Expressions of Matrix Metalloproteinases in Early-Stage Oral Squamous Cell Carcinoma as Predictive Indicators for Tumor Metastases and Prognosis

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ABSTRACT

Purpose: Matrix metalloproteinase (MMP)-2 and MMP-9 are considered to play an important role in the metastasis of malignant tumors. Membrane type 1-MMP (MT1-MMP) and tissue inhibitor of metalloproteinase 2 (TIMP-2) are essential factors for the activation of pro-MMP-2. There are some reports about expressions of MMP family in relationship to clinical features of head and neck squamous cell carcinoma (SCC), but the results were not uniform and the prognostic value of their expressions remains unclear.

Experimental Design: The study group consisted of 53 Japanese patients with oral SCC of early stage (T1-2 N0 M0). Expressions of MMP-2, MMP-9, MT1-MMP, and TIMP-2 were examined using immunohistological methods on the sections of tumor biopsy samples. The intensity of MMP expression was categorized into four grades (score 0–3) by semiquantitative analysis using a computer with NIH image, and correlation between this grade and clinical aspects such as tumor recurrence, metastasis, and prognosis were examined.

Results: The expression score of MMP-2 correlated with that of MMP-9 (r = 0.291; P = 0.036), MT1-MMP (r = 0.286; P = 0.039), and TIMP-2 (r = 0.257; P = 0.050). Patients who developed regional lymph node and/or distant metastasis showed significantly higher scores in the expressions of MMP-9 and TIMP-2 than patients without any tumor metastases (P = 0.036 and P = 0.043, respectively). Kaplan-Meier analyses as well as univariate analyses using the Cox proportional hazards model showed that expression of MMP-9 (P = 0.0143 and P = 0.0418, respectively) and marked expression of TIMP-2 (P < 0.0001 and P = 0.0004, respectively) correlated with worse-cause-specific survival. Multivariate analysis confirmed that marked expression of TIMP-2 was the only independent factor for cause-specific death (hazard ratio, 7.543; confidence interval, 1.693–33.610; P = 0.0080).

Conclusions: Expressions of MMP-9 and TIMP-2 have predictive value for tumor metastases and cause-specific survival. High expression of TIMP-2 is the most independent factor for worse prognosis in early-stage oral SCC.

INTRODUCTION

Tumor invasive process is thought to involve the multiple proteolytic enzyme matrix metalloproteinases (MMPs; Ref. 1). Among the MMPs, MMP-2 and MMP-9 have been thought to be key enzymes in this process, because they degrade type IV collagen, which is one of the important components of extracellular matrix (2, 3). Membrane type 1-MMP (MT1-MMP) has been originally identified as an activator of Pro-MMP-2. On the other hand, tissue inhibitor of metalloproteinase 2 (TIMP-2) is an inhibitor of MMP-2. Recent in vitro studies about the mechanism of cell-mediated MMP-2 activation showed that pro-MMP-2 binds to TIMP-2 in combination with MT1-MMP on the cell surface, forming a ternary complex. Then pro-MMP-2 in the complex is activated by adjacent MT1-MMP that is free from TIMP-2 (4–6).

The expressions of MMP family members in head and neck squamous cell carcinoma (SCC) tissues are reported widely; however, the correlation of these expressions with clinical features is still controversial (7–14). Some studies demonstrated that the expressions of MMP family correlated with histological grade (8, 10, 13), tumor invasion (12), clinical stage (14), and/or lymph node involvement (7–12, 14), although their results were not uniform. Furthermore, a majority of previous studies analyzed these expressions in the patients with a wide variety of tumor (T1-4) and node (N0-3) classifications. There is little known about the predictive value of expression of MMP family members for clinical outcomes and prognosis.

The purpose of this study is to investigate whether expression of MMP family members has predictive value for a clinical course and prognosis in early clinical stage and whether they are useful to select the manner of treatment. For these reasons, we recruited a series of oral SCC patients with early-stage I-II (T1-2 N0 M0) and analyzed the expression of a wide variety of MMP family members, MMP-2, MMP-9, MT1-MMP, and TIMP-2, to determine their predictive values for tumor recurrence, metastases, and prognosis.

