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*International Journal of Current Research*
*Vol. 8, Issue, 05, pp.30496-30501, May, 2016*

**INTERNATIONAL JOURNAL OF CURRENT RESEARCH**

**ISSN: 0975-833X**

## RESEARCH ARTICLE

# SALIVARY CONCENTRATION OF MMP-9 IN COMPARISON WITH THE TISSUE EXPRESSION IN PATIENTS WITH ORAL SQUAMOUS CELL CARCINOMA

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**ARTICLE INFO**

***Article History:***
Received 06th February, 2016
Received in revised form
29th March, 2016
Accepted 22nd April, 2016
Published online 10th May, 2016

***Key words:***
Saliva,
MMP-9,
Oral Squamous Cell Carcinoma,
Immunohistochemistry

**ABSTRACT**

Oral squamous cell carcinoma (OSCCs) is one of the most prevalent types of all oral neoplasms that highly require early diagnosis. Salivary evaluation is non-invasive, simple, and rapid.The aim of the current study was to investigate the relationship between salivary MMP-9 concentration and its tissue expression in patients with OSCC.Materials and Methods: Saliva samples were collected from 24 patients with primary diagnosis of OSCC and 24 healthy age-and-gender- matched. The unstipulated saliva was collected with spitting method. After saliva collection, the tissue samples were obtained by biopsy. Salivary concentration and tissue expression of MMP-9 were evaluated using ELISA and immune histochemistry assays, respectively. Data were analyzed using t-test, chi-square, and ANOVA tests with the confidence interval of 95%. Results: A significant increase in salivary concentration of MMP-9 was evident in OSCC patients in comparison to healthy individuals (P-value < 0.05).In addition, the MMP-9 salivary concentration was significantly higher in males in comparison with females (P-value < 0.05). There was a positive and significant correlation between salivary concentration of MMP-9 and its tissue expression (Pearson Correlation = 0.669, P-value< 0.05). Conclusion: Salivary evaluation of MMP-9 concentration could be a valuable tool to detect OSCC.



**Citation: Dr. Gholamreza Jahanshahi, Dr. Laleh Nazemi,Dr. Ardeshir Talebi, 2016.** "Salivary concentration of mmp-9 in comparison with the tissue expression in patients with oral squamous cell carcinoma", *International Journal of Current Research*, 8, (05), 30496-30501.

## INTRODUCTION

It has been demonstrated that oral cancers consist 2–4 % of all cancer cases worldwide (Siegel *et al*., 2013; Markopoulos, 2012; Ferlay *et al*., 2008; Parkin *et al*., 2005; Oral health in America, 2000). Oral squamous cell carcinoma (OSCCs) accounts for one of the most prevalent types of all oral neoplasms which has consisted nearly 90% of all oral neoplasm (Choi and Myers, 2008; Jahanshahi and Sabaghian, 2012). There exist no consensus regarding the exact risk factors in development of OSCC; however, it has been reported that OSCC is acquired via a combination of genetic and environmental risk factors (Decker and Goldstein, 1982; Sugimura *et al*., 2006; Brennan *et al*., 1995).

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The early detection of OSCC is of high importance in the success of treatment and its survival rate(Siegel *et al*., 2013; Jahanshahi and Sabaghian, 2012). The primary line of treatment protocol in OSCC includes surgery and radiotherapy; the side effects of which are disturbances in swallowing,speech, and physical appearance. Although treatment outcome may affect the patient'squality of life, patients are at risk of recurrence which occurs in 15–33 % of cases (Mücke *et al*., 2009; Sklenicka *et al*., 2010). As a result, development of an appropriate diagnostic tool is of high importance both to patient and clinician (Mücke *et al*., 2009; Sklenicka *et al*., 2010; Gonzalez-Garcia *et al*., 2009).The current concept of OSCC detection is based on a thorough clinical and oral examination along with biopsy for a histologicalexamination. Biopsy has two downfalls; firstly it is a relatively invasive approach which could further stimulate the malignant cells. On the other hand it is highly dependent on the location of which biopsy has been taken. As the appearance of cancerous and precancerous lesions is somehow nonuniform, it

