Tissue Inhibitor of Matrix Metalloproteinase-1 Is Prognostic in Head and Neck Squamous Cell Carcinoma: Comparison of the Circulating and Tissue Immunoreactive Protein

Henni Ruokolainen,1 Paavo Pääkkö,2 and Taina Turpeenniemi-Hujanen1

Abstract

Purpose: Tissue inhibitors of metalloproteinases (TIMP) are capable of inhibiting the matrix metalloproteinases, but they also possess other biological functions. Little is known about the role of TIMP-1 in the progression and spreading of cancer cells among patients with head and neck squamous cell carcinoma (HNSCC). In this study, the pretreatment serum levels of TIMP-1 or the overexpression of TIMP-1 immunoreactive protein in the primary tumor was correlated to the clinical course in patients with HNSCC.

Experimental Design: The TIMP-1 immunoreactive protein was studied in 74 cases representing HNSCC. The tissue immunoreactive protein was evaluated from paraffin-embedded tumor sections in 68 cases using immunohistologic staining with a specific antibody, and in 68 cases the pretreatment serum levels of TIMP-1 were quantitatively measured by ELISA assay. The results were compared with the clinicopathologic factors of the disease and the patients’ outcome.

Results: A positive correlation was found between the size of the primary tumor (T) and the circulating TIMP-1 level (P = 0.021) or the positive immunoreaction of TIMP-1 in tumor (P = 0.039). The 5-year cause-specific survival was significantly lower in patients presenting with a high serum TIMP-1 level than in those with a low level of TIMP-1 (38% versus 64%, P = 0.034). Similarly, the expression of TIMP-1 in tumor was prognostic for shortened survival, the 5-year cumulative relapse-free survival being 42% in patients with a TIMP-1–positive tumor versus 75% in cases with a negative tumor (P = 0.035). Tissue TIMP-1 positivity also seemed associated to the cause-specific survival (P = 0.075) and to be connected with later lymph node or hematogenic relapses.

Conclusions: This study shows for the first time that both circulating and tissue TIMP-1 immunoreactive protein predicts the clinical course and dissemination in HNSCC, suggesting that TIMP-1 might be related to both tumor growth and metastasis in HNSCC.

Carcinomas of the head and neck represent 4% of all newly diagnosed cancers in the United States and 5% in the United Kingdom, and over 90% of the cases represent squamous cell carcinoma [head and neck squamous cell carcinoma (HNSCC)]. Worldwide, >500,000 new cases are diagnosed each year. Tobacco and alcohol consumption are widely documented risk factors, and the incidence of HNSCC has increased in western countries during the last few years, partly because of the increased use of tobacco and alcohol (1, 2).

The long-term survival rates among HNSCC patients have improved only marginally during the last decade, despite the fact that diagnostic methods and treatment strategies have improved. The major cause of death among these patients is a local-regional recurrence. At present, the only prognostic factor that is regularly used in clinical work is the stage of the disease, particularly the presence of lymph node metastases (1–3). It is recognized that the prognosis of patients in each stage group of the disease may also differ greatly, suggesting that some other, possibly biological, factors may also play a role in the progression of HNSCC. It is known that HNSCC emerges after the accumulation of genetic changes in epithelial cells exposed to carcinogenesis. This multistep process has led to the search of biomarkers that might introduce new strategies making it possible to better identify the patient groups in need of more aggressive treatment modalities or other treatment options.

Matrix metalloproteinases (MMP) form a family of zinc-dependent endopeptidases that are able to degrade connective tissue, among other substrates the basement membrane collagen, which seems to play a key role in the invasion and metastasis of SCCs (4–8). In various cancer types, this has been translated to clinical correlation between overexpression of gelatinases and survival in particular (9–12). There are growing amount of data suggesting that circulating MMP-9 or MMP-2

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levels could be valuable in assessing prognosis or diagnosing a relapse during follow-up (13–17).