PATIENTS AND METHODS

Patients. The study group consisted of 53 Japanese patients (41 males and 12 females) who were diagnosed with oral...
Table 1 Clinical and histological features in 53 patients with early-stage oral squamous cell carcinoma

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No. of cases (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>41 (77%)</td>
</tr>
<tr>
<td>Female</td>
<td>12 (23%)</td>
</tr>
<tr>
<td>Age (yr): median, 59, range, 42–84</td>
<td></td>
</tr>
<tr>
<td>&lt;65</td>
<td>33 (62%)</td>
</tr>
<tr>
<td>≥65</td>
<td>20 (38%)</td>
</tr>
<tr>
<td>Primary sites</td>
<td></td>
</tr>
<tr>
<td>Tongue</td>
<td>44 (83%)</td>
</tr>
<tr>
<td>Floor of mouth</td>
<td>9 (17%)</td>
</tr>
<tr>
<td>Clinical stage</td>
<td></td>
</tr>
<tr>
<td>I (T1aN0M0)</td>
<td>23 (43%)</td>
</tr>
<tr>
<td>II (T2N0M0)</td>
<td>30 (57%)</td>
</tr>
<tr>
<td>Tumor differentiation</td>
<td></td>
</tr>
<tr>
<td>Well</td>
<td>41 (77%)</td>
</tr>
<tr>
<td>Moderately</td>
<td>10 (19%)</td>
</tr>
<tr>
<td>Poorly</td>
<td>2 (4%)</td>
</tr>
<tr>
<td>Initial therapy</td>
<td></td>
</tr>
<tr>
<td>Surgery alone</td>
<td>22 (42%)</td>
</tr>
<tr>
<td>Radiotherapy alone</td>
<td>6 (11%)</td>
</tr>
<tr>
<td>Combined with surgery and radiotherapy</td>
<td>25 (47%)</td>
</tr>
</tbody>
</table>

RESULTS

Expressions of MMP-2, MMP-9, MT1-MMP, and TIMP-2. MMP-2, MMP-9, MT1-MMP, and TIMP-2 were expressed mainly on the cell surface and in the cytoplasm of tumor cells (Fig. 1). They were also detected on some endothelial cells or on the stromal fibroblasts surrounding tumor cells. The expression scores of the MMP-2, MMP-9, MT1-MMP, and TIMP-2 are shown in Table 2.

Relationships among MMP-2, MMP-9, MT1-MMP, and TIMP-2 Expression. The expression score of MMP-2 positively correlated with that of MMP-9 \((r = 0.291; P = 0.036)\), MT1-MMP \((r = 0.286; P = 0.039)\), and TIMP-2 \((r = 0.257; P = 0.050)\). However, there was no significant correlation between MT1-MMP and TIMP-2 \((r = 0.013; P = 0.923)\), MMP-9 and MT1-MMP \((r = 0.158, P = 0.255)\), or MMP-9 and TIMP-2 \((r = 0.154; P = 0.266)\) expression scores.

Clinical Outcome. Histological evaluation showed that tumor cells were not present on the surgical margin in any of the 47 patients that underwent surgery. On this evaluation, there were no tumor cells in the biopsy samples obtained from any of the six patients after radiotherapy alone. Therefore, all of the patients experienced a disease-free period. However, seven (13.2%) patients developed local recurrence, and 20 (37.7%) developed regional lymph node metastasis. During the follow-up period, 7 (13.2%) of 20 patients with regional lymph node metastasis developed distant metastasis as well. Fifteen (28.3%) patients died during follow-up; 9 (17.0%) of these died of tumor-related disorder and 6 (11.3%) were tumor free and were excluded because counterstaining of nuclei with hematoxylin would have artificially increased gray-level values. By using this system, the intensity of staining was quantifiable between zero (white) and 225 (black). To adjust the day-to-day and the section-to-section variation of results, cytoplasmic regions of 100 normal epithelial cells were used as an internal control in the same section and were analyzed. The intensity of expression in the tumor cells was standardized by subtraction of the mean gray level of internal control from that of 10 tumor fields. These values were categorized into four grades as follows: score 0 (negative) = value of 0–20; score 1 (mild) = value of 21–50; score 2 (moderate) = value of 51–100; score 3 (marked) = value over 100.