could be challenging to select the right location. In recent years attention has regarded toward a more easy ways for detection of OSCC specially in early stage. So assay of some tumor markers Like matrix metalloproteasein body fluid like saliva has come to consideration as a non-invasive method (Tabor *et al*., 2004; Tabor *et al*., 2002; Tabor *et al*., 2001.Matrix metalloproteinases (MMPs) are a family of zinc and calcium-dependent proteolytic enzymes. In humans, 23 MMPs are described, and they are either membraneanchoredor secreted. MMP-9 has been investigated over its role in metastasis of OSCC. While it can degrade the principle constitutes of basal membrane (collagen IV), it may have role in OSCC metastasis and progression. The concentration of MMP-9 in tissue samples has found to be in correlation with the stage and status of the OSCC (Hadler-Olsen *et al*., 2013). However, Shpitzer *et al* (2009) found that the salivary concentration of MMP-9 has also the ability to predict the status of OSCC.As the salivary biomarkers are appropriate tools in initial screening of oral and systemic diseases within a safe and repeatable sample collection, the aim of the current study was to investigate the relationship between salivary MMP-9 and its tissue in patients with OSCC. Our null hypotheses were that there would be no differences in the salivary concentration of MMP-9 of study and control groups and also there would be no association between salivary and tissueexpression of MMP-9 in OSCC.

## MATERIALS AND METHODS

### Patients

This study was performed in Esfahan University of Medical Sciences. Patients who were referred to Esfahan dental clinic which had been primarily diagnosed with oral squamous cell carcinoma (OSCC) were participated in the present study. Patients who had been biopsied or received any treatment prior the saliva collection and were excluded from the study. The control group included healthy subjects with identical age and gender of study group.

### Saliva Collection

Saliva can be collected in two ways: unstimulated and stimulated. Unstimulated saliva is collected by draining or drool, spitting, suction, or swab while stimulated saliva is collected by providing a stimulant agent (i.e. citric acid, paraffin, or a gum base). While stimulated saliva is obtained primarily from the parotid gland, unstimulated saliva is produced primarily by the submandibular gland (de Almeida *et al*., 2008; Dodds*et al*., 2005). Unstimulated saliva collected from 24 patients with primary diagnosis of OSCC and 24 healthy subjects using the spitting technique. In this technique patients were asked to maintain the saliva for 5 to 15 minutes and spit it every 60 seconds. Patients were instructed to prohibit brushing, smoking, eating, and drinking 90 minutes prior to saliva collection.

### MMP-9 Concentration in Saliva

Saliva samples were centrifuged for 10 minutes and the pellets were suspended in 150 ml of lysis buffer (45mM HEPES, 0.4M KCl, 1mM EDTA, 10% glycerol). Following 30 minutes incubation at room temperature the samples were centrifuged for 10 minutes. The concentration of protein in the supernatants was determined. A volume containing 50 mg of protein was transferred to a 1.5 ml vial. All samples were brought to the same volume of 500 ml using Sorbent PBS solution. Following mixing the solutions well, 100 ml of each sample was added to ELISA-plate wells (Fig 1a). The plate was covered and stored overnight at 4 degrees of centigrade. The other day each well was washed with 100 ml PBS–Tween solution for three times. Then a volume of 100 ml of 1% BSA PBS-T blocking solution was added to each well. After 60 minutes incubation at room temperature, 100 ml of primary antibody was added to each well. In order to identify and measure the concentration of MMP-9, rabbit monoclonal antihuman antibody (Mousa; 1:1000; sigma- aldrish, Saint Louis, USA) was used.

