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南台灣口腔腫瘤樣本中普遍表現有第一型基質金屬蛋白酵素的情形

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關鍵詞：基質金屬蛋白酵素，口腔癌，第一型基質金屬蛋白酵素

註：前三位作者對本文有相同的貢獻。

摘要

本研究旨在分析與腫瘤相關的第一型基質金屬蛋白酵素〔matrix metalloproteinase-1，MMP-1〕，在南台灣的口腔腫瘤中所扮演的角色。我們利用半定量性的反轉錄 DNA 聚合酵素連鎖反應〔reverse transcription-polymerase chain reaction〕以及第一型基質金屬蛋白酵素與甘油醛-3-磷酸去氫酵素〔glyceraldehyde 3-phosphate dehydrogenase〕基因的啓動子〔primers〕，在 39 個口腔腫瘤樣本與腫瘤旁的組織中偵測 MMP-1 的表現情形。結果顯示在所有 39 對組織中，與腫瘤周圍的組織相較之下，MMP-1 的轉錄本均偏好表現於腫瘤塊中。此外，其中有 5 對組織當初在冰存前已切成數塊樣本，因而可進一步製作成組織萃取液並以西方轉印法〔Western blot〕偵測 MMP-1 蛋白。結果在這 5 對組織的腫瘤樣本中，MMP-1 蛋白均有表現且表現量亦較周圍組織更高。我們因此結論在南台灣的口腔腫瘤中 MMP-1 可能普遍均有表現且集中於腫瘤塊中，此外，MMP-1 也可能在整個口腔腫瘤癌化的發展過程中扮演了重要的角色。

The Universal Expression Pattern of the Matrix Metalloproteinase-1 in Oral Tumor Specimens from Southern Taiwan

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Running title: MMP-1 expression in all oral cancers

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Key words: matrix metalloproteinase; oral cancer, MMP-1

Note: The first three authors contributed equally to this work.

Abstract

The objective of this study is to analyze roles of tumor-associated matrix metalloproteinase-1 (MMP-1) in oral tumor patients from southern Taiwan. Thirty-nine surgical specimens of oral tumors (Ts) and their neighboring tissues (Ns) were subjected to the semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) with the primers of MMP-1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) genes. All of the 39 specimen pairs showed preferential expression patterns of MMP-1 messages in Ts than in Ns (100%). Furthermore, 5 pairs of them, originally stored with more than two pieces of Ts and Ns, were available for further homogenate preparation and MMP-1 protein detection by Western blot. The results demonstrated that MMP-1 proteins were also predominantly expressed in Ts than in Ns in all the 5 specimens tested. We conclude that MMP-1 may be universally and preferentially expressed within tumor masses and plays important roles throughout the entire developmental process of oral cancers in southern Taiwan.

Introduction

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases collectively capable of degrading essentially all components of extracellular matrix (ECM) (1, 2). Dozens of MMP members constitute a large proteinase family, and the MMP-28 has recently been identified (3). Functions of MMPs are required for normal tissue reconstructions, e.g., developmental tissue morphogenesis, wound healing, and angiogenesis (4). Among these functions, disruption of basement membrane by MMPs is thought to be an important feature that is endowed by tumors for their local and distant spreading (5, 6).

MMP-1, also designated as collagenase-1, and fibroblast collagenase, is one of the interstitial collagenases that can be barely detected in intact healthy skin. However, it is prominently and invariably expressed by basal keratinocytes at the migratory front in various forms of cutaneous wounds, and its expression diminishes progressively away from the wound edge (7). It is postulated that for invading the surrounding tissue and/or migrating through the wall of blood vessel, tumors may constantly require the expression and activity of MMP-1. Indeed, over-expression of MMP-1, for examples, has been reported in colorectal and lung carcinomas (8, 9). Enhanced expression of MMP-1 was correlated with the poor prognosis of both colorectal and esophageal cancer patients (10, 11). Furthermore, disruption of MMP-1

activity reduced the malignancy of a colon cancer cell line, DHD/K12 (12). These features make MMP-1 as possible candidate, as a tumor marker, for predicting the malignancy of a tumor and the prognosis of a cancer patient (13).

