Expression of Matrix Metalloproteinases and Their Inhibitors Correlates With Invasion and Metastasis in Squamous Cell Carcinoma of the Head and Neck

Pornchai O-charoenrat, MD; Peter H. Rhys-Evans, FRCS; Suzanne A. Eccles, PhD

Background: Matrix metalloproteinases (MMPs) have been implicated in the invasion and metastasis of head and neck squamous cell carcinoma (HNSCC). However, a detailed analysis of MMPs and tissue inhibitors of MMPs (TIMPs) in relation to the biological behavior of HNSCC has yet to be performed in clinical material.

Objectives: To study a comprehensive profile of MMPs and their 2 main inhibitors in HNSCC tissue samples and to correlate the patterns of expression with clinicopathological characteristics, invasion, and metastasis.

Design: This study included 54 consecutive patients with primary HNSCC, 27 of which showed lymph node metastasis. Expression of MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, MMP-10, MMP-11, MMP-13, TIMP-1, and TIMP-2 was simultaneously analyzed in tissue homogenates using semiquantitative reverse transcription–polymerase chain reaction assay. Where feasible, levels of protein and enzyme activity were confirmed by Western blot, enzyme-linked immunosorbent assay, and substrate zymography. Conventional clinicopathological features, including mode of tumor invasion, were also examined.

Results: Significantly higher MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, MMP-10, MMP-11, MMP-13, and TIMP-1 levels were found in tumors vs specimens of matched normal mucosa. No difference in the distribution of MMPs and TIMPs in relation to age, sex, tumor site, or histological grade was observed. A significant correlation was demonstrated between levels of MMP-1, MMP-9, and TIMP-1 and advanced T stage and between MMP-9 expression and an infiltrative pattern of growth. Enhanced expression of MMP-9 was strongly correlated ($P < .001$) and levels of MMP-2, MMP-7, and MMP-11 were weakly correlated ($P = .03-.05$) with lymph node involvement.

Conclusions: Overexpression of multiple MMPs and TIMPs is characteristic of HNSCC, and analysis of specific MMPs, MMP-9 in particular, might be useful for evaluating the malignant potential in individual HNSCC.


ORIGINAL ARTICLE

From the Division of Head and Neck Surgery, Department of Surgery, Siriraj Hospital Medical School, Bangkok, Thailand (Dr O-charoenrat); the Head and Neck Unit, Royal Marsden Hospital, London, England (Dr Rhys-Evans); and the Section of Cancer Therapeutics, Institute of Cancer Research, Sutton, England (Dr Eccles).
account for a separate class based on the presence of a fibronectinlike domain. Gelatinases are able to cleave both the denatured forms of collagen and type IV collagen found in basement membrane. Matrix metalloproteinase 2 and MMP-9 also contain a gelatin-binding domain that endows them with high affinity for gelatin. The last group of MMPs contains the membrane-type MMPs (MT-MMPs), which are composed of MMP-14 (MT1-MMP), MMP-15 (MT2-MMP), MMP-16 (MT3-MMP), MMP-17 (MT4-MMP), and MMP-24 (MT5-MMP). Membrane-type MMPs have the unique property of possessing a hydrophobic sequence at the C-terminus, which allows insertion of the protein into the cell membrane. Some MMPs cannot be grouped into any of these classes, including MMP-12 (metalloelastase), MMP-18, MMP-19, MMP-20 (emelysin), and MMP-23. These enzymes differ in substrate specificity, regulation, tissue-specific expression, and potential interactions with additional MMP and TIMP family members. Expression of MMP activity can be controlled at the level of gene transcription, by proenzyme activation and by broad-spectrum and specific inhibitors. Tumor cells might induce the host cells within the surrounding stroma to secrete these enzymes or vice versa. Most MMPs are secreted as latent proenzymes that undergo proteolytic cleavage of an amino-terminal domain during activation. The net activity of MMPs is determined by the amount of proenzyme expressed, the extent to which the proenzyme is activated, and the local concentration of specific tissue inhibitors of MMPs, i.e., TIMPs.

