CLINICAL SIGNIFICANCE OF MMP-2 AND MMP-9 IN PATIENTS WITH ORAL CANCER

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Accepted 6 October 2006
Published online 24 January 2007 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/hed.20561

Abstract: Background. Factors that represent the potential for invasion and metastasis, such as matrix metalloproteinases (MMPs), could predict prognosis of cancer. Therefore, the authors studied plasma and tissue levels of MMP-2 and MMP-9 in oral cancer, the leading malignancy in India.

Methods. Enzyme-linked immunosorbent assay and gelatin zymography were used for the MMP analysis from plasma and tissue samples, respectively.

Results. Latent, active, and total forms and activation ratio of MMP-2 and MMP-9 were significantly elevated in malignant tissues as compared with adjacent normal tissues. Activation of MMP-2 was higher than MMP-9 in malignant tissues. Activation ratio was significantly higher in malignant tissues of the patients with lymph node metastasis as compared with those without lymph node metastasis (p = .005). Plasma MMP-9 levels were significantly lower in responders as compared with pretreatment levels (p = .002).

Conclusion. The data indicate that MMP-2 and MMP-9 can be useful to identify metastatic phenotype as well as for treatment monitoring in oral cancer.

Keywords: matrix metalloproteinases; squamous cell carcinomas; oral cancer; invasion; metastasis

Cancer is a multifactorial, multifaceted, and multimechanistic disease requiring a multidimensional approach for its diagnosis, treatment, and prevention. Oral cancer is the sixth most frequent cancer in the world, accounting 300,000 new cases annually. The incidence of oral cancer is comparatively very low in Western countries, 2% to 6% of all malignancies, whereas it constitutes nearly a third of all cancer cases in India. Annually, 75,000 to 80,000 new oral cancer cases are registered in India. Various cancer registries have documented that the high incidence is due to widespread habits of tobacco chewing and smoking in the Indian population.

Oral cancer cases in India frequently present with local or regional metastasis at the time of diagnosis. The overall survival rate for patients with oral cancer is among the lowest (less than 50%) and has not changed during the past 2 decades. Only 15% of the patients are diagnosed when the disease is at a localized stage. The patients with advanced disease most often reflect the spread of the disease to local, regional, and distant sites, events that are poorly controlled by
combined surgery/irradiation. The unfavorable prognosis of oral squamous cell carcinomas (SCCs) is mainly due to extensive local invasion and frequent spread to lymph node. It is also known that habit of tobacco consumption is responsible for field cancerization in oral mucosa. Further, field cancerization hypothesis predicts that multiple cells form independent epithelial tumors, because carcinogenic exposure in the form of tobacco carcinogens affects multiple cells in the field. Thus, it could be possible that adjacent normal tissues of oral tumors might be clinically normal; however; molecular and biochemical changes can reveal vital information for prediction of tumor behavior, recurrence, and metastatic potential. It is also documented that second primary tumors occur in the “field cancerization” at a rate of 20% to 30% despite improvement in locoregional control. Therefore, identification of better prognostic markers would allow selection of high-risk patients for closer monitoring and surveillance or more intensive treatment. During recent years, efforts have been made to understand the molecular and cellular mechanisms involved in metastasis. It is documented that factors that represent the potential for invasion and metastasis, such as matrix metalloproteinases (MMPs), could predict prognosis of oral cancer.