There are some preliminary studies establishing the relationship between MMPs and oral cancers. For example, activation of MMP-1 gene induced the invasion of a cell line of oral squamous cell carcinoma (OSCC) (14). Furthermore, down-regulation of this gene restrained tumor's invasion capacity in the three-dimensional raft culture and nude mice implantation models (15). Indeed, the enhanced expression of MMP-1 was shown in many cases of OSCC (16, 17). These facts also render MMP-1 as the possible therapeutic target of oral cancers. There were indeed some MMPs inhibitors (MMPIs), shown to inhibit tumor-associated angiogenesis, invasion, and metastasis in the *in vitro* model systems, developed in the past decade. For instances, a potent MMP-1 (and MMP-2, -7, and -9) inhibitor, BMS-275291, was illustrated to inhibit the angiogenesis and metastasis of tumor cells in two murine models (18). Marimastat (BB-2516), the water-soluble form of batimastat, was also shown to inhibit cervical lymph node metastasis in an orthotopic oral squamous cell carcinoma implantation model (19). However, the effectiveness of MMPIs may depend on MMPs expressed in each individual tumor as well as in its surrounding stromal cells. The third generation of MMPIs, ONO-4817, was demonstrated to suppress lung metastasis of

tumor cells expressing MMPs, but not those that did not express MMPs (20).

It is, therefore, important to elucidate the expression pattern of the documented and tumor-associated MMPs in each tumor patient. In this study, we analyzed the expression pattern of MMP-1 in 39 oral tumor specimens from southern Taiwan.

Materials and Methods

Subjects

The major sites, stages, sizes and differentiation states of the 39 tumor specimens are listed in Table 1. Tumor masses and its surrounding tissues were separately collected, soaked in liquid nitrogen, and kept at -80℃ for subsequent RNA or tissue homogenate extraction.

RNA Isolation

Cellular RNA was isolated from frozen tissue specimens using the Ultraspec™ RNA isolation system (Biotecx Laboratories Inc., Huston, Texas, USA). Tissue was homogenized with 1 ml of Ultraspec™ reagent in a handhold glass-Teflon. Following homogenization, the homogenate was stored at 4℃ for 5 min, then extracted with 0.2 ml of chloroform and centrifuged at 12,000 g (4℃) for 15 min. RNA in the aqueous phase was precipitated with an equal volume of isopropanol and washed twice with 75% ethanol. The content of RNA was assessed (1 OD of A260 equal to 40 μg/ml RNA) spectrophotometrically, and the purity of the extract was assessed using the A260/280 ratio, which in all cases was above 1.75.

Synthesis of First-strand Complementary DNA (cDNA)

First-strand cDNA was synthesized using oligo-dT priming of total RNA with SuperScript™ II RNase H^- reverse transcriptase (Life Technologies Inc.,

Gaithersburg, Maryland, USA). The reverse transcription reaction was performed in a final volume of 20 μl that consisted of 15 mM of $MgCl_2$, 50 mM of Tris-HCl (pH 8.3), 375 mM of KCl, 10 mM of dithiothreitol, 1 mM of dNTP mix (10 mM of each dATP, dGTP, dCTP and dTTP), 20 units of rRNasin ribonuclease inhibitor, 200 units of reverse transcriptase, 2 μM of Oligo $(dT)_{15}$, and 0.1 μg of total RNA. The reaction was allowed to proceed at 42℃ for 60 minutes. The samples were heated at 70℃ for 15 min, and then were stored at -20℃.

Amplification of cDNA by Polymerase Chain Reaction (PCR)

The nucleotide sequences of the PCR primers specific to MMP-1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) genes are listed in Table 2. These primers were designed according to the full-length cDNA sequence of specific genes in Genbank and their specificity was confirmed by BLAST (National Library of Medicinem Bethesda, Maryland, USA) Internet software-assisted search of nonredundant nucleotide sequence database. The PCR reaction was performed with one tenth of the reverse-transcribed cDNA as template in a final volume of 20 μl with DNA polymerase reaction buffer [10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 50 mM KCl and 0.1% Triton X-100] containing 200 μM of dNTP mix, 1 unit of DyNAzyme^{TM} II DNA Polymerase (Finnzymes Inc., Finland), and 0.5 μM of sense and anti-sense primers. PCR

amplifications were carried out in a thermalcycler (Whatman, Biometra Inc., Germany) with temperature profiles as follows: initial melting at 94℃ for 4 min, then 30 cycles of one-minute melting at 94℃, 1 min of annealing at 60℃, and extension at 72℃ for 1 minute. RNA sample without reverse transcription was routinely used as a negative control in PCR amplification. Amplified products were separated by electrophoresis with 0.1 μg GeneRuler^{TM} 100 bp DNA Ladder (MBI Fermentas, Lithuania) in 1.5% agarose gels and then visualized under UV light after being stained with 0.5 μg/ml ethidium bromide.

