Original Article

Diagnostic Value of Serum and Saliva Matrix Metalloproteinase13 (MMP13) in Oral Squamous Cell Carcinoma

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ABSTRACT

BACKGROUND AND OBJECTIVE: Matrix metalloproteinases (MMPs) are a group of enzymes responsible for extracellular matrix breakdown. Increased activity of type 13 is involved in invasion of oral squamous cell carcinoma. This study was performed to compare the level of matrix metalloproteinase13 (MMP13) in saliva and serum of patients with oral squamous cell carcinoma and healthy controls.

METHODS: In this experimental in vitro study, 24 saliva and 24 serum samples were collected from patients with primary oral squamous cell carcinoma after histopathologic confirmation, whereas 21 saliva samples and 21 serum samples were collected from healthy subjects with mucosal health confirmation. Clinical examination was done for MMP-13 levels in saliva and serum, and the results were compared by ELISA.

FINDINGS: MMP-13 levels in serum (7.47±2.36) and saliva (8.42±2.69) of patients with oral squamous cell carcinoma compared to healthy subjects increased respectively by 5.39±1.90 and 6.72±2.11 (P= 0.002 and P= 0.025, respectively). The levels of MMP13 in saliva and serum to determine the existence of oral squamous cell carcinoma has a "good" diagnostic value, with sensitivity and specificity of 88% and 86% respectively for serum, and 88% and 71% respectively for saliva.

CONCLUSION: According to the results of this study, the levels of MMP13 in saliva and serum of patients with oral squamous cell carcinoma was higher than in healthy individuals and this biomarker had good diagnostic value in the diagnosis of oral squamous cell carcinoma.

KEY WORDS: *Oral squamous cell carcinoma, Matrix metalloproteinase 13, Saliva, Serum.*

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Introduction

Oral squamous cell carcinoma has the highest incidence of malignancy in the oral cavity, accounting for more than 90% of oral neoplasms. Oral carcinoma is one of the most common cancers and one of the 10 most common causes of death worldwide (1). According to statistics of 2009, cancer is the third leading cause of death in Iran. Oral cancers account for 1.24% of cases of cancers in women and 0.93% of cases of cancers in men. Overall, Iran has a high incidence of cancer compared to world statistics, and this trend is increasing (2).

The five– year absolute survival rates at the early stages of the disease is 60% to 80%, and at the advanced stages of the disease is reduced to 20% (3,4). However, more than 50% of patients with oral cancer are diagnosed at more advanced stages of the disease, which results in lower prognosis and lower survival rates (3). This is probably due to the absence of pain in the early stages of the disease. 72.9% of patients experience pain only in advanced stages of the disease (5). These issues highlight the importance of diagnostic methods for early detection of oral squamous cell carcinoma (3).

Recently, tumor markers have been increasingly used and have helped to detect oral squamous cell carcinoma in very early stages, even before the smallest clinical signs in the patient. These markers are a set of specific proteins and mRNAs known to be present in saliva and serum, whose alteration from normal size may indicate oral squamous cell carcinoma (OSCC) in the person (3,6). Many studies have shown that the salivary and serum protein profile of patients with oral squamous cell carcinoma differs from that of healthy individuals because the salivary protein products are altered by internal and external factors (3).

Matrix metalloproteinases (MMPs) are a group of enzymes responsible for extracellular matrix degradation and play a key role in phenomena such as proliferation, differentiation, apoptosis, angiogenesis, morphogenesis, and repair of body tissues. The basement membrane that separates the epithelium from the mesenchymal tissue is the first barrier against tumor expansion. Decomposition of the basement membrane and extracellular matrix requires the activity of matrix metalloproteinase (7).

The expression of matrix metalloproteinase gene and its activity in normal body tissue are usually low, but matrix metalloproteinase activity is highly elevated at times such as pathological changes that lead to tissue destruction, inflammatory disease, tumor growth, and metastasis (8). So far, 25 members of the matrix metalloproteinases family have been discovered, each responsible for the breakdown of a particular group of collagens. For example, MMP13 is responsible for the isolation and breakdown of collagen types 1 & 4. Matrix metalloproteinase is said to be a reliable biomarker for the diagnosis of oral squamous cell carcinoma, even before clinical manifestations (7,9). Some recent studies have found the measurement of MMP13 to be a more reliable factor than other MMPs for diagnosis and prognosis of oral squamous cell carcinoma (8,10,11).