Tissue inhibitors of metalloproteinases (TIMP) are known to have at least two different functions. They inhibit the catalytic activity of MMPs and they are also able to act as growth factors (18). Four members of this protein family, TIMP-1 to TIMP-4, have been determined. TIMP-1 plays a role during the activation of MMP-9 (19, 20). It is also able to inactivate the active forms of both MMP-2 and MMP-9. In accordance with these findings, TIMPs have been shown to have the capability to inhibit tumor dissemination animal models (21). According to the literature, there is discrepancy as to whether TIMP-1 has a role in promoting cancer cell dissemination or whether it inhibits tumor progression and formation of metastases. Strong TIMP-1 has, however, been associated with an unfavorable prognosis in some tumor types, such as lymphomas, lung cancer, colorectal, and gastric carcinoma (14, 22–24).

Previous studies have shown that gelatinases are expressed in head and neck carcinoma cells, and they may take part in the progression and invasion of these tumors (25–29). We have recently found that MMP-9 positivity in immunohistochemical staining is associated with unfavorable prognosis in HNSCC (30). However, there are limited and partly conflicting data concerning the association between TIMP-1 and tumor progression or dissemination in HNSCC.

In this study, the role of TIMP-1 has been explored in the progression of HNSCC. Both cancer cell–associated and circulating immunoreactive protein of TIMP-1 has been evaluated with respect to whether they may predict survival among these patients. The circulating pretreatment TIMP-1 levels were also correlated to TIMP-1 immunohistochemical staining in the corresponding primary tumor.

**Materials and Methods**

**Patients.** The patients referred to Oulu University Hospital for treatment of a primary SCC of head and neck between the years 1994 and 1996 were consecutively included in the study. Serum samples were collected during the first visit to the hospital and taken before operation or radiotherapy. Serum samples were stored frozen in −70°C until used for the study. The patients were followed for a minimum of 5 years and there were no dropouts from the follow-up. There were 74 cases in this series; TIMP-1 immunoreactive protein was analyzed from the tumor sections of 68 patients and pretreatment serum samples were available from 68 patients. There were 65 patients from whom both paraffin-embedded tissue samples from the primary tumor and pretreatment serum samples were available. The project protocol was accepted by the Ethical Committee of Oulu University in March 1994 and renewed by the ethical committee of Oulu University Hospital in July 2002. As control values, the serum samples were taken from 44 healthy volunteers (27 female and 17 male), without history of a known malignant disease or recent surgery.

The mean age of the patients was 64 years (minimum 28 years and maximum 88 years). The stage of the disease, tumor size, and lymph node involvement was determined according to the International Union Against Cancer tumor-node-metastasis classification (31). The histologic grade of the tumors was reviewed and classified according to the WHO classification of head and neck tumors (32).

In this study, the treatment strategies for patients were carried out according to the local protocol for treatment, and the treatment line depended on the stage of the tumor. Radical surgical operation with postoperative radiotherapy (50-60 Gy) was the treatment for 25 of the 74 patients, 20 patients were treated with radical surgical operation without other treatments, and 11 patients were inoperable and received only radiotherapy (50-64 Gy). Sixteen patients received preoperative radiotherapy (50 Gy). No patients were treated with adjuvant chemotherapy. Two of the patients had an advanced carcinoma and received only palliative treatment. In the clinical follow-up, the patients were seen every 3 to 6 months to evaluate locoregional tumor control and survival.