Western Blot Analysis

Frozen tissues stored at -80℃ were melted on ice, cutting to pieces and suspended in NET buffer (0.5% NP-40, 2 mM EDTA, 50 mM Tris-HCl, pH 7.4) containing 1X protease inhibitor cocktail set I (CALBIOCHEM-NOVABIOCHEM Corp., San Diego, California, USA). The suspensions were sonicated at 25% amplitude for 1 min at 1-second interval by the sonicator (SONICS & MATERIALS, Inc., Newtown, Connecticut, USA). Protein concentrations were then determined by Bio-Rad Protein Assay reagent (Bio-Rad Laboratories, Hercules, California, USA) and 20 μg of total proteins of each tissue homogenate were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were transferred onto nitrocellulose (NC) paper and immunoblotted with 1 μg ml^{-1} of

anti-MMP-1 monoclonal antibody (SIGMA, Saint Louis, Missouri, USA) in TBS buffer (50 mM Tris-HCl, pH 7.35, 0.85% NaCl) containing 1% lipid-extracted milk at room temperature for 1 hr. After washing three times with washing buffer (50 mM Tris-HCl, pH 7.35, 0.85% NaCl, 0.5% Tween 20), the NC paper was further incubated with 10^4 diluted Goat-anti-mouse-IgG monoclonal antibody coupled with horse radish peroxidase (HRP) (CHEMICON, International, Temecula, California, USA) in the same buffer as the first antibody at room temperature for 1 hr. The NC paper was then extensively washed, and signals of MMP-1 protein were detected with WESTERN LIGHTENING^{TM} Chemiluminescence Reagent (PerkinElmer Life Sciences, Inc., Boston, Massachusetts, USA) as instructed by the manufacturer. To serve as the protein loading control, the NC paper was stained with Ponceau S solution (Sigma Diagnostics, Inc., St. Louis, Montana, USA) before immunoblotting with MMP-1 antibody, and destained with water to the conditions with clear and visible bands.

Results

RT-PCR

Tumors and their neighboring tissues from oral tumor patients were continuously collected and stored at -80℃ in the past two years. Reverse transcription-polymerase chain reaction (RT-PCR) was utilized for the measurement of MMP-1 messages expressed in these tissue samples. Total cellular RNAs extracted from surgical specimens of 39 oral tumor patients were used as the templates for the subsequent cDNA preparation. After the reactions of reverse transcription, signals of MMP-1 cDNA were further amplified by PCR with specific MMP-1 primers (Table 1). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), an intracellularly housekeeping gene, was included in the RT-PCR and served as a control for cDNA amount. Firstly, to make sure that the PCR products were linearly correlated to the PCR cycles, optimal numbers of PCR cycles for MMP-1 and GAPDH genes were determined. The cDNA from one of these 39 tumor specimens was subjected to PCR analysis, and products of MMP-1 and GAPDH were detected at 24 and 21 cycles and reached the maximal level at 30 and 27 cycles, respectively (Figure 1). Accordingly, 27 and 24 PCR cycles may represent the ideal conditions for the quantitation of MMP-1 and GAPDH genes, respectively. With these PCR conditions, surgical specimens from all the 39 patients showed similar results and the results of 5 oral

squamous cell carcinomas (OSCCs) are shown in Figure 2. Relatively equivalent levels of GAPDH signals in both tumors (T) and their neighboring tissues (N) indicate that the starting mRNA of T and N from patients 1-5 used for RT-PCR were about the same amount. However, the stronger signals of MMP-1 in T than in N suggest that MMP-1 mRNA is overexpressed in these tumor masses (Figure 2).

Western Blot Analysis

To access whether MMP-1 protein molecules are also preferentially expressed in tumors, tissue homogenates, prepared from the specimens originally preserved with more than 2 tissue masses, of the above 5 OSCC tissue pairs were subjected to immunoblotting with MMP-1 monoclonal antibody. As shown in Figure 3 (upper panel), both latent 53 kDa (Pro-MMP-1) and active 51 kDa forms of MMP-1 proteins could be recognized by this antibody. The expressions of MMP-1 proteins are higher in T than in N in all the 5 tissue pairs tested.