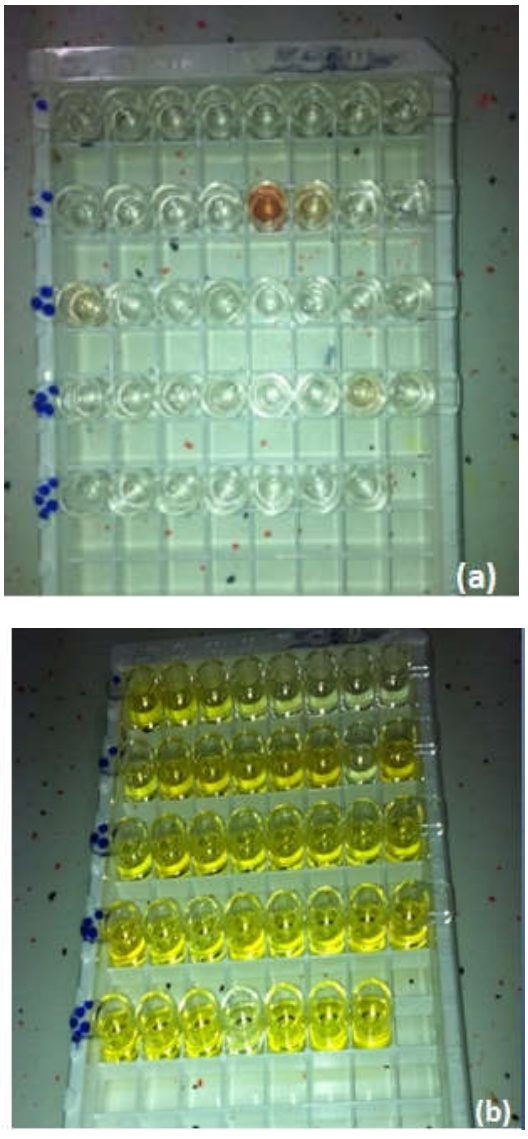



**Fig. 1. Changes in the color of ELISA wells (a) prior and (b) after adding antibody**

After 120 minutes incubation at room temperature, the plates were washed with aforementioned technique. Then 10 ml of secondary antibody attached to Peroxidase enzyme (1:5000; Jackson I; west, Grove, Pa, USA) was added to each well.

After further 120 minutes incubation at room temperature the plate was washed as described above. In order to develop color in samples 100 ml of 3,30,5,50-tetramethylbenzidine solution (Southern Biotech, Birmingham, AL, USA) was added to each well (Fig 1b). After 1–2 min 100 ml of stopping reagent to each well (10% sulphuric acid) was added. Absorbencies of the samples were measured at the wavelength 450nm using a Zenith 200 ELISA reader (Anthos,Eugendorf, Austria).

**Tissue Sample Preparation**

Following saliva collection, patients with primary clinical diagnosis of OSCC were underwent a biopsy to establish the diagnosis. After 24 houers fixations in 10% formalin and ordinary tissue processing for samples,The samples were embedded in paraffin blocks and were cut into 3 μm slices. One of the slices was used in H&E staining and the other in immunohistochemistry (IHC) staining. Slices were mounted on poly-L-lysine-coated slides. Briefly, the sections were dewaxed with xylene, and rehydrated in graded ethanol.Prepared slides were put in hematoxylin and eosin staining. Then the slides were rinsed with water and were dehydrated in gradual alcohol. For IHC staining slides were dehydrated and were immersed in EDTA (Novacastra, Germany) for 20 minutes (95 centigrade degree). Slides were remained in cold condition for 30 minutes and rinsed in hydrogen peroxidase. Then slides were preserved with the secondary antibody for 30 minutes, conjugated using the conjugation solution (Novacastra, Germany) for 30 minutes, and finally rinsed in DAB solution (Novacastra, Germany) for 5 minutes. Then the slides were rinsed with water and stain with H&E staining.

**MMP-9 Concentration in Tissue Samples**

The stained tissue samples were evaluated under light microscope with 40× magnitude. Two blind pathologists counted the number of stained cells and degree of staining in 5 random fields to calculate the SID criteria. The number of stained cells was categorized into 1: 0-25%; 2: 26-50%; 3: 51-75%; and 4: 76-100%. In addition the degree of staining was categorized into 0: no staining; 1: very low; 2: low; 3: moderate; 4: high. The SID criteria calculated with multiplying the number of stained cells with degree of staining.

**Statistical Analysis**

Data were collected in SPSS software version 11.5 (Chicago, IL). In order to analyze data, t-test, chi-square, and ANOVA tests were performed with the confidence interval of 95%.

## RESULTS

In the current study 24 (17 males and 7 females) healthy and 24 (17 males and 7 females) patients with OSCC participated. The mean age and gender distribution of both groups was identical. Based on the Kolmogrov-Smirnov test the data were distributed normally (P-value > 0.05). The mean concentrations of salivary and tissue MMP-9 is presented in Table 1.

**Table 1. Mean and standard deviation of MMP-9 concentration**

| Variable | N | Mean | Standard deviation |
|---|---|---|---|
| Salivary (control group) | 24 | 10049.50 | 4773.00 |
| Salivary (study group) | 24 | 18539.19 | 8926.73 |
| Tissue (study group) | 24 | 12.96 | 4.03 |