**Immunohistochemical staining and evaluation of tissue inhibitor of metalloproteinase-1 immunostaining.** Paraffin-embedded sections (4 µm) from the primary tumors of head and neck carcinomas were stained using the avidin-biotin-immunoperoxidase technique. Paraffin sections were incubated at 37°C for at least 4 hours, dewaxed (Histoclear, National Diagnostic, Atlanta, GA), and hydrated. Endogenous peroxidase activity was blocked by incubating the slides in 3% hydrogen peroxide/methanol for 10 minutes, and nonspecific binding was blocked with 10% goat serum for 15 minutes. A mouse monoclonal antibody to TIMP-1, 17.5 µg/ml [R&D Systems, Minneapolis, MN; in 0.01 mol/L phosphate buffer, 0.9% NaCl (pH 7.5)] was used as a primary antibody mixed with Antibody Diluent (Dako, Glostrup, Denmark). The specimens were incubated overnight at room temperature in a humidity chamber, and the immunohistochemical staining was continued using the Histostain bulk kit (Zymed, San Francisco, CA) according to the manufacturer’s protocol. Biotinylated antimouse immunoglobulin G was used as a second antibody. The peroxidase was introduced after that with a streptavidin conjugate. The slides were washed thoroughly with PBS between all stages of the procedure. The antibody reaction was visualized by using a fresh substrate solution containing romulin aminoethyl carbazol (Romulin AEC-Chromogen, Biocare Medical, Walnut Creek, CA). The sections were counterstained with hematoxylin, dehydrated, and mounted (Histomount, National Diagnostics, Manville, NJ). For the negative controls, the primary antibody for TIMP-1 was replaced with mouse non-immunoglobulin G and each set of staining always included a separate known positive control.

The slides were analyzed by two independent observers blinded from clinical data, and the immunoreactivity in the malignant cells in each section was graded from 0 to 3 according to the extent of positive staining. The case was considered positive when >1% of the tumor cells showed a positive staining. Weak positivity was marked as + (1% < tumor cells with a positive reaction <25%) and moderate positivity was ranked as ++ (25% < tumor cells with a positive reaction <50%). Cases showing a positive staining in >50% of the tumor cells were considered extensively positive for TIMP-1 (+++).

**Quantitation of tissue inhibitor of metalloproteinase-1 in ELISA.** ELISA was done on 8-well E.I.A microtiter plates (Corning, NY) using the standard protocol. When measuring the total TIMP-1, a mouse monoclonal anti-TIMP-1 antibody (Diabor Ltd., Oulu, Finland) that recognizes both the free TIMP-1 and TIMP-1 in complex with MMP-9, was coated onto the microtiter plates. Samples and standards were added and the bound analytes were recognized with a polyclonal anti-TIMP-1 antibody produced in chicken against TIMP-1 (Diabor Ltd., Oulu, Finland) and visualized by using a fresh substrate solution containing romulin aminoethyl carbazol (Romulin AEC-Chromogen, Biocare Medical, Walnut Creek, CA) according to the manufacturer's protocol. For the negative controls, the primary antibody for TIMP-1 was replaced with mouse non-immunoglobulin G and each set of staining always included a separate known positive control.

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**Statistical analyses.** All statistical analyses were done by using the SPSS software system (SPSS for Windows, version 11.0, Chicago, IL). The correlations of tumor stage, tumor-node-metastasis classification, histologic grade, and the primary anatomic site were analyzed separately according to the TIMP-1 immunoreactivity and to the TIMP-1 immunoreactive protein level measured by ELISA. The statistical significance of these correlations was determined with Fisher’s exact test. To compare the difference of the means between groups, the Mann-Whitney U test was used. The cause-specific and relapse-free survival rates were analyzed using the Kaplan-Meier method, and the statistical differences in survival among subgroups were compared by a log-rank test (33). The cause-specific survival was defined as the time...
from the date of diagnosis to the date of death due to head and neck carcinoma. The cases were censored on the date of the last control or at the time of death to another disease. Similarly, the relapse-free survival was calculated from the date of diagnosis to the date of relapse, and the case was censored at the time of the last follow-up visit.

Results

Tissue inhibitor of metalloproteinase-1 expression in squamous-cellular carcinoma of head and neck. Paraffin-embedded tissue sections were available in 68 patients. Overexpression of the TIMP-1 immunoreactive protein was found in 55 (81%) cases (Table 1). Extensive positive staining for TIMP-1 (+++, >50% of the tumor cells appearing as positive) was found in 24 of 68 cases (35%), moderate positivity (++, 25% < tumor cells with a positive reaction <50%) was found in 16 cases (24%), and weak positivity (+, 1% < tumor cells with a positive reaction <25%) in 15 cases (22%). The staining was negative in 19% (13 of 68) of the cases. All positivity for TIMP-1 appeared as a diffuse staining in the cancer cell cytoplasm (Fig. 1A). No granular staining was seen. The adjacent uninvolved mucosa remains negative for TIMP-1 immunoreactive protein (Fig. 1C).