The 20 μg homogenate proteins separated by SDS-PAGE and transferred onto NC paper were stained by Ponceau S solution and photographed prior to MMP-1 immunoblotting (Figure 3, lower panel). Results indicate that the total proteins applied in each of N and T tissue pair were relatively identical.

Discussion

Signals representing MMP-1 mRNA could be detected in the N of patients 1-4 (Figure 2), however, its protein molecules were not detected in all of the patients (Figure 3, upper panel). This may be due to the differential sensitivity between these two methods. Although RT-PCR is often suspected to bring about false positive results, there were no any detectable MMP-1 or GAPDH PCR products of every tested tissue specimens whenever the RT reactions were skipped in the RT-PCR process (data not shown). In addition, the universal expression of MMP-1 proteins in 5 OSCC homogenates eliminates and also minimizes the possibility of their own and in the rest of 34 tumor specimens' false RT-PCR positivity, respectively (Figure 2 and Figure 3). We therefore, conclude that both MMP-1 mRNA and proteins are commonly expressed and concentrated in the tumor masses.

Within tumor masses, whether MMP-1 molecules were expressed by tumor cells or the surrounding stromal cells remains unclear. Most likely, it is the stromal cells of these oral tumors that synthesize and secrete MMP-1 molecules as described by Nelson *et al* in other cancers (21). They employed the *in situ* hybridization analysis and localized MMP-1 mRNA primarily in stromal fibroblasts, especially in proximity to invading cancer cells, but not in the carcinoma cells of human breast, colorectal, lung, prostate, and ovarian cancers.

In this study, we have demonstrated the 100% preferential expression pattern of MMP-1 in oral tumor tissues by RT-PCR. These results suggest the functions of MMP-1 are important in the tumorigenesis of oral tumors in Taiwan. The unexpected universal expression pattern of this molecule, although the underlying mechanism is not clear, might be related to the common betel-chewing habit in these oral tumor patients. Among these 39 oral tumor patients, 36 (92.3%) of them had fallen into this oral habit. Sixteen and 12 of them chewed more than 20 and 10-20 betel nuts a day, respectively. It may provide a continuous stimulus to the buccal mucosa and gingiva, which resulted in chronic inflammatory conditions in these locations, such as leukoplakia, submucous fibrosis, and premalignant lesion. Since MMP-1 is known to be involved in wound healing processes (7, 22), it may be constantly expressed in these betel nut users. This speculation is further strengthened by the observation that there are high incidences of buccal and gingival inflammations as well as carcinomas in the betel users in southern Taiwan (23). In this study, all of these 39 patients came from southern Taiwan, and tumors from 27 (69.2%) of them localized to these two sites (Table 1).

Finally, the 39 specimens collected from different sites, stages, differentiation states, and sizes of oral tumors were not correlated to MMP-1 expression (Table 1). We propose that MMP-1 may be constantly and commonly expressed in

betel-chewing oral tumor patients. It is not known, but possible, that MMP-1 might play more important roles in this type of tumors than those without betel-chewing oral habit. This may provide an insight for a new therapeutical strategy by interfering MMP-1 activities in these oral cancers, although some clinical trials with MMPs inhibitors have been discouraging (24). As both tumors and their neighboring stromal cells may express a variety of MMPs, blocking one of these MMPs may not be sufficient to attenuate their invading and/or metastatic potential. However, as more of the important and common factors having been identified, such as MMP-1, they may serve as the targets for the new drugs or agents capable of intervening their functions. If one or more capacities vital for the unlimited growth, invasion, and metastasis of cancers can be inhibited, the improved prognosis and survival rate of these patients may then be expected.

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Table 1 The sites, stages, differentiation states, sizes of the 39 oral tumors patients

Sites[1]		Stages[2]		Differentiation states		Sizes	
BM	18[3]	PML	7	Well	17	T1	2
G	9	I	2	Moderate	14	T2	14
T	7	II	9	Poor	2	T3	1
MF	2	III	2			T4	15
SP	2	IV	19				
R	1						

[1]BM: buccal mucosa; G: gingiva; T: tongue; MF: mouth floor; SP: soft palate; R: retromolar trigone.