MMP13 is not found in normal epidermal keratinocytes, whereas it is expressed as an effective proteinase in altered epidermal keratinocytes in malignancies such as squamous cell carcinoma (12). MPMP13 breaks down collagen, basement membrane and other extracellular matrix components in an uncontrolled way, which may be related to tumorigenesis. Given these issues and recent studies, increased MMP13 levels may be an important marker for determining changes of epithelial cell toward malignancy and an important prognostic factor (2,12).

Assurance of increased MMP13 expression in oral squamous cell carcinoma will point to the role of this proteinase in the pathogenesis, and by conducting extensive studies with high sample size in this regard, it may be possible to establish serum and saliva MMP13 as a reliable marker for early detection and can also be used to determine prognosis, behavior, and ultimately to treat the tumor. The aim of this study was to measure and compare the levels of MMP13 in saliva and serum of patients with oral squamous cell carcinoma and healthy controls.

Methods

In this experimental in vitro study, after approval of the Ethics Committee of Babol University of Medical Sciences (Code IR.MUBABOL.REC.1397.009), 24 saliva samples and 24 serum samples were collected from patients with oral squamous cell carcinoma with pathologic confirmation who received no treatment for their disease. In addition, 21 saliva samples and 21 serum samples from healthy individuals were selected with confirmation of mucosal health and clinical examination, which were matched for age and gender

with the patient group. All subjects in the two groups had no systemic disease, no medication at the time of study, no active periodontal disease or severe dental caries, and no lesion was observed elsewhere in their mouth. 0.5 cc fasting blood samples were prepared by disposable sterile syringes. Within a maximum of one hour, the samples were centrifuged at 3000 rpm for 5 minutes, after coagulation and clotting. During this phase, the serum is removed from the clot. They were then frozen and stored at -80 °C. "Spitting" method was used to prepare non-stimulated saliva samples. Patients were asked to refrain from eating and drinking and brushing for 90 minutes before sampling. Samples were collected between 9 and 11 am. The patient sat comfortably, while slightly bent forward, and discharged saliva was poured into the sterile test tube every 1-2 minutes for 10 minutes. After encoding and sealing the tube with Parafilm, it was centrifuged at 2000 rpm for 10 min.

To evaluate the salivary cytokines, Enzyme-linked immunosorbent assay (ELISA) Eastbiopharm kit was used by biotin/avidin double-antibody sandwich technique. In this method, MMP13 was added to wells previously coated with "MMP13 monoclonal antibody". It was then incubated, and anti-MMP13 antibody (labeled as biotin) was added to the wells to form a bond with " Streptavidin-HRP". The enzymes that did not form a bond were isolated during the wash out process. "Chromogen A&B" and "Stop" solutions were then added to the wells.

The color change in the wells was read with respect to the blank wells (as control and zero reference). The resulting color change showed the MMP13 levels of each sample. First, the reactants, samples and standard solution were prepared. Then, the second Antibody (labeled as biotin), and the ELISA solution were prepared and added to the standard solution, and were allowed to react at 37 °C for 60 min. Plate were washed 5 times, Chromogen A&B was added, and solution was incubated at 37 °C for 10 min to obtain color change.

Then, stop solution was added and the "OD" value was read 10 minutes later. Finally, the data were entered into SPSS software and analyzed by Chi-Square, T-test and Pearson correlation coefficient at significance level of $P<0.05$.

Results

The mean age of patients with oral squamous cell carcinoma was 61.2 ± 12.1 years. 14 samples (58.3%) were related to women and 10 samples (41.6%) were for men. The healthy group consisted of 12 women (57.1%) and 9 men (42.8%) with mean age of 65.2 ± 14.7 years. There was no significant difference between the two groups in terms of age and gender; (P=0.936) and (P=0.330), respectively. The levels of MMP13 in the saliva and serum samples of these subjects were determined by ELISA test, and the difference between the two groups was significant in terms of the levels of MMP13 in saliva and serum (p<0.05) (Table 1). MMP13 levels in saliva and serum in both healthy and patient groups were significantly correlated (P<0.001), and according to the Pearson correlation coefficient calculations, r=0.905 was measured, indicating a direct linear relationship (Figure 1).