A multigene family of proteins named TIMPs has been demonstrated to inhibit fully activated MMPs. Tissue inhibitors of MMPs comprise at least 4 members, and, together, they provide a tightly regulated mechanism for control of MMP activation and function. Tissue inhibitor of MMP-1 and TIMP-2 have molecular weights of 28.5 and 21.0 kD, respectively, and seem to act by forming 1:1 stoichiometric complexes with the active MMP. Tissue inhibitor of MMP-1 can inhibit the collagenases, MMP-3, and the gelatinases. Tissue inhibitor of MMP-2 binds preferentially to MMP-2 but also inhibits the activities of MMP-1, MMP-3, MMP-7, and MMP-9. The
sulfate–polyacrylamide gels copolymerized with 0.1% (wt/vol) gelatin or 0.03% β-casein. Gelatinolytic or caseino-
ytic enzymes were detected as transparent bands on the
blue background, and the intensity of the bands was mea-
sured using image analysis software (Quantiscan, Cam-
bridge, England), as described previously.20 Results were
expressed in arbitrary units per 40 µg of total protein.
Conditioned medium from 12-O-tetradecanoyl-phorbol-
13-acetate (TPA)–treated HT-1080 fibrosarcoma cell line
and TPA-treated MDA-MB 231 mammary carcinoma cells
served as a positive control and a standard for intergell
variations for gelatin zymography and casein zymography,
respectively.

QUANTITATION OF MMP-1, TIMP-1, AND TIMP-2
BY ENZYME-LINKED IMMUNOSORBENT ASSAY

Concentrations of MMP-1, TIMP-1, and TIMP-2 in the same
tissue homogenates (300 µg per sample) as used in zymog-
raphy were measured using commercially available enzyme-
linked immunosorbent assay (ELISA) kits (Chemicon In-
ternational Inc, Temecula, Calif). The values measured
represent pro–MMP-1, TIMP-1, and TIMP-2 concentra-
tions, with a range of detection at 0.16 to 10.0, 1.2 to 49.0,
and 20.0 to 320.0 ng/mL, respectively. Results were cal-
culated as nanograms per 1 mg of total protein tissue ex-
tracts. Two independent experiments were performed. In
each experiment, tissue lysates were prepared from 2 sepa-
rate pieces of the same tissue specimen, and ELISA values
were measured in duplicate for each sample.

WESTERN BLOT ANALYSIS

Equal amounts of protein (100 µg) from the same tissue
homogenates as used in zymography were resolved un-
der reducing condition in 10% and 15% sodium dodecyl
sulfate–polyacrylamide gel electrophoresis for MMP and
TIMP detection, respectively, and transferred onto a nitro-
cellulose membrane (Highbond-C extra; Amersham Inter-
national Inc, Buckinghamshire, England), then probed with the
appropriate primary antibody. Antibodies to MMP-2,
MMP-3, MMP-7, MMP-9, and MT1-MMP were provided
by British Biotech (Oxford, England). Antibodies to TIMP-1
and TIMP-2 were purchased from Chemicon (Harrow,
England). Blots were washed, incubated with a secondary
antibody coupled to horseradish peroxidase (Serotec, Ox-
ford), and developed using the luminol reagent (Santa Cruz
Inc, Santa Cruz, Calif) and Kodak X-OMAT AR film (East-
man Kodak, New York, NY) with an intensifying screen.
Levels of proteins were determined by image analysis us-
ing Quantiscan software. As a negative control, the pri-
mary antibody, which was preabsorbed with correspond-
ing proteins overnight at a ratio of 1:10, or normal serum
was reacted with the membrane filter. The specific bands
were absent when the preabsorbed antibody or normal se-
rum was used. Purified human MMP-2/MMP-9, MMP-3/
MMP-7, TIMP-1 (28 kd), and TIMP-2 (24 kd) were ob-
erved statistically significant. Unless otherwise stated, each
experiment was performed twice with virtually identical
results.

RESULTS

EXPRESSION OF MMPs AND TIMPs IN HNSCC

Lesions from primary HNSCC (n=54) and LNM (n=27)
and histologically normal adjacent mucosa (n=32) were
examined for mRNA levels of multiple MMPs and TIMPs.

