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**ORIGINAL ARTICLE**

# Association of serum Matrix Metalloproteinase 1, 2 and 3 with Oral Squamous Cell Carcinomas

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## ABSTRACT

**Background:** Squamous cell carcinoma involving the oral cavity (OSCC) is a malignant lesion prone to local dissemination and distant metastasis. It causes severe morbidity in the afflicted patients and has low 5-year survival rate. The current study explored the serum levels of extracellular matrix degrading enzymes, the MMPs 1,2 and 3 in OSCC patients and normal healthy controls.

**Materials and methods:** Blood samples from thirty eight patients suffering from OSCC and thirty eight normal healthy controls were included in the study. The MMP 1 - 3 were estimated by multiplex ELISA.

**Results:** Out of three MMPs analyzed between cases (n=38) and controls (n=38), significant differences were observed in serum MMP levels in MMP 1 and 2. In MMP 1, cases were recorded to have higher values, as evidenced by mean and median levels. ROC curve analysis, used to assess the prognostic and diagnostic worth of the two statistically significant markers (MMP 1, 2) indicated that both markers had area under the curve (AUC) significantly better than 0.5. For MMP 2, control group had higher serum levels than cases.

**Conclusions:** MMP 1 expression was found significantly elevated in cases with 71.0% sensitivity and 56.3 specificity so it can be further evaluated as a drug target as well as diagnostic and prognostic tumor marker.

**Keywords:** Oral squamous cell carcinoma, Matrix metalloproteinase.

## INTRODUCTION

Oral cancer accounts for one of the top 10 cancers in the world. Oral malignancies account for around 3% of all cancer occurrences; they are more prevalent in males than in females; and two-thirds of cases occur in poor nations. It is the second commonest cancer in Pakistan[2]. In terms of occurrence, major oral cancer is the squamous type i.e. oral squamous cell carcinoma (OSCC)[3]. Despite significant advances in the field of OSCC medical care, it still presents as a disease of very late diagnosis and has increased recurrence rate, immensely affecting quality of life and has 5-year survival rate of about 55%[4] .

As with almost all cancers, the pathogenesis of OSCC is also multifactorial, comprising of a myriad of genetic and environmental factors. It's worth noting that this type of cancer has been linked to particular risk factors including cigarette and/or alcohol use[5]. Despite this evident link, a significant number of individuals acquire OSCC without ever having been exposed to them, highlighting the importance of additional risk factors such as genetic vulnerability and oncogenic viruses[6].

The initial stage of tumor invasion involves secretion of elevated titers of proteases leading to the destruction of extracellular matrix (ECM) including the basement membrane, hence giving easy routes to tumor cells for migration and spread via circulatory and lymphatic channels[7]. These ECM degrading proteases are generally divided into four basic categories. One of these, the matrix metalloproteinases (MMPs), serves to enable matrix turnover by destruction of matrix components. Here in this study, we analyze the levels of members of three subclasses of MMPs, MMP 1 collagenase, MMP 2 gelatinase, and MMP 3 stromelysin.

MMP-1, a collegenase, is defined by capacity to breakdown interstitial tissue collagen I, II, and III at specific locations. These enzymes can break a variety of ECM and non-ECM compounds in addition to collagens. MMP2 is also known as gelatinase A. Both of these cleave the denatured collagens and gelatins. The Fibronectin type II domain trirepeat in these metalloproteinases binds to gelatin, laminin and collagen. Only MMP-2, out of gelatinases, cleaves type I, II, and III collagen. MMP-3 (Stromelysin 1) has a higher proteolytic capacity of all stromelysins. In addition to digesting various ECM components, MMP-3 acts on and activates many proMMPs, its action on proMMP-1 being critical in generation of a complete active MMP-1[8].

Various studies retrospectively analyzing the expression of MMPs in cancer patients show that presence or enhanced expression of numerous MMPs, such as MMP 1, 2, and 3, at the original cancer site and/or distant metastases, is linked to poor prognosis, poor cancer cell differentiation, invasive cancer stage with low patient survival, and metastatic dissemination to distant locations.[9].