The ROC curve was used to determine the diagnostic value of the test and the area under the curve was presented with 95% confidence interval. The cut off point for MMP13 in saliva and serum was calculated separately based on ROC curve, while sensitivity, specificity, positive and negative prediction value, and positive and negative likelihood ratios with 95% prediction interval were reported. According to Figure 2, the area under the curve measured for serum was 0.68–0.9 and for saliva was 0.62–0.9, indicating a "good" diagnostic value of MMP13 for the determination of oral squamous cell carcinoma. The sensitivity and specificity were 88% and 86% for serum, and 88% and 71% for saliva, respectively (Table 2).

Table 1: Comparison of MMP13 levels in serum and saliva in patients with oral squamous cell carcinoma and healthy controls (ng/ml)

MMP13 levels	Oral squamous cell carcinoma Mean±SD	Healthy Mean±SD	P-value
Serum	7.47±2.36	5.39±1.90	0.002
Saliva	8.42±2.69	6.72±2.11	0.025

Table 2: Sensitivity, specificity, positive and negative predictive value and positive and negative likelihood ratios with 95% prediction interval for salivary and serum MMP13 in patients with oral squamous cell carcinoma

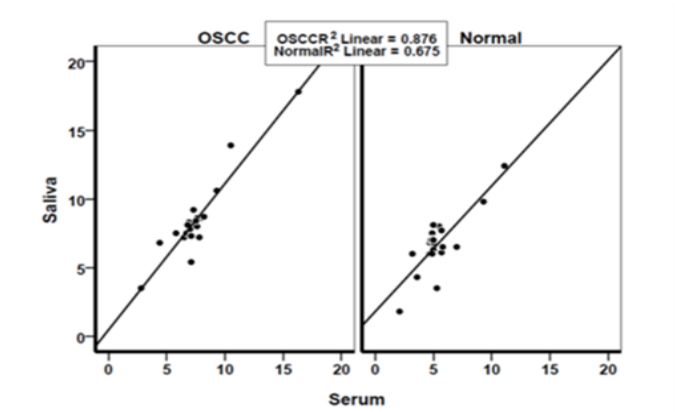|	Serum≥6 With 95% confidence interval	Saliva≥7.2 With 95% confidence interval
Sensitivity	88% (74–100%)	88% (74–100%)
Specificity	86% (71–100%)	71% (52–91%)
Positive predictive value	88% (74–100%)	78% (62–93%)
Negative predictive value	86% (71–100%)	83% (66–100%)
Positive likelihood ratio (LR$^+$)	6.13 (2.13–17.65)	3.06 (1.53–6.12)
Negative likelihood ratio (LR$^-$)	0.15 (0.05–0.43)	0.17 (0.06–0.52)

Figure 1: Relationship between the levels of MMP13 in saliva and serum of healthy individuals and individuals with oral squamous cell carcinoma

Figure 2: ROC curve to determine the diagnostic value of MMP13 in saliva and serum of patients with oral squamous cell carcinoma

Discussion

The results of this study showed that the incidence of MMP13 expression in both saliva and serum of patients with oral squamous cell carcinoma (OSCC) is higher than that of healthy subjects, which is in line with some previous studies (11–13). In the present study, in 90% of salivary and serum samples of oral squamous cell carcinoma patients, the level of MMP13 was higher than the control group. The high incidence of MMP13 in oral squamous cell carcinoma samples indicates the important role of this matrix metalloproteinase in the diagnosis of this disease. The study of Agha-Hosseini et al. showed a weak statistical association between the levels of MMP13 present in "non-stimulated" saliva and the serum of oral squamous cell carcinoma patients, while its levels showed no association in the "stimulated" saliva and serum samples (12). The results of the present study also showed a significant relationship between the levels of MMP13 in "non-stimulated" saliva and the serum of patients with oral squamous cell carcinoma. In the present study, the mean increase of MMP13 in both saliva and serum samples of patients was significant compared to the control group. However, the average increase in serum was greater than the average increase in saliva. This suggests that serum may be a more valid criterion for measuring MMP13 elevation in patients with oral squamous cell carcinoma.
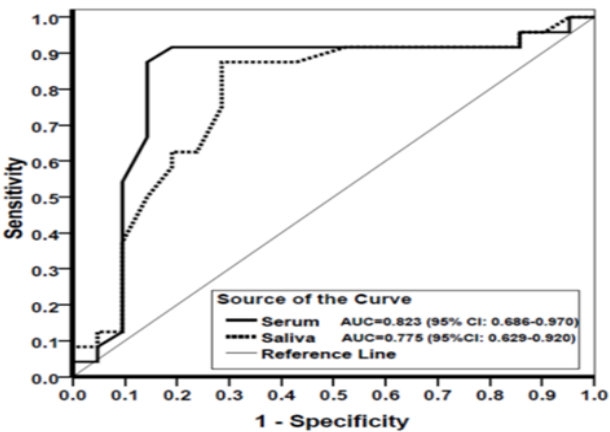
Schiegnitz et al. stated that MMP13 is a potent collagenase that is rarely present in normal tissues, but when high turn over of the extracellular matrix is required (including during local invasion or expansion of metastatic masses), the levels of this enzyme grows in tissue. Studies have shown that MMP13 is secreted directly by cancer tissue, but indirectly contributes to angiogenesis of this tissue. Studies of this group on serum samples of 81 patients with oral squamous cell carcinoma and comparison of MMP13 levels with healthy subjects showed that MMP-13 levels or even lesions increased in patients with oral squamous cell carcinoma compared to normal subjects. But this was not statistically significant (13). The study of Choudhry