Using semiquantitative RT-PCR assay, mRNA expres-
sion of MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, MMP-
10, MMP-13, and TIMP-1 was significantly greater in ma-
lignant tissues (primary tumors and/or LNM) compared
with mRNA levels in histologically normal mucosa (P=.02
to <.001) (Figure 1). No significant differences were
found between mRNA levels of MMP-14 (MT1-MMP) and
TIMP-2 in tumors and control tissues. In addition, no
differences were found between expression of most MMPs
tested in primary HNSCC vs LNM, except for the MMP-3
gene, where the levels in primary tumors (14.55±3.91)
were significantly higher than those in LNM (1.09±0.32
(P=.03).

Levels of MMP-1, TIMP-1, and TIMP-2 proteins were
also measured from tissue homogenates using the ELISA
kit (Figure 2). Compared with control tissues, (1)
MMP-1 protein levels were 4.1-fold greater in primary
tumors and 3.8-fold greater in LNM, except for the MMP-3
gene, where the levels in primary tumors (1.45±0.32)
were significantly higher than those in LNM (1.09±0.32
(P=.03).

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control tissues, but no significant differences were observed, corresponding to the RT-PCR results.

We used substrate zymography to determine the activity of MMP-2/MMP-9, MMP-3/MMP-7, and TIMP-1/TIMP-2. Gelatin zymography revealed a varied profile of MMPs: lysis zones corresponding to molecular weights of 92, 84, 72, and 62 kd and high-molecular-weight (approximately 120-kd) gelatinases were seen (Figure 3A). Gelatinases of 92 and 84 kd correspond to pro–MMP-9 and an active form of MMP-9, respectively. Those of 72 and 62 kd are considered to be pro–MMP-2 and its active form, respectively. These results are supported by the profile of TPA-treated HT-1080 cells, which express all of these enzyme species and whose gelatinolytic zones matched those of the HNSCC samples. The high-molecular-weight gelatinase detected in some samples might be due to a complex of MMP and TIMP. Indeed, 2 inhibitors tested, TIMP-1 and TIMP-2, were detected in most HNSCC tissues by ELISA (Figure 2) and gelatin reverse zymography (data not shown). Casein zymography demonstrated lytic zones corresponding to molecular weights of of approximately 57, 45, 29, and 21 kd and several small bands between 57 and 45 kd and 29 and 21 kd (Figure 3B). The 57- and 45-kd bands correspond to latent and active forms of MMP-3, respectively. The 29- and 21-kd bands are considered to be pro–MMP-7 and its active form, respectively. The nature of gelatinolytic (MMP-2, MMP-9, and MMP-14) and caseinolytic

<table>
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<th>Characteristic</th>
<th>Patients, No. (%)</th>
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<tr>
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<td>Oropharynx</td>
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<td>Hypopharynx</td>
<td>13 (24)</td>
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<td>T4</td>
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<td>Localized (grades 1 and 2)</td>
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<td>Infiltrating (grades 3 and 4)</td>
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<td>Positive</td>
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<tr>
<td>II</td>
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<tr>
<td>III</td>
<td>15 (28)</td>
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<td>IV</td>
<td>21 (39)</td>
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Figure 1. Semiquantitative reverse transcription–polymerase chain reaction analysis of matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs). Each value is the ratio of MMP or TIMP to β-actin messenger RNA level and is the mean of 3 independent RNA samples. The MMPs and TIMPs for which no messenger RNA was detected by 50 or more polymerase chain reaction cycles are indicated by zeros in the graphs. Horizontal lines indicate mean values; LNM, lymph node metastasis; and MT1, membrane-type 1.
(MMP-3 and MMP-7) enzymes or inhibitors (TIMP-1 and TIMP-2) was confirmed by Western blotting of the same tissue homogenates with specific antibodies (only data of MMP-14 are shown in Figure 3C).

Using computerized image analysis of transparent bands, we quantitated the gelatinolytic and caseinolytic activities in tissue homogenates. The results (Figure 4) showed that the MMP-9 (92 and 84 kd, respectively) and MMP-2 (72 and 62 kd, respectively) activities were significantly greater in primary tumors and LNM compared with the levels in control tissues. Compared with control tissues, the levels of high-molecular-weight gelatinase (approximately 120 kd), MMP-3 (45-51 kd), and MMP-7 (21-29 kd) were also greater in the primary HNSCC, although the levels in LNM did not reach statistical significance (Figure 4).