Tissue inhibitor of metalloproteinase-1 serum levels. The pretreatment serum sample was available from 68 patients. The median serum level for TIMP-1 immunoreactive protein was 527 ng/mL, varying from 304 to 1,074 ng/mL. The mean value was 565 ng/mL. In analyses, high level was defined as values >510 ng/mL. This cutoff value was found when the effect of different cutoff values to log-rank analysis was studied. This value was more powerful in differentiating the cases according to their prognosis than the median or the mean levels. In contrast, the TIMP-1 serum levels in the 44 healthy controls ranged from 259 to 661 ng/mL (median, 409 ng/mL; mean, 432 ng/mL). TIMP-1 serum levels were significantly higher in the HNSCC patients than in healthy controls (P < 0.001, Mann-Whitney test).

Correlation between the circulating and the tumor immunoreactive tissue inhibitor of metalloproteinase-1 protein. There was a trend of association between TIMP-1 immunohistochemical staining and the serum level of the TIMP-1 immunoreactive protein (Table 2). In cases with a positive TIMP-1 immunostaining in the primary tumor, 33 of 51 patients (65%) presented with a high level (>510 ng/mL) of serum TIMP-1 immunoreactive protein. On the other hand, 42% of patients presenting with a negative TIMP-1 staining in the primary tumor had a high serum level for TIMP-1 (P = 0.13, Fisher's exact test).

Correlation with clinical variables. The tissue TIMP-1 positivity or serum TIMP-1 level did not correlate with the

### Table 1. Tissue and circulating TIMP-1 in different subgroups of head and neck carcinoma

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>n</th>
<th>TIMP-1 positive, n (%)</th>
<th>P</th>
<th>n</th>
<th>TIMP-1 high*, n (%)</th>
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<td>16 (73)</td>
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<td>9</td>
<td>5 (56)</td>
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<td>11</td>
<td>7 (64)</td>
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<td>3 (33)</td>
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<tr>
<td>II</td>
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<td>14</td>
<td>9 (64)</td>
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<tr>
<td>III</td>
<td>22</td>
<td>16 (73)</td>
<td></td>
<td>21</td>
<td>11 (52)</td>
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</tr>
<tr>
<td>IV</td>
<td>25</td>
<td>23 (92)</td>
<td></td>
<td>24</td>
<td>17 (71)</td>
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</tr>
</tbody>
</table>

Abbreviation: TNM classification, tumor-node-metastasis classification.

* >510 ng/mL.
stage of the disease ($P = 0.36$, $P = 0.30$, Fisher’s exact test) or with the grade of the tumor ($P = 0.59$, $P = 0.56$, Fisher’s exact test). Additionally, the anatomic region of the primary tumor did not have any effect on the positive immunoreaction for TIMP-1 or on the level of the serum immunoreactive protein for TIMP-1 ($P = 0.53$, $P = 0.16$, Fisher’s exact test). Instead, there was a positive correlation between the size of the primary tumor (T) and TIMP-1–positive immunoreaction ($P = 0.031$, Fisher’s exact test). Additionally, the same correlation was seen between the circulating TIMP-1 and the tumor size ($P = 0.021$, Fisher’s exact test). No correlation was however detected with the degree of lymph node invasion ($P = 0.41$, $P = 0.53$, Fisher’s exact test). A statistically significant correlation between the TIMP-1 positivity or the serum value of TIMP-1 and patients’ sex was seen in this study: 22 of 23 cases of the female patients had a positive TIMP-1 immunoreaction ($P = 0.023$, Fisher’s exact test). The same trend was seen when comparing the high level of serum TIMP-1 and the female gender ($P = 0.088$, Fisher’s exact test). No statistical differences were found with circulating TIMP-1 levels or TIMP-1 immunohistochemical staining among the groups of patients with different treatment.

