

## ARTICLE

## Expression of Membrane type 1-Matrix Metalloproteinase in Laryngeal Carcinoma

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Membrane type 1-matrix metalloproteinase (MT1-MMP) is a member of the recently identified unique membrane-type subgroup in the matrix metalloproteinase (MMP) family. MT1-MMP has proteolytic activity against components of the extracellular matrix and activates progelatinase A (MMP-2) at the cell surface. In this study, we analyzed the expression of MT1-MMP mRNA in 45 cases of laryngeal carcinoma by RT-PCR and investigated the relationship between MT1-MMP expression and survival in 18 cases. The result showed that the expression of MT1-MMP mRNA was higher in tumor tissue than in corresponding normal tissue.

The tumoral expression in clinical stage III was higher than in stage II. The tumoral expression level of MT1-MMP mRNA in patients with lymph node metastasis was significantly higher than those with negative lymph nodes. The patients with high expression level showed significantly poorer 5 year survival than those with low expression level. Collectively, our findings suggest that the high level of MT1-MMP expression is closely related to the invasion and metastasis of laryngeal carcinoma, and indicates poorer prognosis. (Pathology Oncology Research Vol 5, No 3, 214–217, 1999)

*Keywords:* laryngeal carcinoma, MT1-MMP, invasion, metastasis

### Introduction

Cancer cell metastasis to distant organs is the major cause of death in cancer patients. Metastasis is a multi-step process, and the initial step is considered to be the invasion of surrounding stromal tissue by cancer cell. The degradation of extracellular matrix (ECM) components is essential for tumor cells to invade surrounding tissue and to form metastatic colonies in other distant organs and lymph nodes. This invasive process is thought to involve multiple proteolytic enzymes including matrix metalloproteinases (MMPs), which are secreted by tumor cell.<sup>1,2</sup>

Overexpression of MMPs in tumor tissues is associated with invasion and metastasis in many different carcinoma

types. MMP-2 and MMP-9 are believed to play a major role in tumor invasion by degradation of type IV collagen, which is the main component of the epithelial ECM.<sup>3,4</sup> MMP-9 is activated by other proteases such as trypsin, stromelysin or collagenase and MMP-2 has been shown to be activated by membrane type 1 MMP (MT-MMP). MT1-MMP has been studied most extensively among the MT-MMPs. MT1-MMP can introduce specific cleavage in the propeptide domain of proMMP-2 and induces autocatalytic activation on the cell surface.<sup>5,6</sup> Because MMP-2 is one of the most important type IV collagenase to degrade the basement membrane in tumors, MT1-MMP is believed to enhance invasive potential when it is expressed on tumor cell.<sup>7</sup>

In the present study, we analyzed the expression of MT1-MMP mRNA in 45 cases of laryngeal carcinoma and investigated the relationship between MT1-MMP mRNA expression and the survival in 18 cases of laryngeal carcinoma. We reported that MT1-MMP expression may be a good indicator to determine the malignant behavior of laryngeal carcinoma.

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## Materials and Methods

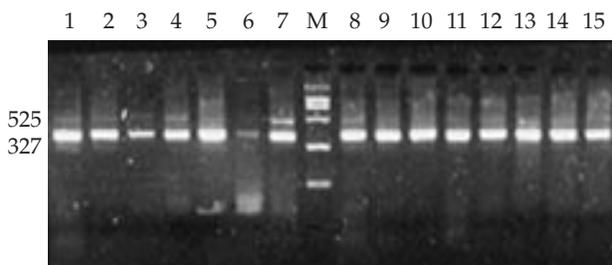
### Clinical specimens

Fresh surgical specimens of 45 primary laryngeal squamous cell carcinoma and their paired adjacent normal laryngeal tissues were used for our study. After resection, all tissue specimens were frozen in liquid nitrogen and kept at  $-90^{\circ}\text{C}$  until use. The specimens were prepared for RNA extraction.