Our results imply that several members of the MMP and TIMP family are present in primary and secondary HNSCC. Apart from MMP-14 and TIMP-2, all the molecules tested (including TIMP-1) were up-regulated in primary HNSCC and/or LNM compared with adjacent histologically normal mucosa.

LEVELS OF MMPs AND TIMPS IN RELATION TO CLINICOPATHOLOGICAL VARIABLES

Relationships between the mRNA expression of MMPs and TIMPs in 54 primary tumors and their clinicopathological variables were analyzed. As shown in Table 2, MMP-1, MMP-9, and TIMP-1 mRNA expression in primary HNSCC showed a statistically significant relationship with a higher T classification (T3-T4) (P = .004, .001, and .003, respectively). A significant correlation was found between MMP-9 expression and an infiltrating pattern of growth (P = .002). In addition, enhanced mRNA expression of MMP-9 was strongly correlated with the presence of lymph node involvement (P < .001), whereas MMP-2, MMP-7, and MMP-11 levels were weakly correlated (P = .04, .03, and .049, respectively). Comparing primary tumors with early lesions (pathological stages I and II) and advanced diseases (stages III and IV), higher expression levels of MMP-2 and MMP-9 were observed in the latter group (P = .01 and < .001, respectively). On the other hand, when patients were separated into 4 groups according to the cutoff values for MMP-2 and MMP-9 expression obtained from the mean mRNA levels determined by RT-PCR in the primary tumors, groups with high MMP-2 and high MMP-9 expression showed the highest incidence of LNM (100%; P = .002; odds ratio, 28.24) and advanced pathological stages (100%; P = .02; odds ratio, 12.78), whereas groups with low MMP-2 and low MMP-9 expression showed the lowest incidence of nodal metastasis (23%; P < .001) and advanced stage (38%; P < .001) compared with other groups (Figure 5). In contrast, there was no association between MMP-1, MMP-2, MMP-9, MMP-11, or TIMP-1 levels and age, sex, site of primary tumors, or histological grade (Table 2). Furthermore, no association between expression of MMP-3, MMP-10, MMP-13, MMP-14, TIMP-2, ratio of MMP-9 to TIMP-1, or ratio of MMP-2 to TIMP-2 and any clinicopathological variables was observed (data not shown).

A number of studies have attempted to delineate which, if any, of the MMPs and TIMPs are required for HNSCC to grow and spread. Until now, the predictive value of the MMPs and TIMPs in invasion and metastasis of HNSCC has been controversial, partly because of the varying methods used to detect MMP expression. Because the components of the extracellular matrix are complex, the combined action of various MMPs is essential for the efficient degradation of the structure. Thus, a comprehen-
sive study of the expression of multiple MMPs and their inhibitors (TIMPs) is important for understanding the complex processes by which tumors acquire their invasive and metastatic potential. In the present study, we quantified the expression of a comprehensive set of MMPs and TIMPs previously identified in HNSCC and studied their relationship with clinicopathological variables in an attempt to determine whether overexpression of certain specific proteases could be particularly relevant to progression in this disease.

Using the highly sensitive RT-PCR assay, we studied the expression of all genes of interest within the same tissue samples. Where available, we also confirmed the presence of proteins and enzyme activities using immunoblot, enzyme immunoassay, and substrate zymography. We used tumor margins in the present studies based on the hypothesis that the cellular events in the tumor-stromal interface might be more closely related to the metastatic potential of the tumor than the (often necrotic) center. Coexpression of several members of the

**Table 2. Correlation Between Clinicopathological Variables and Messenger RNA Levels of MMPs and TIMPs in Primary HNSCC Tissues**