**Tissue inhibitor of metalloproteinase-1 as a prognostic factor.** The survival analysis showed a statistically significant correlation between the pretreatment serum level of TIMP-1 and the cause-specific survival. Patients with serum TIMP-1 levels of $\leq 510$ ng/mL survived longer than patients with TIMP-1 levels of $>510$ ng/mL. The Kaplan-Meier analysis showed that the cause-specific cumulative survival of the patients with a low level of TIMP-1 was 64% after 5 years of follow-up, whereas the survival was only 38% among the patients presenting with a high serum TIMP-1 level ($P = 0.034$, log-rank analysis; Fig. 2A). High TIMP-1 serum levels also associated with shorter relapse-free survival (Fig. 2B), but the difference did not quite reach statistical significance in the log-rank analysis ($P = 0.090$). The cumulative 5-year relapse-free survival rate was 56% in patients presenting with a low serum level for TIMP-1, whereas in patients with a high serum TIMP-1 level the rate was only 37% (Fig. 2B).

A similar trend towards a more favorable survival in TIMP-1–negative cases was observed when analyzing whether the TIMP-1 overexpression would predict the survival. Ten of the 13 patients with a primary tumor negative for TIMP-1 were free of relapse after 5 years of follow-up. The cumulative relapse-free survival rate was 75%. On the other hand, the cumulative 5-year relapse-free survival rate for patients with TIMP-1–positive tumors was 42%. This difference was statistically significant ($P = 0.036$) in the log-rank analysis (Fig. 3A). A difference in 5-year cause-specific survival in Kaplan-Meier analysis was also found between the patient groups presenting with TIMP-1–positive and TIMP-1–negative tumors (47% and 75%, respectively; $P = 0.075$, log-rank analysis; Fig. 3B).

When evaluating the relapses, it was noticed that in 34 of the 37 relapsed cases, the TIMP-1 immunostaining appeared as positive in the primary tumor. This difference was statistically significant ($P = 0.013$, Fisher’s exact test). Additional analyses showed that in cases with a later lymph node or hematogenic
relapse, 27 of the 29 patients (93%) had a positive immunostaining of TIMP-1 immunoreactive protein when compared with the cases that emerged later with a local relapse or did not face a relapse at all (Table 3). This difference was also statistically significant \((P = 0.026, \text{Fisher’s exact test})\). It is notable that 20 of the 29 (69%) patients that relapsed with lymph node or hematogeni metastases had a high pretreatment serum TIMP-1 compared with 51% of the patients with a local relapse or no relapse. A trend was found, but it was not statistically significant \((P = 0.112, \text{Fisher’s exact test})\).

**Discussion**

The role of TIMP-1 in tumor progression and invasion is currently far from clear. Despite being named for its ability to inhibit MMP activities, the TIMP-1 protein has multifunctional actions. Traditionally, TIMP-1 has been associated with growth and metastasis inhibiting capacity \((18–21)\). However, various recent studies have indicated that TIMP-1 is linked with aggressive behavior and poor prognosis among patients with different tumor types \((14, 22–24)\). In HNSCC, there are only limited data concerning TIMP-1 and the progression of the cancer, and there are no survival analyses. In this study, we show that high preoperative serum TIMP-1 could be a prognostic factor for shortened survival in HNSCC patients. Only 38% of the patients with a high serum level of TIMP-1 \((>510 \text{ ng/mL})\) survived for 5 years after the diagnosis of HNSCC, whereas in patients with a low TIMP-1 level \((\leq 510 \text{ ng/mL})\) a 64% cause-specific 5-year survival rate was observed. This difference was statistically significant. A notable difference was also found in 5-year cause-specific survival in the Kaplan-Meier analysis between patient groups presenting with positive or negative TIMP-1 immunoreactivity in the primary tumor \((47\% \text{ or } 75\%, \text{respectively, } P = 0.075)\). To our knowledge, this is the first study to show such a difference in survival according to circulating or tissue TIMP-1. Elevated levels of circulating TIMP-1 have previously been shown to be associated with poor prognosis in lung, ovarian, transitional cell or colorectal carcinoma, and tissue TIMP-1 has been reported to predict independently shortened survival in multivariate analysis in breast carcinoma \((14, 34–37)\). Very recently, Aljada et al. also published a study on non–small cell lung cancer showing that patients with a tumor showing a high TIMP-1 expression had an increased risk of death compared with patients presenting with a low expression of TIMP-1 \((38)\).