[2]PML: premalignant lesion.

[3]Sample numbers are listed after each item.

Table 2. PCR primer sequences and size of expected products for MMP-1 and GAPDH

Gene	Forward Primer	Reverse Primer	Size of Product (bp)
MMP-1	CATGACTTTCCTGGAA TTGGCCAC	TTGAGCTGCTTTTCCTC CGGCAAA	540
GAPDH[1]	GTGAAGGTCGGAGTC AACG	CAATGCCAGCCCCAGC G	895

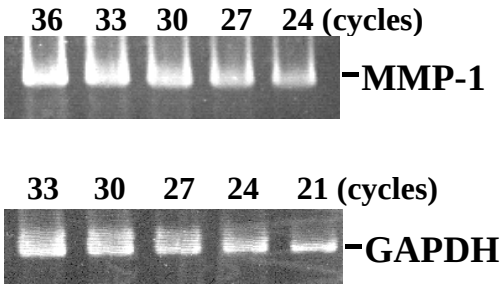
[1]GAPDH is the glyceraldehyde 3-phosphate dehydrogenase.

Legend

Figure 1. Determination of optimal PCR cycles for MMP-1 and GAPDH genes. Total cellular RNA from one of the tumor specimens was subjected to RT-PCR analysis as described in Materials and Methods. Different numbers of PCR cycles are listed on the upper panel (MMP-1) and lower panel (GAPDH).

Figure 2. Semi-quantitative RT-PCR analysis. Total cellular RNAs from tumors (T) and their adjacent tissues (N) were analyzed by RT-PCR as described in Materials and Methods. PCR products of MMP-1 (540 bp) and GAPDH (895 bp) from 5 patients are indicated on the right side of each panel. M: marker, 100 bp-DNA ladder (from 100 bp to 1000 bp).

Figure 3. Western blot analysis of tissue homogenates. For each tissue sample, 20 μg of homogenate proteins were applied to SDS-PAGE and transferred to NC paper. Upper panel: NC paper was immunoblotted with anti-MMP-1 monoclonal antibody as described in Materials and Methods. Lower panel: Before the incubation with MMP-1 antibody, the NC paper was firstly stained with the Ponceau S solution and photographed.



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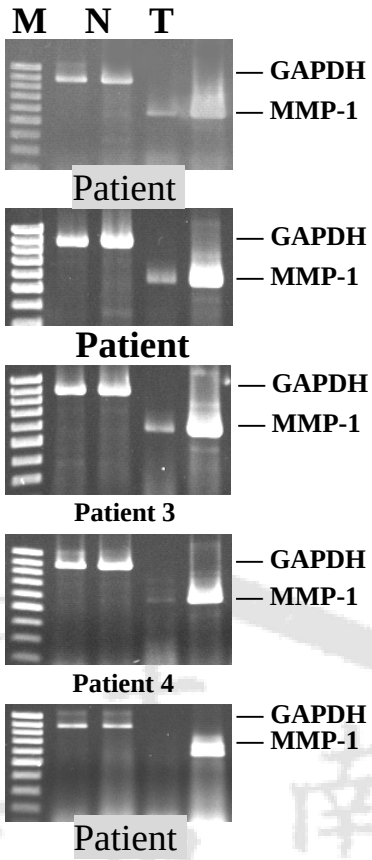

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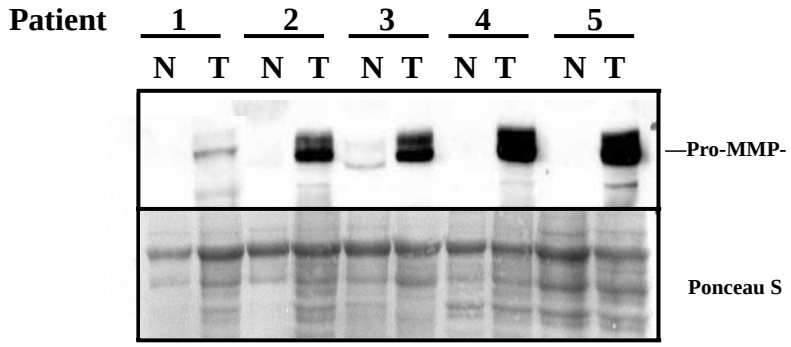

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