<table>
<thead>
<tr>
<th>Clinicopathological Variable</th>
<th>MMP-1</th>
<th>MMP-2</th>
<th>MMP-7</th>
<th>MMP-9</th>
<th>MMP-11</th>
<th>TIMP-1</th>
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<tr>
<td>≤60</td>
<td>10.27 ± 1.24</td>
<td>5.67 ± 1.54</td>
<td>10.73 ± 1.17</td>
<td>65.58 ± 9.88</td>
<td>10.95 ± 1.05</td>
<td>14.15 ± 1.77</td>
</tr>
<tr>
<td>&gt;60</td>
<td>10.03 ± 1.00</td>
<td>5.63 ± 2.13</td>
<td>9.67 ± 1.78</td>
<td>43.28 ± 6.06</td>
<td>10.52 ± 1.44</td>
<td>11.17 ± 1.78</td>
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<td></td>
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<tr>
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<td>9.56 ± 0.90</td>
<td>5.79 ± 1.55</td>
<td>10.95 ± 1.05</td>
<td>55.79 ± 7.07</td>
<td>11.33 ± 0.95</td>
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<td>Female</td>
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<td>8.27 ± 2.33</td>
<td>57.17 ± 15.83</td>
<td>9.04 ± 1.71</td>
<td>13.85 ± 2.33</td>
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<td>Location of primary tumor</td>
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<tr>
<td>Oral cavity</td>
<td>9.64 ± 1.54</td>
<td>4.39 ± 2.17</td>
<td>9.49 ± 1.60</td>
<td>45.35 ± 5.27</td>
<td>10.85 ± 1.66</td>
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<td>12.03 ± 1.23</td>
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<td>47.44 ± 12.66</td>
<td>8.64 ± 1.87</td>
<td>13.85 ± 3.01</td>
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<td>1.32 ± 0.55</td>
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<td>14.46 ± 0.92</td>
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<td>8.13 ± 2.83</td>
<td>11.80 ± 1.98</td>
<td>69.67 ± 14.31</td>
<td>10.86 ± 1.15</td>
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<td>Differentiated</td>
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<td>5.79 ± 1.86</td>
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<td>3.50 ± 1.29</td>
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<td>T3-T4</td>
<td>11.78 ± 0.93$\dagger$</td>
<td>6.88 ± 1.78</td>
<td>11.12 ± 2.22</td>
<td>69.43 ± 8.31$\dagger$</td>
<td>11.41 ± 0.91</td>
<td>15.25 ± 1.72$\dagger$</td>
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<tr>
<td>Localized (grades 1 and 2)</td>
<td>10.07 ± 1.13</td>
<td>5.61 ± 1.49</td>
<td>8.64 ± 1.93</td>
<td>36.70 ± 5.89</td>
<td>9.29 ± 1.48</td>
<td>12.13 ± 1.93</td>
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<td>Infiltrating (grades 3 and 4)</td>
<td>9.71 ± 1.21</td>
<td>5.69 ± 2.02</td>
<td>12.36 ± 0.97</td>
<td>70.44 ± 9.14$\dagger$</td>
<td>12.29 ± 0.60</td>
<td>15.19 ± 1.89</td>
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<td>Lymph node metastasis</td>
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<tr>
<td>Negative</td>
<td>10.46 ± 1.19</td>
<td>3.68 ± 0.36</td>
<td>7.86 ± 1.81</td>
<td>32.60 ± 5.80</td>
<td>9.04 ± 1.41</td>
<td>10.04 ± 1.49</td>
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<td>Positive</td>
<td>10.00 ± 1.11</td>
<td>6.78 ± 1.41$\dagger$</td>
<td>12.69 ± 1.07$\dagger$</td>
<td>69.56 ± 8.34$\dagger$</td>
<td>12.33 ± 0.82$\dagger$</td>
<td>14.51 ± 1.74 $\dagger$</td>
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<tr>
<td>I/II</td>
<td>0.82 ± 0.23</td>
<td>1.07 ± 0.16</td>
<td>10.65 ± 4.08</td>
<td>15.30 ± 4.53</td>
<td>9.64 ± 3.29</td>
<td>8.79 ± 2.82</td>
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<tr>
<td>III/IV</td>
<td>1.04 ± 0.09</td>
<td>6.28 ± 1.38$\dagger$</td>
<td>10.34 ± 0.98</td>
<td>61.75 ± 6.68$\dagger$</td>
<td>10.97 ± 0.85</td>
<td>13.82 ± 1.36</td>
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</tbody>
</table>

*The relative levels of matrix metalloproteinase (MMP) and tissue inhibitor of MMP (TIMP) transcripts normalized to β-actin expression were determined by computerized image analysis. Results are shown as mean ± SEM. HNSCC indicates head and neck squamous cell carcinoma.