### RNA extraction and RT-PCR

**Isolation of total RNA.** – Total cellular RNA from laryngeal tumors was isolated by a modified phenol-guanidinium isothiocyanate method. Laryngeal tumor were homogenized in lysis buffer 500  $\mu\text{l}$  of GITC (4 M guanidinium isothiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, 0.1M  $\beta$ -mercaptoethanol). All subsequent steps were performed on ice and pre-chilled solutions were used. Homogenates were centrifuged at 900  $g$  for 15 min and supernatants were transferred to a fresh microfuge tube. Subsequently, 50  $\mu\text{l}$  of sodium acetate (3 M pH 4.0), 250  $\mu\text{l}$  of water-saturated phenol and 250  $\mu\text{l}$  of chloroform-isoamyl alcohol (49:1,v/v) were added and mixed well by vortexing. The mixture was left on ice for 10 min, after which it was centrifuged at 10000  $g$ . The supernatant was transferred to a fresh microfuge tube and after adding double volume of cold isopropanol and it was placed at  $-20^{\circ}\text{C}$  for 24h. RNA was pelleted by centrifugation at 15000  $g$ , re-precipitated in 150  $\mu\text{l}$  of GITC and isopropanol. RNA was pelleted by centrifugation as described above, and washed with 70% ethanol. The pellet was dissolved in 30  $\mu\text{l}$  of DEPC-treated water. The integrity of the RNA samples was assessed on formaldehyde-containing agarose gels.

**Reverse transcription.** – First-strand cDNAs were synthesized by reverse transcription of 1  $\mu\text{g}$  of total RNA in 20  $\mu\text{l}$  of a AMV active buffer containing 100 ng random oligonucleotide primers, 10 U AMV (Promega), 1U



**Figure 1.** Rt-PCR analysis of MT1-MMP mRNA expression in laryngeal carcinoma. 1,2 = moderately differentiated tumors; 3,4,7 = LN+ tumors; 5,9,10,11,15 =LN- tumors; 6 = normal tissue; 8,12,13 = poorly differentiated tumors. Marker: DL2000

RNasin, 0.5 mM dNTP. Following a 1h incubation at  $42^{\circ}\text{C}$ , the reverse transcriptase was heat-inactivated at  $94^{\circ}\text{C}$  for 4 min.

**DNA amplification.** – MT1-MMP and  $\beta$ -actin cDNAs were amplified using 2  $\mu\text{l}$  cDNAs as template in 50  $\mu\text{l}$  amplification buffer containing 10 mM Tris-HCl, 50 mM KCl, 50  $\mu\text{M}$  each of the four deoxyribonucleoside triphosphates, 3 mM  $\text{MgCl}_2$ , 0.1 mg/ml bovin serum albumin, 1 U Taq DNA polymerase (MBI), and 200 nM each of a pair of oligonucleotide primers specific for MT1-MMP and  $\beta$ -actin. These sequences were as follows.

MT1-MMP: sense: 5-ACGGAGGTGATCATCATTG-AGG-3, antisense: 5-AGATGGGGGCTGGACAGACA-CA-3.

$\beta$ -actin: sense: 5-GATGGCCAGGTCATCACCATTG-GC-3, antisense: 5-GAAGGTGGACAGTGAGGCCAG-GAT-3.

Amplification was performed in 60-well plates using a PTC-100 programmable thermal controller (MJ Research). Amplification were cycled for 30 (denaturing at  $94^{\circ}\text{C}$  for 45s, annealing at  $55^{\circ}\text{C}$  for 45s, elongation at  $72^{\circ}\text{C}$  for 90s). Before amplification all samples were subjected to an initial denaturation for 5 min. The final elongation step was extended by 10 min. Amplification products were analyzed by agarose gel electrophoresis, and ethidium bromide staining. The level of MT1-MMP mRNA was quantitated using the ratio of OD value of PCR products of MT1-MMP and  $\beta$ -actin.