Statistical Analysis. Two groups were compared using the Mann-Whitney \(U\) test, \(\chi^2\) test, or Fisher’s exact test and were summarized with their appropriate \(P\). Spearman’s regression coefficient was used to examine the magnitude of selected association. Time was defined as the period from diagnosis to the target event or last follow-up. For overall survival (OS), the target event was death. For cause-specific survival (CSS), it was cause-specific death. For local control (LC), it was local failure. For regional and distant control (RDC), it was regional lymph node and/or distant metastases. The probability of OS, CSS, LC, and RDC were calculated using the Kaplan-Meier method and were compared using a log-rank test. For determination of factors related to OS, CSS, LC and RDC, a Cox proportional hazards model was used. The final results of these analyses are hazard ratios, their 95% confidence intervals, and \(Ps\). A \(P\) less than 0.05 was considered to be statistically significant.
died from intercurrent diseases. The 5-year LC, RDC, OS, and CSS rates were 87%, 62%, 72%, and 84%, respectively (Table 3).

Local Recurrence and Regional Lymph Node and Distant Metastases According to Variables. Gender, age, histology, and tumor-node-metastasis classification at diagnosis did not influence local recurrence or regional lymph node and distant metastases. Among the three groups of patients with different initial therapy, the 5-year LC rate was significantly lower for the patients who were treated with radiotherapy alone (63%) than for the patients who had radiotherapy and surgery (100%; \( P = 0.0012 \)). However, the 5-year RDC and CSS rates were not different among the three groups (Table 3).

There was no difference in MMP expressions between patients with local recurrence and patients without any recurrence and metastases. Patients who developed regional lymph node and/or distant metastases showed significantly higher scores in the expressions of MMP-9 and TIMP-2 than patients without any tumor metastases (MMP-9: 1.500 ± 1.100 versus 0.862 ± 0.953, \( P = 0.036 \); TIMP-2: 1.800 ± 0.834 versus 1.314 ± 0.733, \( P = 0.043 \); Fig. 2). The expression scores of MMP-2 and MT1-MMP were not different between patients with or without metastases.

Table 2  Expression score of matrix metalloproteinase (MMP) families in 53 patients with early-stage oral squamous cell carcinoma

<table>
<thead>
<tr>
<th></th>
<th>Score 0</th>
<th>Score 1</th>
<th>Score 2</th>
<th>Score 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-2</td>
<td>7 (13.2%)</td>
<td>11 (20.8%)</td>
<td>26 (49.1%)</td>
<td>9 (16.9%)</td>
</tr>
<tr>
<td>MMP-9</td>
<td>22 (41.5%)</td>
<td>10 (18.9%)</td>
<td>16 (30.2%)</td>
<td>5 (9.4%)</td>
</tr>
<tr>
<td>MT1-MMP</td>
<td>13 (24.5%)</td>
<td>15 (28.3%)</td>
<td>17 (32.1%)</td>
<td>8 (15.1%)</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>6 (11.3%)</td>
<td>13 (24.5%)</td>
<td>31 (58.5%)</td>
<td>3 (5.7%)</td>
</tr>
</tbody>
</table>

\( ^a \) Intensity of the expressions in carcinoma cells was scored into four grades according to gray level values digitalized by a computer with NIH image; score 0: value of 0–20; score 1: value of 21–50; score 2: value of 51–100; score 3: value over 100.

\( ^b \) MT1, membrane type 1; TIMP, tissue inhibitor of MMP-2.
The 5-year LC and RDC rates according to expression of MMP family members are listed in Table 3. The RDC rates of patients with MMP-9 expression (score 1–3) were significantly shorter than that of patients without MMP-9 expression (score 0; \( P = 0.0247; \) Fig. 3A). The RDC rates of patients with marked expression (score 3) of TIMP-2 were also significantly shorter than those of patients with negative or moderate expression (score 0–2) (\( P = 0.0019; \) Fig. 3B).

**Survival According to Variables.** The 5-year OS and CSS rates according to variables are shown in Table 3. Survival was not affected by histology, gender, age, tumor-node-metastasis classification, or initial therapies.

With regard to correlation between expression of MMP family and survival, the expressions of MMP-2 and MT1-MMP did not influence OS or CSS. However, patients with MMP-9 expression (score 1–3) showed significantly shorter CSS than patients without MMP-9 expression (score 0; \( P = 0.0143; \) Fig. 3C). Univariate analysis also showed that the expression of MMP-9 (score 1–3) as a significant indicator for poor CSS (\( P = 0.0418; \) Table 4).

Patients with marked expression (score 3) of TIMP-2 showed significantly shorter OS and CSS than patients with negative or moderate expression (score 0–2) of TIMP-2 (\( P = 0.0004 \) and \( P < 0.0001 \), respectively; Fig. 3D). Univariate analysis also showed that marked expression of TIMP-2 was a significant indicator for poor OS (\( P = 0.0028 \)) and poor CSS (\( P = 0.0004 \); Table 4). Multivariate analysis for CSS confirmed that marked expression of TIMP-2 was the only independent factor for cause-specific death (hazard ratio, 7.543; confidence interval, 1.6930–33.610; \( P = 0.0080 \)).