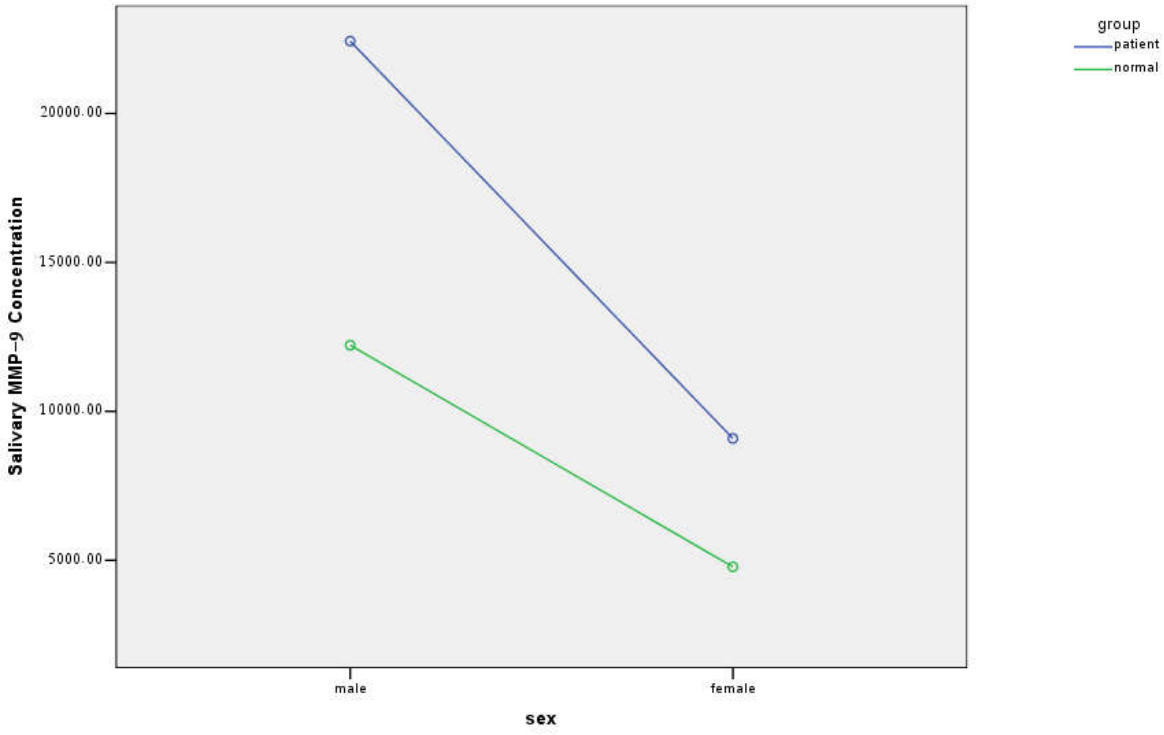
The distribution of study variables based on gender is presented in Table 2. According to the independent sample t-test, no significant difference observed between males and females regarding age (P-value > 0.05). However, a significant difference between two genders observed regarding tissue and salivary concentration of MMP-9 (P-value < 0.05) (Table 2)In order to investigate the correlation between salivary concentration of MMP-9 and its tissue expression, Pearson test was performed. The outcome of the mentioned analysis revealed a significant association between these two concentrations (Pearson Correlation = 0.669 ،P-value = 0.000). To evaluate the effect of gender (male and female) and group (study and control) on the salivary MMP-9 concentration, Univariate Analysis of Variance was performed. The results of this analysis revealed a significant difference between two genders (P-value = 0.000) and groups (P-value = 0.000). In addition In addition, no significant interaction was found between two variables (gender and group) (P-value = 0.082) (Graph 1).

## DISCUSSION

Early diagnosis and detection result in greater rate of survival and is crucial in successful treatment. Most of the OSCC cases are detected in the advanced stages of the disease. The OSCC lesions are usually painless in the early stages without any alarming symptoms(Markopoulos, 2012). In addition there is possibility that some lesions could not be found in a general examination if located in hard-to-find regions.The analysis of saliva is challenging as the concentration of potential biomarkers is small which varies from milligrams to picograms per milliliter (Chiappin, 2007; Humphrey and Williamson, 2001; Amerongen *et al*., 2004; Liu and Duan, 2012).MMP-9 has a role in angiogenesis as a fundamental procedure in the progression of cancer. MMP-9 releases and activates various pro-angiogenic factors including vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and transforming growth factor beta (TGF-β) (Gialeli *et al*., 2013; Yu and Stamenkovic, 2000; Belotti *et al*., 2003). In addition, MMP-9 has the ability to degrade the basement membrane within disintegration of its main component (collagen IV). This enables the metastasis of cancerous cells to reach further sites in the body through blood or lymph stream (Sorsa *et al*., 2004). Some studies have found that a generally high expression level of a number of MMPs including MMP-9 in tumor associated fibroblasts or macrophages were associated with increased risk of distant metastases (delCasar *et al*., 2009; Vizoso *et al*., 2007). Most of the previous studies were aimed to evaluate the presence MMP-9 in tissues and few studies had evaluated the serum or salivary concentration of MMP-9. Wang *et al* (Wang *et al*., 2013) investigated the serum concentration of MMP-9 in the serum of patients with HNSCC.