MMPs are a family of zinc dependent proteinases, which are secreted as proenzyme (latent enzyme) and require proteolytic cleavage for activation. Digestion of the subendothelial basement membrane is the first step toward invasion and metastasis. Type IV collagen is the main component of basement membrane, and degradation of this structural protein is favored by 2 metalloproteinases, namely, the gelatinase A (MMP-2) and gelatinase-B (MMP-9). MMP-2 and MMP-9 are known to be closely associated with the malignant potential of tumor cells. To the best of our knowledge, there are no reports on MMP-2 and MMP-9 activation in patients with oral cancer from India. Considering the problems of late presentation, spread of the disease, and low overall survival of the patients with oral cancer in India as well as prevalence of tobacco habits, it is of utmost importance to document parameters that can identify the metastatic potential of disease at an early stage. Therefore, the objectives of the present investigation were to study the following: (1) zymographic analysis of latent, active, and total form and activation ratio of MMP-2 and MMP-9 in malignant and adjacent normal tissues of patients with oral cancer, (2) correlation of MMP-2 and MMP-9 activation with other clinicopathological parameters, (3) comparison of MMP-2 and MMP-9 activation in malignant tissues, (4) correlation of MMP-2 and MMP-9 with lymph node metastasis, and (5) comparison of plasma total MMP-2 and MMP-9 levels between pretreatment and follow-up samples.

**MATERIALS AND METHODS**

**Subjects.** Prior consent was taken from all the subjects to participate in the study.

**Patients.** The study included 60 untreated patients with SCC of the oral cavity enrolled from out patients’ department of the Gujarat Cancer & Research Institute, Ahmedabad. Staging was done according to American Joint Committee on Cancer norms. Clinical details of the patients are shown in Table 1. In most of these patients, surgery was primary treatment of choice. However, large number of patients were treated by combinational therapy involving surgery, radiotherapy, and chemotherapy (n = 35; surgery + radiotherapy [RT] and/or chemotherapy).

**Samples**

**Tissue Samples.** Surgically resected malignant (n = 60) and adjacent normal tissue (N) (n = 60) samples of patients with oral cancer were collected from operation theater. Adjacent normal tissues at least 1 cm away from malignant tissues were taken from free margins. Tissue samples were washed with cold phosphate buffer saline, pH 7.4, and immediately kept at −70°C. Tissue samples were homogenized in phosphate buffer saline (pH 7.4) with glass mortar and pestle on ice. The cytosols were separated by centrifugation at 20,000 rpm in cooling centrifuge and stored at −20°C. Cytosolic proteins were estimated by the method of Lowry et al.

**Blood Samples.** Blood samples from the patients were drawn by venipuncture in heparinized vacutettes before initiation of anticancer therapy and these were termed as pretreatment patients (n = 12). The patients were clinically evaluated at various intervals after initiation of anticancer treatment. Follow-up blood samples (n = 38) were also collected from the patients during their visits to the hospital. The follow-up blood samples from patients with no evidence of disease after surgical removal of tumor were classified as complete.
responders (n = 30), whereas the follow-up samples from patients with locoregional failure of disease, stable/progressive disease, metastasis, or recurrence were classified as nonresponders (n = 8). Plasma were separated by centrifugation of whole blood at 3000 rpm for 10 minutes and stored at −20°C until analysis.

**Methods**

**Zymography.** Latent and active MMP-2 and MMP-9 from adjacent normal and malignant tissues of patients with oral cancer were studied by gelatin zymography as described by Lorenzo et al.\(^{11}\) Commercially available pure human MMP-2 and MMP-9 standards (Calbiochem, USA) were used to construct standard plots. Activation ratio of MMP-2 and MMP-9 were calculated as the ratio of active form to that of total form of MMP-2 or MMP-9.

**Enzyme-Linked Immunosorbent Assay.** Total MMP-2 and MMP-9 activity from untreated and follow-up plasma samples of patients with oral cancer were studied by enzyme-linked immunosorbent assay (ELISA) assay using commercially available Kits (R & D systems, USA).