**DISCUSSION**

Our patients were treated in three different ways. The patients with radiotherapy alone showed the worst LC rate. However, neither the metastasis in regional lymph nodes and/or distant sites nor the patient’s survival was influenced by the treatment modality. Furthermore, there was no difference in expressions of MMP family members among the three treatment groups. Therefore, analyses for correlation between MMP ex-
pression and the metastasis or patient’s prognosis showed that they were not influenced by treatment modality.

Recent in vitro studies (4–6) have made the mechanism of cell-mediated MMP-2 activation in tumor tissue more understandable, i.e., pro-MMP-2 secreted by fibroblasts binds to TIMP-2 in combination with MT1-MMP on the cell surface, and the pro-MMP-2/TIMP-2/MT1-MMP ternary complex is formed. The pro-MMP-2 in the ternary complex is activated by adjacent MT1-MMP that is free from TIMP-2 (4–6), and the activated MMP-2 degrades extracellular matrix components. Therefore, on the basis of this theory, the significant association of MMP-2 expression with expression of either MT1-MMP or TIMP-2 observed here is very reasonable. On the other hand, it is reported that different transcriptional regulation of MMP-2 and MMP-9 genes may be due to the presence of different promoter elements (18). We observed significant correlation between expressions of MMP-2 and MMP-9. Although we cannot explain this reason accurately, this result seems to suggest unknown concurrent events that activate transcriptions of both MMP-2 and MMP-9 genes in oral SCC.

In this study, we found significant correlation of MMP-9 expression with regional lymph node and/or distant metastasis and poor prognosis. Univariate analysis also showed that MMP-9 expression was a significant indicator for poor prognosis. This result agrees with previous reports about MMP-9 expression in head and neck SCC (3, 10, 11). O-charoenrat et al. (11) reported that mRNA level of MMP-9 correlated with lymph node involvement. Imanishi et al. (8) reported that expression of MT1-MMP correlated with lymph node metastasis but that of MMP-2 did not. In oral SCC, Kusukawa et al. (12) reported that expression of pro MMP-2 correlated with lymph node involvement. Kurahara et al. (7) reported that expressions of MMP-2 and MT1-MMP correlated with tumor invasion and lymph node involvement in oral SCC.

Therefore, MMP-9 expression may be a useful marker for predicting tumor metastasis and for prognosis in head and neck SCC including early-stage oral SCC.

In contrast to MMP-9 expression, clinical association and prognostic values of MMP-2 and MT1-MMP are still controversial in head and neck SCC. In head and neck SCC recruited from different anatomical sites, O-charoenrat et al. (11) reported that mRNA level of MMP-2 correlated with lymph node involvement. In oral SCC, Kurahara et al. (7) reported that expressions of MMP-2 and MT1-MMP correlated with tumor invasion and lymph node involvement. However, these previous studies analyzed only the status at diagnosis and did not examine the predictive values for tumor recurrence and prognosis. Recently, Yoshizaki et al. (14) reported that marked expressions of MMP-2 and MT1-MMP correlated with lymph node recurrence and worse survival as well as with clinical stage at the time of diagnosis in tongue SCC. On the other hand, we failed to determine the correlation of MMP-2 and MT1-MMP expressions with tumor metastases and prognosis. Although we cannot accurately resolve the discrepancy, this may be caused by differences in the clinical stages of patients. Previous studies were composed of cases with a wide variety of tumor (T1–4) and lymph node (N0–3) classifications (7, 8, 11, 12, 14), whereas our study was composed of cases with T1–3N0M0 classification. Therefore, the predictive value of MMP-2 and MT1-MMP expressions for tumor recurrence or metastases and prognosis may vary according to primary sites, tumor sizes, and/or lymph node status at the time of diagnosis in head and neck SCC. Alternatively, this discrepancy may be due to difference in specificity of the antibody used. The
antibody for MMP-2 used in this study recognizes both pro MMP-2 and activated MMP-2 (19). The expression of MMP-2 observed here might not correctly reflect the activated MMP-2 that degrades extracellular matrix components of tissues.