### Statistical analysis

Association between the variables were tested by t-test. Survival analysis was done by Kaplan-Meier method and Log-Rank test.

## Results

### MT1-MMP mRNA levels in laryngeal carcinoma

The MT1-MMP mRNA could be detected in all of the laryngeal carcinoma specimens and 29 of 45 (64.44%) adjacent tissues also showed MT1-MMP mRNA expression (Figure 1). As shown in Table 1, the expression of MT1-MMP in tumor specimens was similar to the adjacent tissues and there was no difference between MT1-MMP expression and histological differentiation of the tumors.

However, when tumors were grouped into staging categories we have found that the tumoral expression of MT1-MMP in stage III and IV was higher than in stage I and II ( $P < 0.05$ ) (Figure 2). Furthermore, the expression level of MT1-MMP mRNA in the patients with lymph node metastasis was significantly higher than those with negative lymph nodes ( $P < 0.01$ ) (Figure 3).

**Table 1. MT1-MMP expression and clinicopathologic data**

Variable	N	MT1-MMP/ $\beta$ -actin
<i>Tissue type</i>		
Laryngeal carcinoma	45	0.4511 $\pm$ 0.1052
Adjacent tissue	29	0.4018 $\pm$ 0.0680
<i>Histological differentiation</i>		
Good	8	0.3825 $\pm$ 0.1004
Moderate	27	0.4014 $\pm$ 0.0992
Poor	10	0.3927 $\pm$ 0.1235

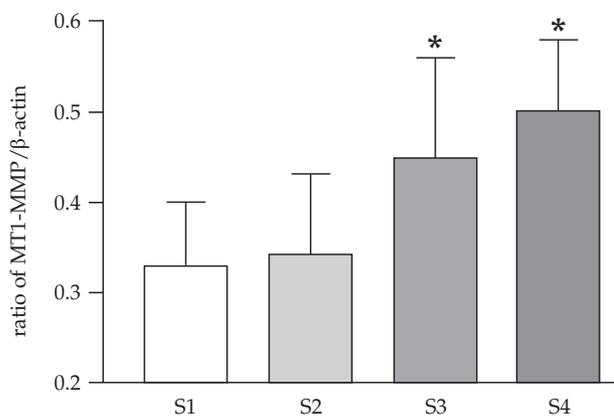
Data are expressed as ratio of the densities of the respective bands.

#### MT1-MMP expression and prognosis

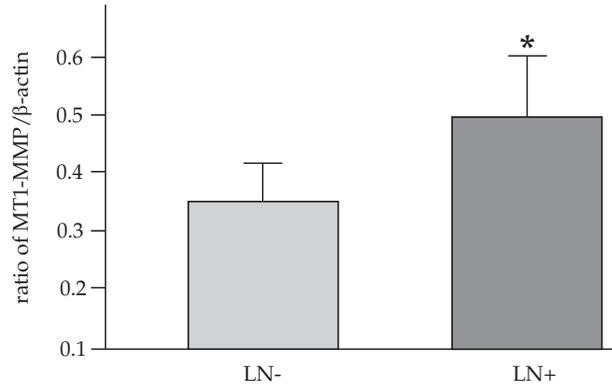
18 of 45 cases were analyzed for the relationship between MT1-MMP mRNA expression and 5-year survival. Samples were divided into two groups by the average rate of MT1-MMP/ $\beta$ -actin. The patients with high expression level (n=9) showed a significantly poorer prognosis than those with low expression level (n=9,  $p < 0.01$ ) (Figure 4).