**Statistical Analysis.** Data were analyzed statistically using the SPSS statistical software (Version 10). Paired t test was performed to assess level of significance of MMP-2 and MMP-9 between normal and malignant tissues. Receiver operating characteristic curves were constructed to evaluate discriminatory efficacy of MMP-2 and MMP-9 between patients with and without lymph node involvement. Student paired t test was also performed to assess level of significance between pretreatment and follow-up plasma samples for total MMP-2 and MMP-9 levels. Multivariate analysis was performed to assess level of significance for MMP activity with different clinicopathological parameters including sex, tumor stage, grade, differentiation, and lymph node involvement etc. Odds ratio were calculated to evaluate risk of lymph node metastasis development in node-negative patients. Overall survival was estimated by Kaplan–Meier method. The log-rank test was applied to compare survival curves with the activation ratio of MMP-2 and MMP-9 in malignant tissues. \(p\) values < .05 were considered statistically significant.

**RESULTS**

**Standards for Latent and Active MMP-2 and MMP-9.** Densitometric analysis of latent and active forms of MMP-2 and MMP-9 from 0 to 20 ng and 0 to 2 ng, respectively, revealed a linear correlation of density/cu. mm area with concentration of gelatinases (Figures 1A and 1B).

**Zymogram of Malignant and Adjacent Normal Tissue Cytosols.** Figure 2 shows representative zymogram of malignant and adjacent normal tissue cytosols of oral SCC tissues samples. Fifty micrograms of cytosolic proteins were loaded into each lane. Concentrations of both the forms of MMP-2 and MMP-9 were calculated as ng/50 \(\mu\)g of protein. It is evident from the figure that malignant tissues exhibited more prominent gelatinolytic activity.
activities of MMP-2 and MMP-9 as compared with adjacent normal tissues.

**Latent, Active, Total, and Activation Ratio of MMP-2 and MMP-9 in Oral Cancer.** Latent, active, and total forms of MMP-2 and MMP-9 were significantly elevated in malignant tissues as compared with adjacent normal tissues ($p \leq .02$). Activation ratio of MMP-2 and MMP-9 were also significantly elevated in malignant tissues as compared with adjacent normal tissues ($p = .016$ and .049, respectively) (Figure 3).

**Percentage Activation of MMP-2 and MMP-9.** Gelatinase activities of latent as well as active forms were calculated as percentage activity for each tissue sample considering total activity as 100% (Figure 4). Activation of MMP-2 was 11% higher and MMP-9 was 5% higher as compared with their respective adjacent normal tissues. Activation of MMP-2 was 6% higher as compared with MMP-9 in malignant tissues.

**MMP-2 in Regional Lymph Node Metastasis.** Figure 5 shows activation ratio of MMP-2 in malignant oral SCCs tissues with and without lymph node metastasis. Activation ratio of MMP-2 was significantly elevated ($p = .005$) in malignant tissues with lymph node metastasis as compared with malignant tissues without lymph node metastasis.

**Odds Ratio Analysis.** Odds ratios were calculated to evaluate risk of lymph node metastasis development in node-negative patients. Cut-off levels of activation ratio of MMP-2 (0.3754 ng/mL) and MMP-9 (0.1216 ng/mL) were calculated as mean + SD of MMP-2 and MMP-9 in malignant tissues of patients without lymph node metastasis. The odds ratios were calculated between

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**FIGURE 1.** Representative zymogram of human standard matrix metalloproteinase (MMP)-2 and MMP-9. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

**FIGURE 2.** Representative zymogram of oral squamous cell carcinoma tissues and adjacent normal tissues. N, adjacent normal; M, malignant; —LN, no Lymph node metastasis; 9N, patient #9 adjacent normal tissue; 9M, patient #9 malignant tissue; 8-LN, patient #8 negative lymph node tissues; 8M, patient #8 malignant tissue; 8N, patient #8 adjacent normal tissue. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
patients with and without lymph node metastasis with activation ratio of MMP-2 and MMP-9 above and below cut-off levels in malignant tissue (Table 2). The data revealed that activation ratio of MMP-2 in malignant tissues above cut-off levels were significantly associated with higher risk of developing lymph node metastasis in node-negative patients (OR = 2.74, 95% C.I. = 1.075–46.67, p = .042).

