamsters from this group sacrificed every 2 weeks. No pathological changes were observed in the untreated group using hematoxylin and eosin staining. Thus, our previous study had no effect on the current study and could be run concurrently.

Levels of elastic fibers during different stages of carcinoma progression. Numerous AF-positive elastic fibers were distributed throughout the lamina propria of the normal tongue. The elastic fibers within atypical hyperplastic tissues did not differ

significantly in morphology compared with the normal tongue mucosa (Fig. 1A and B). AF-positive elastic fibers in the lamina propria demonstrated intermittent fracturing, shortening and distribution sparseness in the tissues from *in situ* carcinomas (Fig. 1C). Additionally, fewer AF-positive elastic fibers were found in the lamina propria layer of the invasive carcinoma tissues (Fig. 1D).

Correlations between per-area staining of elastic fibers and different tumor progression stages were analyzed using Spearman's correlation test. The results showed that the expression levels of elastic fibers decreased gradually with the malignant progression of hamster tongue carcinoma ($r=-0.566$; $P<0.01$).

Levels of collagenous fiber during different stages of carcinoma progression. Masson's trichrome-positive collagenous fibers were long and thin, with a straight, flat orientation in the normal lamina propria (Fig. 1E). In the atypical hyperplastic tissues, the morphology of the collagenous fibers did not change significantly (Fig. 1F). Moreover, the *in situ* carcinoma tissues exhibited thicker, compact collagenous fiber

Table I. Association between collagenous fiber and elastic fiber levels, and MMP and TIMP expression levels in hamster tongue carcinoma by Spearman's correlation.

Component	MMP-1		MMP-2		MMP-1/TIMP-1		MMP-2/TIMP-2	
	R	P-value	R	P-value	R	P-value	R	P-value
Elastic fiber	-0.257	0.047 ^a	-0.267	0.039 ^a	-	-	-0.613	0.002 ^b
Collagenous fiber	0.364	0.004 ^b	0.297	0.021 ^a	-0.549	<0.001 ^b	-	-

R represents the coefficient of correlation. ^aCorrelation is significant at the 0.05 level (two-tailed). ^bCorrelation is significant at the 0.01 level (two-tailed).

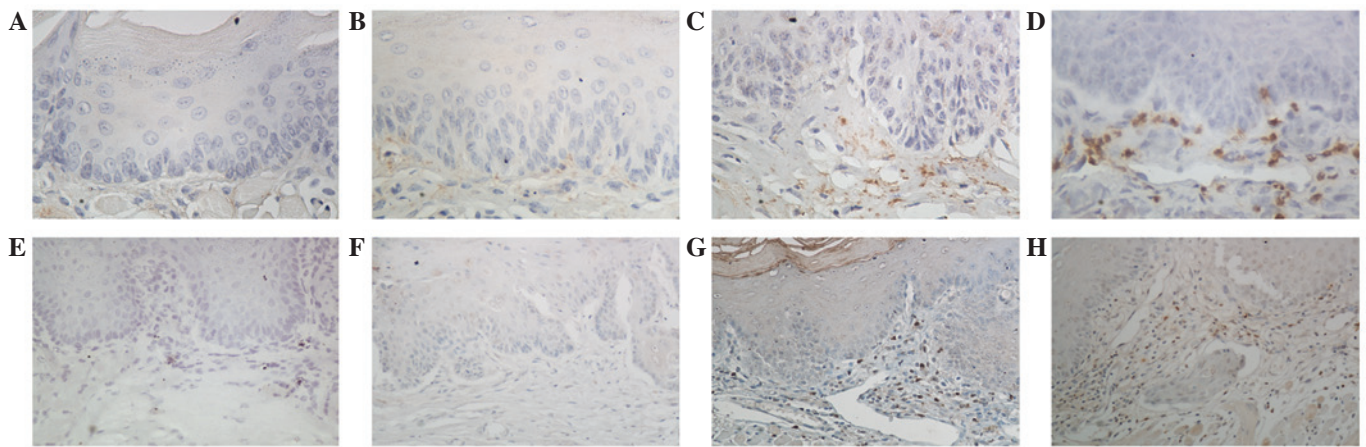


Figure 3. Immunohistochemical staining of (A-D) matrix metalloproteinase-2 (MMP-2; magnification, x400) and (E-H) tissue inhibitors of metalloproteinase-2 (TIMP-1; magnification, x200) during different stages of tongue squamous cell carcinoma. (A and E) Normal tongue mucosa, (B and F) atypical hyperplastic tissues, (C and G) carcinoma *in situ* and (D and H) invasive carcinoma. Positive expression of MMP-1 and TIMP-1 were predominantly observed in the cytoplasm of stromal cells and proliferating epithelial cells as brownish granules.

bundles in the lamina propria (Fig. 1G). In invasive carcinoma, collagenous fiber levels were increased, and fibers appeared compactly distributed, with a deeper staining color (Fig. 1H). The results showed that the expression levels of collagenous fiber was positively correlated with the progression of the tumor ($r=0.619$, $P<0.01$).