$\dagger$ P < .01, significant difference between groups in the same variable.

$\ddagger$ P < .05, significant difference between groups in the same variable.

§P < .001, significant difference between groups in the same variable.
MMP family seems to be a general characteristic of human HNSCC. The simultaneous expression of several MMPs is consistent with the concept that extracellular matrix remodeling during tumor progression requires the synergistic action of several proteolytic enzymes produced by tumor cells or by stromal cells. Evidence suggests that each of these MMP genes might have a distinct role in tumor progression. Some members of the MMP family, such as MMP-2 and MMP-11, are expressed mainly in stromal fibroblasts and might be regarded as paracrine, stroma-derived factors necessary for the progression of HNSCC. Both MMP-7 and MMP-9 were expressed exclusively in epithelial cells, MMP-1 and MMP-10 were found principally within fibroblasts surrounding tumor, in endothelial cells, and also in neoplastic cells. Muller et al. reported that increased MMP-11 gene expression might be a useful marker for defining subpopulations of aggressive HNSCC. Increased expression of MMP-14 was detected at the tumor cell surface, especially at the invasive edge of tumor cell nests, in most HNSCC tissues assayed.

Although inhibition of in vitro and in vivo tumor invasion by TIMPs has been demonstrated, increased, rather than decreased, TIMP levels have been shown to be related to poor outcome in several malignant tumors, such as bladder cancer. The present finding of increased TIMP-1 expression in HNSCC might be explained by the growth-promoting activity of TIMPs on a variety of cell types or the induction of TIMPs by secreted MMPs (or vice versa) from tumor-host interaction in the extracellular milieu. The correlation between increased TIMP-1 and TIMP-2 levels with less aggressive tumors was found in some studies, although the opposite pattern was also reported (and confirmed in the present study).

Several studies have examined relationships between the expression of gelatinases and malignant potential in HNSCC, but the results are still inconclusive. In oral cancers, MMP-2, but not MMP-9, was found to correlate with LNM and poor clinical outcome. One study found that high levels of MMP-2 and MMP-9 were related to the invasiveness of oral SCC, whereas another showed no difference in MMP-2 and MMP-9 levels between primary HNSCC and LNM. Most recently, several studies have shown that MMP-9 might play a more important role than MMP-2 in the invasive and metastatic potential of HNSCC. Among 9 MMPs and 2 TIMPs tested in the present study, MMP-9 overexpression showed the strongest correlation with the presence of neck nodal metastases and advanced pathological stages. The underlying roles that some MMP family members play in the process of lymphatic metastasis remain to be elucidated. In addition, it is possible that some MMPs and TIMPs did not show statistical significance because of a type II error (relatively small sample size with high variability), and another study with a larger sample size might be required.

The up-regulation of several MMPs in lymph node–positive patients suggests that the evaluation of MMPs, MMP-9 in particular, in HNSCC tissues at the time of presentation might allow identification of a subset of patients with HNSCC who are more susceptible to metastatic spread via lymphatic pathways and permit therapy to be offered accordingly. With the application of highly sensitive RT-PCR analysis, preoperative assessment from small tissue biopsies or even needle aspirates will become more useful in assessing the malignant potential of HNSCC. Aggressive neck management in tumors showing multiple MMPs positive, MMP-9 in particular, might be considered to avoid later lymphatic spread. Because of the relatively short follow-up, we are unable to demonstrate yet whether MMP/TIMP expression is related to survival. This awaits confirmation in a longer follow-up period.

In conclusion, the results of these studies suggest that expression of multiple MMPs and TIMPs is characteristic of HNSCC and that no specific member of the MMP family is solely responsible for HNSCC progression. The correlation studies of MMP/TIMP expression in human head and neck tumor tissues suggest the potential role of MMP-2, MMP-7, MMP-9, and MMP-11 in progression and metastasis of human HNSCC. Combined analysis of these MMPs, MMP-9 in particular, might be useful in evaluating the malignant potential in individual HNSCC.

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