Activation Ratio of MMP-2 and MMP-9 in Malignant Tissues and Overall Survival. Overall survival of the patients using activation ratio of MMP-2 and MMP-9 in malignant tissues were analyzed. The median values obtained for activation ratio of MMP-2 (0.174) and MMP-9 (0.064) from the adjacent normal tissues was used as cut off. The data did not reveal any significant difference in survival of the patients with regards to activation ratio of MMP-2 and/or MMP-9 (Figure 6).

Plasma Total MMP-2 and MMP-9 Levels in Pretreatment Patients, Complete Responders, and Nonresponders. Figure 7 shows plasma total MMP-2 and MMP-9 levels in pretreatment patients, complete responders, and nonresponders. To compare the MMP-2 and MMP-9 levels between the
pretreatment and follow-up samples, student’s paired $t$ test was performed. MMP-2 and MMP-9 levels in untreated (pretreatment) patients were paired and compared with the patient’s own follow-up MMP-2 and MMP-9 values. The analysis revealed no significant difference in MMP-2 levels between pretreatment and complete responders (paired $t = 0.072, p = .943$) as well as pretreatment patients and nonresponders (paired $t = 1.032, p = .349$). Although plasma total MMP-9 levels were significantly decreased in complete responders as compared with pretreatment levels (paired $t = 3.433, p = .002$), the MMP-9 levels were comparable between pretreatment patients and nonresponders (paired $t = 0.276, p = .791$).

**Plasma Levels of MMP-2 and MMP-9 as Treatment Monitors of Patients with Oral Cancer.** Figures 8A and 8B shows representative patterns of circulating MMP-2 and MMP-9 levels before and after anticancer treatment in the patients. Figure 8A shows variations in circulating total MMP-2 and MMP-9 levels in the patient who was initially classified as a responder in postsurgery period. However, the patient developed recurrence during follow-up and was treated with radiotherapy and chemotherapy also. The alterations in MMP-2 levels increased initially and were decreased during recurrent disease, whereas MMP-9 levels were decreased initially at 1 month and 3 months follow-up as compared with pretreatment values. Further, MMP-9 levels were elevated above pretreatment levels with recurrent disease during follow-up. Figure 8B shows variations in circulating total MMP-2 and MMP-9 levels in a patient who was classified as a responder and there was no evidence of disease during postsurgery follow-up. MMP-2 values failed to show any correlation with disease status during follow-up period. However, MMP-9 levels were sharply decreased after surgery and remained below pretreatment level during the whole follow-up duration.

**DISCUSSION**

The role played by MMPs in the progression of oral cancer appears increasingly interesting. Many studies have shown that gelatinases have significant clinical usefulness in tumor progression. In view of this, the study of MMP-2 and MMP-9 activation in oral SCCs could be a valuable approach for management of these patients.

MMPs can occur in different forms in biological samples, such as latent enzyme, active enzyme, complexed with inhibitors. Although different types of assays such as immunohistochemistry, ELISA, and zymography are used to detect MMPs, immunohistochemistry cannot distinguish be-

<table>
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<tr>
<th>Malignant Tissue</th>
<th>Ratio</th>
<th>95% CI</th>
<th>Significance</th>
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<tbody>
<tr>
<td>Activation ratio of MMP-2</td>
<td>2.74</td>
<td>1.075 - 46.678</td>
<td>.042</td>
</tr>
<tr>
<td>Activation ratio of MMP-9</td>
<td>1.611</td>
<td>1.212 - 2.141</td>
<td>.278</td>
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**FIGURE 6.** Survival curves for activation ratio of matrix metalloproteinase (MMP)-2 and MMP-9 in malignant tissues. Overall survival curves plotted according to Kaplan–Meier method. Median level of activation ratio of MMP-2 (0.174) and MMP-9 (0.064) in adjacent normal tissues was used as cut-off.
between latent and active forms of MMPs,14 while ELISA is sensitive but costly on routine basis. Gelatin zymography is a cost-effective and easily reproducible technique, which can separate and quantitate latent as well as active forms of MMP-2 and MMP-9.15 Reproducibility and cost-effectiveness of gelatin zymography make it possible for routine analysis for each patient. In the present study, we developed zymograms of both the forms of MMP-2 and MMP-9 standards, which revealed a linear correlation with concentration against gelatinolytic activity in terms of optical density per cu. mm area. Similar standards were also observed using zymography by Parsons et al.16