**Table 2. Distribution of study variables based on gender**

| Variable | Gender | N | Mean | Standard deviation | P-value |
|---|---|---|---|---|---|
| Salivary MMP-9 (control group) | male | 17 | 12219.58 | 3256.22 | 0.000 |
| | female | 7 | 4779.28 | 3647.93 | |
| Salivary MMP-9 (study group) | male | 17 | 22430.05 | 5600.07 | 0.000 |
| | female | 7 | 9089.92 | 8647.10 | |
| Tissue MMP-9 (study group) | male | 17 | 14.59 | 1.25 | 0.040 |
| | female | 7 | 9.00 | 5.67 | |
| Age (both groups) | male | 17 | 61.76 | 11.92 | 0.070 |
| | female | 7 | 71.71 | 10.76 | |




**Graph 1. Salivary concentration of MMP-9 according to gender and group**

They found that the MMP-9 was significantly higher in the serum of patients when compared with control individuals. Cheng *et al* (Cheng *et al*., 2014) found that the serum concentration of MMP-9 was significantly higher in patients with nasopharynx SCC in comparison to healthy individuals. In addition, it has been demonstrated that MMP-9 could be prognostic marker in colorectal, breast, and prostate cancer (Ring *et al*., 1997; Stankovic *et al*., 2010; Zhong *et al*., 2008).The aim of the current study was to investigate the relationship between salivary concentration of MMP-9 and its tissue expression patients with OSCC. Our first null hypothesis was that there would be no differences in the salivary concentration of MMP-9 of study and control groups. The second null hypothesis was that there would be no association between salivary and tissue concentration of MMP-9 in OSCC. The first null hypothesis was rejected as the salivary concentration of MMP-9 was significantly higher in OSCC patients when compared with control group. The second null hypothesis was also rejected as there was significant association between salivary and tissue concentration of MMP-9 in patients with OSCC. Based on the findings of the present study, the salivary concentration of MMP-9 was significantly higher in patients with OSCC when compared with healthy subjects. In accordance with our findings, Shpitzer *et al* (2007) evaluated the salivary concentration of 25 OSCC and 25 healthy –age and gender-matched individuals and found a significant increase in MMP-9 concentration in saliva of OSCC patients. In addition, Shpitzer *et al* (2009) performed another study and found significant increase in salivary concentration of MMP-9 in 19 patients with OSCC. As a result the need for accessible screening sample is essential (Zini *et al*., 2010; Warnakulasuriya, 2009; Peacock *et al*., 2008). The results of the present study unveiled that salivary concentration of MMP-9 could be promising assessment to diagnose OSCC in early stages.In the current study the unstimulated saliva was collected with the spitting technique. Shpitzer *et al* (2009) were also used the same method. Saliva collection is non-invasive, simple, and rapid method when compared with other diagnostic tools. It should be noted that stimulation of saliva changing the composition of the saliva in favor of larger molecules as it decreases the concentration of small molecules (including myoglobins) (Mohamed *et al*., 2012). Hence, it is obvious that unstimulated saliva is more favorable when the study aims to evaluate trace biomarkers (Principe *et al*., 2013). To evaluate

the salivary concentration of MMP-9, sandwich ELISA technique was used in the present study. Similar to our study, Shpitzer *et al*. (2007) were also used the identical methodology to assess the MMP-9 in saliva. In the current study individuals in control group were adjusted according to their age and gender with the OSCC patients. Hence, the effect of the mentioned demographic variables was eliminated. Similar to our study, Shpitzer *et al*. (2009) (Shpitzer *et al*., 2007) were also matched the age and gender of their participants in study and control groups. One of the limitations of the current study was the lack of knowledge regarding the stage of tumor. It was due to the deficiency of patients' documents. In case of knowing the stage of tumor, we would be able to evaluate the changes of salivary concentration of MMP-9 to predict the stage of OSCC. Hence, it is recommended to consider mentioned data in future studies.

The other limitation of the present study was its sample size; it is highly recommended to perform prospect studies on higher number of participants to ensure the effectiveness of salivary MMP-9 as a diagnostic tool in OSCC. Patel *et al.* (Patel *et al*., 2007) found that the changes in serum concentration of MMP-9 were in line with the progress of patients' treatment. In addition, Shpitzer *et al*. (2007) found that the salivary assessment of MMP-9 concentration has high sensitivity (100%) and specificity (79%);az mentioned above insufficient number of participations in our study did not allow us to calculate sensitivity and specifityalong with the results of the current study regarding the strong relationship between the tissue and salivary concentration of MMP-9, it could be concluded that this method is a valuable tool to diagnose OSCC in early stages. According to the limitations and results of the present study, it is recommended to perform further studies on higher number of patients while considering the stage of the OSCC.

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