Expression of MMP-1 and TIMP-1 during different stages of carcinoma progression. In the normal and atypical hyperplastic tissues, MMP-1 was only expressed in a few epithelial and stromal cells as brownish granules (Fig. 2A and B). In the *in situ* carcinoma tissues, the expression of MMP-1 was mainly found in stromal cells surrounding the epithelial nests of the carcinoma (Fig. 2C). In tongue invasive carcinoma, MMP-1 was expressed in significantly increased levels in the cytoplasm of the stromal cells of cancer nests and around the blood vessels (Fig. 2D). Similarly, the expression of TIMP-1 was extremely weak in the normal tongue mucosa and atypical hyperplastic tissues (Fig. 2E and F). In the *in situ* carcinoma tissues, TIMP-1 expression was mainly observed in the stromal cells surrounding the epithelial nests. Positive expression of TIMP-1 was mainly observed in the cytoplasm of the cancer and stromal cells (Fig. 2G and H).

The expression of MMP-1 increased with the progression of hamster tongue carcinogenesis ($P>0.05$). Additionally, the

expression of TIMP-1 was highly correlated with carcinogenic progression ($r=0.705$, $P<0.01$; $r=0.759$, $P<0.01$).

Expression of MMP-2 and TIMP-2 during the progression of hamster tongue carcinoma. The expression of MMP-2 in the normal tongue mucosal tissues was negative (Fig. 3A). In the atypical hyperplastic and *in situ* carcinoma tissues, the expression of MMP-2 was significantly increased in the epithelial and cancer cells (Fig. 3B and C). In invasive carcinoma, the expression of MMP-2 was mainly distributed in the stromal and cancer cells of the lamina propria, and relatively weak expression was found in the basement cells around the cancer nests (Fig. 3D). TIMP-2 expression was negative or weakly positive in the normal tongue mucosa and atypical hyperplastic tissues (Fig. 3E and F). In the *in situ* carcinoma tissues, the expression of TIMP-2 was mainly found in the stromal cells of the lamina propria (Fig. 3G). In the invasive tissues, TIMP-2 expression was found mainly in the cytoplasm of the cancer and stromal cells as brown or dark-brown granules (Fig. 3H).

Moreover, it was found that the expression levels of MMP-2 and/or TIMP-2 were positively correlated with the progression of the tongue tumor ($r=0.633$, $P<0.01$; $r=0.751$, $P<0.01$, respectively). Additionally, by analyzing the associations between type IV collagen expression (substrate of MMP-2) and the

progression of hamster tongue cancer, it was found that the expression of type IV collagen was gradually reduced with carcinogenic progression ($r=-0.881$, $P<0.001$).

Correlation of elastic and collagenous fibers with MMP and TIMP expression during the progression of hamster tongue carcinoma. High levels of proteases facilitate the degradation of the BM and ECM, providing channels that allow tumor cells to migrate and metastasize through the vasculature system (36). For this reason, the present study next determined whether the levels of the components of the ECM were correlated with the expression of MMPs and/or TIMPs. As shown in Table I, the level of elastic fibers was negatively correlated with the expression of MMP-1 and MMP-2 ($r=-0.257$, $P<0.05$; and $r=-0.267$, $P<0.05$, respectively). The results also showed that the level of collagenous fibers was positively correlated with MMP-1 and MMP-2 protein expression ($r=0.364$, $P<0.01$; and $r=0.297$, $P<0.05$, respectively). The results also showed type IV collagen expression to be negatively correlated with MMP-2 protein expression ($r=-0.552$, $P<0.01$).

MMP activity is controlled by changes in the delicate balance between the expression and synthesis of MMPs and their major endogenous inhibitors, TIMPs. The catalytic competence of MMPs is controlled through the activation of MMP pro-enzymes and their inhibition or activation by TIMPs (37). Therefore, the associations between the ratios of MMP1/TIMP1, MMP2/TIMP2 and ECM components were analyzed (Table I). The results showed that a correlation existed between collagenous fiber level and the ratio of MMP-1/TIMP-1 ($r=-0.549$, $P<0.01$). The associations between elastic fiber level and the MMP-2/TIMP-2 ratio were also significant ($r=-0.613$, $P<0.01$).