In general, TIMP-2 had been believed to suppress tumor invasion and metastasis by inhibiting MMP-2, because TIMP-2 inhibits collagenolysis activity of MMP-2 in vitro (20). However, according to the model of cell-mediated MMP-2 activation, pro-MMP-2 bound to the TIMP-2/MT1-MMP complex is activated by MT1-MMP that is free from TIMP-2. The activated form of MMP-2 is inactivated by the attachment of TIMP-2 (4–6). Recently, high activation of MMP-2 in tongue SCC samples, detected by gelatin zymographic assay, has been reported to be associated with high expression of TIMP-2 in tumor cells (14). Therefore, the immunoreactivity of TIMP-2 is likely to be useful for monitoring MMP-2 activation. Moreover, some recent immunohistological studies reported that TIMP-2 plays the positive role for tumor metastasis (13, 21–24). Kanayama et al. (22) reported that a high mRNA level of TIMP-2 was associated with poor prognosis in bladder carcinoma. Kikuchi et al. (24) reported that high expression of TIMP-2 correlated with lymphatic invasion and lymph node metastasis in colorectal carcinoma. In the present study, we clearly showed that the marked expression of TIMP-2 strongly correlated with lymph

![Fig. 3](image)

**Fig. 3** Regional and distant control (RDC) curves (A–B) and cause-specific survival (CSS) curves (C–D) according to expressions of matrix metalloproteinase 9 (MMP-9; A and C) and tissue inhibitor metalloproteinase-2 (TIMP-2; B and D) in 53 patients with oral squamous cell carcinoma. 

A, the RDC of patients with MMP-9 expression (score 1–3) tended to be shorter than that of patients without MMP-9 expression (score 0; \( P = 0.0247 \)). B, the RDC of patients with marked expression (score 3) of TIMP-2 was significantly lower than that of patients with none or weak expression (score 0–2) of TIMP-2 (\( P = 0.0019 \)). 

C, the CSS of patients with MMP-9 expression (score 1–3) was significantly shorter than that of patients without MMP-9 expression (score 0; \( P = 0.0143 \)). D, the CSS of patients with marked expression (score 3) of TIMP-2 was significantly shorter than that of patients with none-to-weak expression (score 0–2) of TIMP-2 (\( P < 0.0001 \)). The probabilities of RDC and CSS were calculated using the Kaplan-Meier method and were compared using the log-rank test.

### Table 4

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hazard ratio</th>
<th>95% CI</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (≥65)</td>
<td>1.08</td>
<td>0.226–4.385</td>
<td>0.9139</td>
</tr>
<tr>
<td>Gender (male)</td>
<td>2.056</td>
<td>0.381–16.597</td>
<td>0.4953</td>
</tr>
<tr>
<td>Clinical stage (T)</td>
<td>2.984</td>
<td>0.619–14.343</td>
<td>0.1732</td>
</tr>
<tr>
<td>Tumor differentiation (poorly/moderately)</td>
<td>1.03</td>
<td>0.213–4.983</td>
<td>0.9703</td>
</tr>
<tr>
<td>Initial therapy (radiotherapy alone)</td>
<td>1.052</td>
<td>0.282–3.927</td>
<td>0.9402</td>
</tr>
<tr>
<td>MMP-2 (expression score 1–3)</td>
<td>3.561</td>
<td>0.532–34.281</td>
<td>0.1661</td>
</tr>
<tr>
<td>MMP-9 (expression score 1–3)</td>
<td>8.691</td>
<td>1.084–69.715</td>
<td>0.0418</td>
</tr>
<tr>
<td>MT1-MMP (expression score 1–3)</td>
<td>2.832</td>
<td>0.353–22.746</td>
<td>0.3273</td>
</tr>
<tr>
<td>TIMP-2 (expression score 3)</td>
<td>13.68</td>
<td>3.211–58.306</td>
<td>0.0004</td>
</tr>
</tbody>
</table>

\( \text{a CI, confidence interval; MMP, matrix metalloproteinase; MT1, membrane type 1; TIMP-2, tissue inhibitor of MMP-2.} \)
node and distant metastases as well as with poor prognosis in early-stage oral SCC, and was the only independent factor for poor prognosis. Yoshizaki et al. (13) also demonstrated high expression of TIMP-2 as the only independent factor for poor prognosis in tongue SCC.

In summary, expression of MMP-9 and marked expression of TIMP-2 are associated with regional lymph node and/or distant metastasis and poor prognosis. In particular, marked expression of TIMP-2 is the most important indicator and is valuable in identifying the patients at high risk for poor prognosis even in early clinical stage.

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