et al. showed a significant increase in MMP13 in the serum of patients with oral squamous cell carcinoma compared to healthy controls. However, they stated that only MMP12 could be a tumor marker for oral squamous cell carcinoma, as other MMPs, including MMP13, have a "moderate" diagnostic value to determine the presence of oral squamous cell carcinoma (14). However, Nosratizehi et al. identified MMP13 as a tumor marker for diagnosis of oral squamous cell carcinoma. This group compared the salivary levels of MMP-13 in oral squamous cell carcinoma patients and compared it with healthy controls, which was significantly higher in the patient group than in healthy controls. They suggested that MMP13 could be used as a tumor marker to diagnose oral squamous cell carcinoma as well as to determine the prognosis and extent of tumor invasion, especially at higher stages of the disease (11).

A study by Vincent-Chong et al. showed a strong association between increased MMP13 protein levels and the presence of lymph node metastasis, disease progression, and larger tumor size (15). In this study, we found that MMP13 could be a biomarker for the determination of oral squamous cell carcinoma in saliva and serum with good diagnostic value. On the other hand, the study of Agha-Hosseini et al. showed no significant difference between serum and saliva of oral squamous cell carcinoma patients at stage 1, 2 and stage 3, 4 (12).

In addition, the study by Schiegnitz et al. showed no significant association between MMP13 levels and severity of metastasis, recurrence, or prognosis (13). Overall, both Agha-Hosseini and Schiegnitz studies showed that MMP13 is not a good marker for disease stage or prognosis, whereas Vincent-Chong et al. believed that MMP13 levels are independent and very powerful determinant for disease prognosis. In the study of Vincent-Chong et al., 95.5% of the tissue samples of oral squamous cell carcinoma patients showed increased expression of MMP13 gene. The difference in MMP13 protein levels in normal epithelium and oral squamous cell carcinoma was also statistically significant (15). A study by Marcos et al. showed that high levels of MMP13 in serum of patients with squamous cell carcinoma are associated with the likelihood of lymph node metastasis. They determined the sensitivity of MMP13 as a tumor marker at 76% and its specificity at 100% (16).

The study by Marcos et al. found that serum levels of MMP13 to determine oral squamous cell carcinoma were less sensitive than the present study, but specificity was 100% (16). The study of Mishev et al. also stated that MMP13 has a "good" diagnostic value for the diagnosis of oral squamous cell carcinoma, while other MMPs, including MMP9 and MMP2 do not have this property (7). In the present study, measurements below the curve with a 95% confidence interval showed that the level of MMP13 in saliva and serum was of "good" diagnostic value to determine the presence of oral squamous cell carcinoma. The sensitivity and specificity of the test were 88% and 86% for serum and 88% and 71% for saliva, respectively.

Given the significant difference in the levels of MMP13 in saliva and serum of patients with oral squamous cell carcinoma compared to healthy individuals, it seems that this enzyme may be a useful diagnostic criterion for determining this disease. According to the cut off point measured in this study, this test had a "good" diagnostic value, and sensitivity and specificity were 88% and 86% for serum, and 88% and 71% for saliva, respectively.

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