#### Discussion

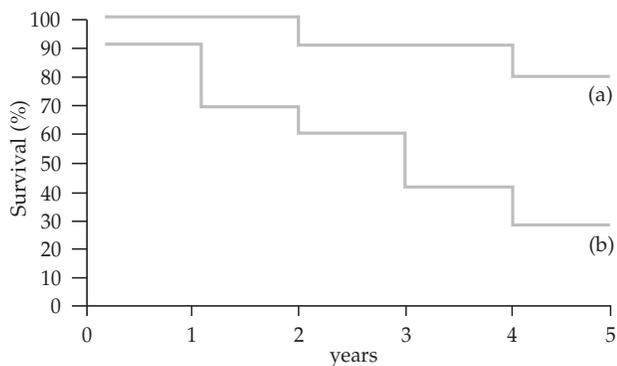
MT1-MMP is a cell surface activator of MMP-2 and is believed to be a key enzyme for tumor cell invasion and metastasis.<sup>3,7</sup> Therefore, the tumors with higher expression of MT1-MMP may be prone to invade tissues through the activation of pro-MMP-2, therefore MT1-MMP could only be an active player in invasion together with MMP-2. On the other hand, few authors have reported the clinical significance of MT1-MMP expression with regard to tumor invasion or metastasis in clinical samples of carcinomas.



**Figure 2.** Expression of MT1-MMP relative to  $\beta$ -actin in laryngeal cancers according to clinical stages. \* =  $p < 0.05$



**Figure 3.** Expression of MT1-MMP relative to  $\beta$ -actin in laryngeal cancers according to nodal status. LN = lymph node; + = presence of metastasis; \* =  $p < 0.05$



**Figure 4.** Survival curve and MT1-MMP expression. Patients with higher MT1-MMP expression (a) (n=9) exhibit a significantly poorer prognosis ( $p < 0.01$ ) than those with lower MT1-MMP expression (b) (n=9).

Bando et al, studied MT-MMP expression in gastric cancer and found that patients with MT-MMP-positive tumor had a significantly worse prognosis than those with MT-MMP-negative tumor.<sup>8</sup> Multivariate analysis showed MT-MMP overexpression as an independent prognostic factor in gastric cancer patients. Mori et al, found that the expression of MT1-MMP may influence prognosis via tumor invasion of the gastric wall and lymph node metastasis and MT1-MMP activation of MMP2 may be clinically relevant in gastric carcinoma tumors.<sup>9</sup> Yamamoto et al, reported that among brain tumors MT1-MMP mRNA levels were significantly higher in malignant astrocytomas than in low-grade gliomas and in normal brain tissues.<sup>10</sup> Gilles et al, reported a higher level of MT1-MMP expression in invasive cervical carcinoma and lymph node metastases compared with its expression in non-invasive lesions.<sup>11</sup> Tokuraku et al, studied 58 cases of lung carcinoma and observed that MT1-MMP was higher in metastasizing tumors.<sup>12</sup> These findings indicate that MT1-MMP is highly

expressed in various tumor tissues and correlates well with the malignant behavior.

With respect to laryngeal carcinoma, Yoshizaki et al, studied the expression of MT1-MMP in head and neck carcinoma<sup>13</sup> and found an increased expression level of MT1-MMP mRNA in all head and neck tumor tissues but not in normal tissues or inflammatory lymph nodes. Our results contradict that observation since the expression levels of MT1-MMP in 45 laryngeal carcinoma were similar to normal tissues. However, tumors at higher clinical stages (III and IV) expressed more MT1-MMP mRNA than low stage tumors. We have also demonstrated that tumors with positive lymph nodes express significantly more MT1-MMP than those without metastasis. On the other hand our results suggest that MT1-MMP expression does not correlate with the level of histological differentiation of tumors. The significance of MT1-MMP expression in laryngeal cancer was further corroborated by analysis of the 5-year survival of 18 patients. This study indicated a significant difference in survival of high MT1-MMP expressing tumors compared to the low expressors. Since the molecular target of MT1-MMP is MMP-2, it is noteworthy to mention in this respect, that increased expression of MMP-2 was demonstrated earlier in a highly aggressive subgroup of laryngeal cancers.<sup>14</sup> In conclusion, we can suggest that the high level of MT1-MMP expression in laryngeal carcinoma correlates to the metastatic potential and influences the progression of the disease.

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