Tissue TIMP-1 overexpression predicted significantly earlier relapse in HNSCC. The cumulative 5-year relapse-free survival rate was 75% for the patients presenting with a TIMP-1–negative tumor, whereas it was only 42% for patients with a TIMP-1–positive tumor \((P = 0.036)\). Moreover, the high TIMP-1 serum levels also associated with a shorter relapse-free survival \((37\% \text{ versus } 56\%),\) although this difference did not reach statistical difference. Our study is the first to show that high TIMP-1 in HNSCC is not only associated with unfavorable cause-specific survival but also with an earlier relapse. These data are in line with those reporting high levels of tissue TIMP-1 to be related to disease progression in patients with breast or colorectal cancer \((39, 40)\). High preoperative TIMP-1 level was very recently found to correlate with a poor relapse-free survival in primary node–negative breast carcinoma \((41)\).

We show here a correlation between the size of the primary tumor and the level of circulating TIMP-1. Seventy-three percent of the patients with a high serum level of TIMP-1 were

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**Table 2. Correlation between the TIMP-1 immunohistochemical staining results in primary tumors and the TIMP-1 immunoreactive protein level in serum**

<table>
<thead>
<tr>
<th>Immunohistochemical staining result (TIMP-1)</th>
<th>Serum TIMP-1 low*, n (%)</th>
<th>Serum TIMP-1 high†, n (%)</th>
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<tr>
<td>Negative</td>
<td>12</td>
<td>5</td>
</tr>
<tr>
<td>Positive</td>
<td>51</td>
<td>33 (65)†</td>
</tr>
<tr>
<td>Weak (+)</td>
<td>15</td>
<td>11 (73)</td>
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<tr>
<td>Moderate (+++)</td>
<td>14</td>
<td>8 (57)</td>
</tr>
<tr>
<td>Extensive (+++)</td>
<td>22</td>
<td>14 (64)</td>
</tr>
</tbody>
</table>

*\(\leq 510 \text{ ng/mL}\).
†\(>510 \text{ ng/mL}\).
\(P = 0.128\).
found to have T stage III to IV. This noteworthy difference was also statistically significant ($P = 0.021$). It was interesting that the same effect was seen when analyzing the expression of TIMP-1 immunoreactive protein and the volume of the primary tumor. Thirty-one of the 34 primary tumors presenting with a T grade 3 to 4 were positive for TIMP-1 ($P = 0.031$). However, there was a lack of correlation between the nodal status of tumor and TIMP-1 expression or level of circulating TIMP-1. Accordingly, we could not observe any correlation between the TIMP-1 and the histologic grade of the tumor or the stage of the disease. Our finding concerning the correlation between TIMP-1 and tumor size is in line with data of O-charoenrat et al., who reported a significant correlation between TIMP-1 and advanced T grade in HNSCC patients (42). In that study, no difference was noticed in distribution of TIMPs in relation to age, sex, tumor site, or grade. On the contrary, Charous et al. reported in 1997 that no statistical correlation was found between the primary stage of the disease and recurrences in HNSCC patients (25). TIMP-1 was detected in 95% of primary tumors when in the present study only 81% of the cases appeared as positive, which is in line with the data published recently in breast carcinoma (37). Recently, Kurokpat et al. reported that there was no correlation between TIMP-1 over-expression and grade, stage, or metastatic potential among HNSCC patients (43). There is only one previous study on HNSCC where TIMP-1 levels have been shown to appear higher in nonmetastatic than in metastatic cases of oral SCC (44). In other malignomas, a correlation between primary tumor size or invasion and TIMP-1 expression has thus far been published at least by Miyata et al. in transitional cell carcinoma of upper urinary tract, by Yukawa et al. in colorectal, and by Yoshikawa et al. in gastric carcinoma (35, 39, 45).