Concentrations of latent, active, as well as total MMP-2 and MMP-9 were significantly elevated in malignant tissues as compared with their adjacent normal tissues. Tokumaru et al17 have found that active MMP-2 was significantly elevated in malignant head and neck SCC tissues as compared with normal tissues. However, the authors did not find significant difference in latent form of MMP-2 between normal and malignant tissues. Tokumaru et al17 have also reported significant elevations in activation ratio of MMP-2 in malignant tissues as compared with normal head and neck SCCs. Our data for activation ratio of MMP-2 are also comparable with the report of Tokumaru et al.17 We found significant elevations in total activity of MMP-2 and MMP-9 in malignant tissues as compared with their adjacent normal tissues.

To compare activation between MMP-2 and MMP-9, we calculated percentage activity of MMP-2 and MMP-9. Activation of MMP-2 was twice as compared with MMP-9 in malignant tissues. These findings suggested that MMP-2 activation in malignant oral SCC tissues was higher as compared with MMP-9. However, in the same study, Hong et al18 observed that gelatinolytic activity determined by gelatin zymography for MMP-2 was 3.64 times higher in malignant tissues than normal mucosa, which was 2.8 times higher in malignant tissues for MMP-9 in oral SCCs as compared with normal mucosa. Our observations are in accordance with these zymographic observations of Hong et al.18

MMP-2 and MMP-9 activities in tumor tissues were compared with various clinicopathological variables. However, we have not observed significant changes of MMP-2 and MMP-9 activities between different clinicopathological findings including sex, early and advanced stage, nuclear grade, tumor differentiation, tobacco habits etc. (data not shown). Similar results were also been noted by Yorioka et al12 in patients with oral SCC. Sutinen et al19 have observed that mRNA expression of MMP-2 in lymph node metastasis correlated with the expression in oral SCCs and thus confirmed the independent role of MMP-2 in tumor invasion.

![FIGURE 8](image-url) Representative patterns of total matrix metalloproteinase (MMP)-2 and total MMP-9 levels during follow-up. PT, pretreatment; NED, no evidence of disease; RT, radiotherapy; NR, nonresponders; NS, not significant.
Odds ratio analysis showed that activation ratio of MMP-2 was significantly associated with risk of lymph node metastasis development in lymph node-negative patients. Therefore, it can be useful in monitoring patients without lymph node involvement. These patients may be at a higher risk of developing regional lymphatic spread and might be followed up closely and shifted to more vigorous treatment. However, such risk prediction analysis has not been reported previously and therefore an extensive study with more patients in this direction is needed for validation of the observations. Further, the survival curves did not show significant association in longer survival of the patients with lower activation ratio of both the MMPs as compared with shorter survival of the patients with higher activation ratio. However, Yorioka et al.\textsuperscript{20} showed that MMP-2 and MMP-9 activities correlated with the disease free survival of patients with oral SCC.