Discussion

In the lamina propria of the tongue, elastic fibers provide lingual tissue flexibility, while collagenous fibers provide toughness and support. Type IV collagen, the main component of the BM, can effectively prevent harmful substances from invading the lamina propria. As the main component of the ECM, collagenous fibers are widely distributed throughout the layers of the tongue tissue, and the changes in the structure and content of the ECM directly affect the physiological and/or pathological processes of tissue repair and tumor metastasis (5,38,39).

The present study observed the gradual reduction, damage, fracturing or even disappearance of elastic fibers with DMBA-induced hamster tongue cancer progression. Additionally, the papillae layer of the lamina propria gradually lost its structure during cancer progression. The results showed that the elastic fiber content was lower in the invasive carcinoma tissues than in the normal tissues, and we hypothesize that fracturing of the elastic fibers in the lamina propria allows cancerous cells to move deeper into the tissue, accelerating the process of cancer formation. At the same time, it was also observed that the expression of MMP-2 in invasive carcinoma tissues, which have low elastic fiber levels, was higher than that in the atypical hyperplastic tissues, which have high elastic fiber levels. TIMP-2 protein

expression also increased; however, the difference was not as significant as that observed for MMP-2. Additionally, the results showed MMP-2 expression to be negatively correlated with the level of elastic fibers. An inverse correlation was also demonstrated between elastic fiber content and TIMP-2 expression, suggesting that the MMP-2/TIMP-2 ratio could represent an independent index with which to evaluate cancer progression and invasion. Moreover, the results suggested that the expression of type IV collagen was gradually reduced with carcinogenic progression, and was decreased with increasing MMP-2 expression. These results suggested that damage of the BM components of the epithelial tissues could provide proper channels for tumor cell invasion, which was similar to previously published results (38,40,41). Thus, we speculate that the overexpression of MMP-2 in cancer tissues can accelerate the degradation of type IV collagen, leading to fractures and defects in the BM, thereby reducing its barrier function and promoting the invasion of cancer cells into the lamina propria. So, from the present study and others, we believe that it is the mutual effects of MMP-2 and type IV collagen that create appropriate conditions for tumor cell invasion and metastasis.

Oral submucous fibrosis, a type of collagenosis, results in a precancerous lesion (6,42). The formation of numerous new collagenous fibers was observed in the present study during early-stage carcinogenesis, and this may be the reason that the lamina propria appeared to thicken. Although the collagenous fiber content in the lamina propria was high, the fibers were damaged as the cancer cells broke through the BM during late-stage carcinogenesis. The degradation substrates of MMP-1 are mainly type I and type III collagen. Moreover, MMP-1 expression has been shown to be increased in oral mucosal fibrosis tissues and oral cancer tissues (43). The present study found that MMP-1 expression increased with the progression of tongue cancer; however during the period between carcinoma *in situ* and invasive cancer, MMP-1 expression was retained at a relatively high level, without continuing to increase. Further analysis showed that the MMP1/TIMP1 ratio was reduced with increased collagenous fiber content. Therefore, we speculate that the increase in MMP-1 activity in the ECM, stimulating the production of high levels of TIMP-1, would lead to the inhibition of MMP-1 in a negative feedback loop. Thus, while changes in the expression of MMP-1 and TIMP-1 may lead to the deposition of collagenous fibers in the oral mucosa, they are not the only reason for this phenomenon.

In the present DMBA-induced tongue cancer hamster model, which reproduces a number of the characteristics of the ECM in tongue tissues during carcinogenesis, elastic fiber and type IV collagen degradation, as well as collagenous fiber deposition, was able to be reproducibly measured in the tongue mucosal tissues. The experiments revealed, for the first time, the occurrence of changes in the morphology and content of collagenous and elastic fibers, and type IV collagen during tongue cancer progression in a hamster model. Changes of these matrix components directly or indirectly altered tissue structures, leading to tissue fibrosis. Indeed, the data demonstrated that MMP-2 and TIMP-2 have significant roles in the degradation of the BM and elastic fibers, and that they are intimately involved in tongue cancer

invasion. In addition, MMP-1 and TIMP1 also appear to be involved in tongue cancer progression. Thus, the present study provides a novel visual field for the study of tongue cancer pathology and suggests the importance of fully understanding the molecular mechanisms of tumorous tissue remodeling.

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