We show here that the patients with TIMP-1–positive tumors had more relapses than patients with TIMP-1–negative tumors ($P = 0.013$). Further analyses showed that 93% of the patients with later lymph node or hematogenic relapses had a tumor positive for TIMP-1, indicating that more aggressive relapses were linked with TIMP-1 positivity. Moreover, there was a trend for high serum pretreatment TIMP-1 to correlate to later lymph node or hematogenic relapses. However, that correlation was not as strong as the one found with high expression of tissue TIMP-1.

Our data together with those published previously seem to indicate that the tissue TIMP-1 protein might be involved in cancer progression. It has been shown previously that SCCs often express TIMP-1 protein in culture (46). We previously showed an overexpression of MMP-9 immunoreactive protein in primary tumors of HNSCC (30). We have correlated also TIMP-1 immunoreactive protein overexpression with the data of MMP-9 expression. We found that 47 of 55 patients (85%) with MMP-9-positive immunostaining were also positive for TIMP-1 immunoreactive protein. On the other hand, 39% of patients presenting with a negative result in both immunostainings. This correlation did not quite reach statistical significance ($P = 0.11$, Fisher’s exact test). Some data associate TIMP-1 with tumor growth and the dissemination potential of the neoplasm, but the association between the expression of TIMP-1 and relapses might show that TIMP-1 could have a role in some mechanisms important for dissemination capacity of a neoplasm or overall tumor progression, such as angiogenesis. TIMP-1 has actually been shown to act as an antiapoptotic agent and a growth-stimulating factor in malignant cell lines (18). On the other hand, there is some evidence based on

![Table 3](https://www.aacrjournals.org/clin-cancer-res/article-pdf/11/9/3262/4461415/clin-cancer-res-2005-11-9-3262.pdf)

<table>
<thead>
<tr>
<th>TIMP-1 Status</th>
<th>No relapse (%)</th>
<th>Local</th>
<th>Lymph node</th>
<th>Hematogenous</th>
</tr>
</thead>
<tbody>
<tr>
<td>TIMP-1 positive</td>
<td>10 (32)*</td>
<td>7 (88)</td>
<td>22 (92)</td>
<td>5 (100)</td>
</tr>
<tr>
<td>TIMP-1 high†</td>
<td>15 (50)</td>
<td>5 (56)</td>
<td>17 (71)</td>
<td>3 (60)</td>
</tr>
</tbody>
</table>

* $P = 0.013$.
† $P = 0.026$.
‡ 3510 ng/mL.
staining in the cytoplasm. This is in line with some previous studies using immunohistochemical methods (37, 38).

The prognosis of SCC of the head and neck is traditionally linked to the stage of the disease. However, it is well known that it is not sufficient for accurate prediction of the outcome. Therefore, using TIMP-1 as a molecular tumor marker seems like an inviting alternative due to the various mechanisms of TIMPs in tumor progression and dissemination. TIMP-1 seems to correlate with unfavorable prognosis in HNSCC and might help in determining the prognosis. It could also be recruited as a follow-up marker in HNSCC or be used in developing new treatment modalities.

In conclusion, we show here for the first time that both circulating and tissue immunoreactive protein of TIMP-1 associate with the shortened cause-specific and relapse-free survival in HNSCC. Further studies with larger patient materials are needed to investigate the role of TIMP-1 as a marker for tumor progression to also evaluate its possible value in the follow-up of patients treated for HNSCC.

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References

22. Kurahara S, Shinozuka M, Ikebe T, et al. Expression of MMPs, MT-MMP and TIMPs in squamous cell carcinoma of the oral cavity: correlations with tumor inva-
26. Shahmugaratnam K. In collaboration with Sobin LH and Pathologists in 8 countries. Histological typing of


Tissue Inhibitor of Matrix Metalloproteinase-1 Is Prognostic in Head and Neck Squamous Cell Carcinoma: Comparison of the Circulating and Tissue Immunoreactive Protein

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