Measurement of serum or plasma levels of MMP-2 and MMP-9 is useful for monitoring treatment response and predicting recurrence of the disease. Therefore, in the present study a subpopulation of the patients were analyzed for plasma levels of total MMP-2 and total MMP-9 before initiation of anticancer treatment and subsequently after and during posttreatment follow-up of the patients. Kolomecki et al.\textsuperscript{20} reported that serum levels of MMP-8 and MMP-9 were significantly decreased after surgery. In the present study, follow-up plasma samples from responders showed significant decrease in total MMP-9 activity as compared with their respective pretreatment levels. Total MMP-2 activity in individual cases showed elevations in MMP-2 levels on recurrence of the disease and decreased in regression of the disease. However, a wide range of variations in total MMP-2 activity within pretreatment and follow-up was noted. To nullify these variations, paired $t$ test was carried out to compare MMP-2 and MMP-9 levels between PT and follow-up levels. In case of total MMP-9, representative cases showed that recurrence or appearance of advanced disease showed elevations in total MMP-9 levels as compared with their respective pretreatment levels, while total MMP-9 levels were decreased in responders as compared with their pretreatment levels. This is the first study showing plasma total MMP-2 and total MMP-9 levels before and during/after treatment in patients with oral cancer. This approach, being noninvasive, is advantageous to assess treatment outcome during posttreatment follow-up in patients with oral cancer. Total MMP-2 and total MMP-9 activities were comparable between pretreatment patients and nonresponders.

When follow-up plasma samples compared between responders and nonresponders, it was found that total MMP-9 activities were significantly decreased in complete responders as compared with nonresponders, while total MMP-2 activities remained comparable between complete responders and nonresponders. The present observations on pretreatment and posttreatment plasma total MMP-2 and total MMP-9 levels in patients with oral cancer are in accordance with observation of Kolomecki et al.\textsuperscript{20} High serum levels of MMP-2 were shown to be correlated with presence of metastasis in lung cancer\textsuperscript{21} or to disease progression in patients with prostate cancer.\textsuperscript{22} Tutton et al.\textsuperscript{23} have reported that plasma level of MMP-2 and MMP-9 significantly decreased upon successful curative resection of colorectal tumors of all stages. Low activities of MMP-2 and MMP-9 have shown to be associated with better response to neoadjuvant chemotherapy in SCC of oral cavity.\textsuperscript{24} The present study also showed that plasma total MMP-9 levels are useful for treatment monitoring of patients with oral SCC. Ylisirnio et al.\textsuperscript{25} showed that plasma MMP-9 as well as tissue inhibitors of metalloproteinase-1 could serve as prognostic markers, for survival in patients with lung cancer. However, serum MMP-2 levels were not found to be associated with survival of patients with lung cancer. Endo et al.\textsuperscript{26} have noticed that patients with gastric cancer with highest value of serum latent MMP-2 and plasma latent MMP-9 were finally diagnosed as cases of advanced cancers. Activation of latent MMPs is mediated through several steps involving serine proteases, tissue inhibitors of metalloproteinase-1, membrane type-MMP, and other activated MMPs. Therefore, it is essential to carry out in-depth work on the pathways and interactions of other components to predict treatment outcome and survival of these patients.

The present study has thrown light on activation of MMP-2 and MMP-9 with oral cancer metastasis, which may be useful for aggressive therapeutic guidelines in patients with metastatic potentials. The study showed that gelatin zymography is sensitive and specific as well as cost-effective technique. Therefore, it can be useful for routine work-up for these patients to evaluate for metastatic potentials. It has been reported that zymographic analysis of the MMP-2 and MMP-9 activities in oral SCC specimens may be useful to
predict the disease free survival period in these patients. Therefore, simultaneous analysis of getatinases by gelatin zymography apart from histopathology of oral SCC could clinically be useful for predicting lymph-node metastasis of oral SCCs. The study of circulating levels of MMP-2 and MMP-9 may be useful for predicting recurrence of the disease. In the present study, activation of MMP-2 and MMP-9 were significantly elevated in malignant tissues as compared with their adjacent normal tissues. However, activation of MMP-2 was more prominent as compared with MMP-9 in malignant oral SCCs. Elevated activation ratio of MMP-2 also correlated significantly with lymph node metastasis in oral SCCs. Accordingly, MMP-2 could be considered as more selective molecular marker for prediction of metastatic potentials of oral SCCs, while plasma levels of total MMP-9 and total MMP-2 could be useful as treatment monitors in patients with oral SCC.

REFERENCES