In normal healthy persons, MMPs are synthesized only as and when required during the course of tissue remodeling in various steps like fetal maturation, reduction in uterus and mammary size following parturition, wound repair, during the process of cartilage replacement with bone in ossification and in placental development – during trophoblast penetration in the endometrium. However these are also released because of/or may result in, pathological conditions like joint disease (rheumatoid arthritis), cancer infiltration and metastasis[10].

In many of the cancers, including OSCC, MMPs have been shown to anticipate the incidence of tumor recurrence, thus elevated MMPs expression in tumor tissue correlates with progressive disease with or without infiltrative growth and lymphatic involvement[11]. This enhanced expression of MMPs is found characteristic for head and neck squamous cell carcinoma[12]. Various global studies have also shown positive relation between OSCC and MMPs[13-14] . Currently, no serological markers or other such laboratory parameters exist that can assist in the discovery of primary OSCC at a stage when there is no detectable cancer tissue or precursor yet[15]. The efforts on this front are based on the premise that displacing the time of detection of tumor sooner in the timeline of carcinogenesis may not only extend the survival but also improve the quality of the patient's life by limiting cancer related morbidity[16].

It has been noted while reviewing the current studies that all of them have exclusively highlighted a few markers, and have rarely emphasized the roles of rare biomolecules like adhesion and proteolytic molecules. Hence, in our study, we used a new multianalyte Bio-Plex® MAGPIX™ Multiplex Reader[17]. which processes simultaneous measurement of a panel of serum MMPs of OSCC patients and controls. Here, we wanted to see if the titres of MMP 1, 2, and 3 in OSCC patients and healthy controls might be utilised to diagnose the disease.

There is presently no study in the Pakistani cohort that measures normal levels of serum MMPs. Hence this study will also

serve to provide an estimate of levels of serum MMPs which can form basis for further studies.

## MATERIAL AND METHODS

Study subjects included OSCC patients (38 of which 19 men and 19 women; with a mean age 51±20 years) & age matched healthy controls (38 of which 19 men and 19 women; mean age 51±21 years). The criteria for inclusion were biopsy positive patients of age range of 20 – 60 years. Exclusion criteria was patients on chemotherapy or radiotherapy, patients with Rheumatoid arthritis[18], Diabetic foot[19] and Myocardial Infarction (acute)[20]. These OSCC patients were selected from Mayo Hospital, Lahore - Oral and Maxillofacial Surgery Department, and they participated willingly with prior consent. In the specified proforma, the individuals' medical histories and biochemical results were documented. Each individual had a 5 mL venous blood sample collected, deposited in a tube, allowing it to clot for 20–30 minutes, and finally centrifuged. For the measurement of serum MMP, the clear serum was stored in labelled eppendroff tubes at -80°C. The analysis performed on the serum samples was MMP measurement using Bio-Plex Pro™ Human MMP Panel, 9-Plex #171-AM001M kit to detect the serum levels of MMP 1, 2, 3 using multianalyte Bio-Plex® MAGPIX™ Multiplex Reader.

Statistical analysis was conducted on Statistical Package for Social Sciences (SPSS) version 21.0. Data was stated as median and interquartile range. Further, to have an idea regarding outliers, box-and-whisker plots were used. Mann Whitney U test was utilised to analyse the MMP levels between case and control subjects and to find out whether the difference between them is statistically significant. Significance was taken at P< 0.05. Finally, receiver operating characteristic (ROC) curve was used to quantify the diagnostic value of the statistically significant markers.

## RESULTS

For MMP 1, as shown below, visually, the levels in controls appear to be low (Figure 1), and certain outliers were also seen both in the case and control groups. Also note that the box-and-whisker plots clearly represent the skewness of the data- that is the data does not conform to a normal distribution.

Median MMP1 levels in case and control groups were 565.85 and 350.54 pg/ml respectively; the distributions in the two groups differed significantly (sample size cases = sample size controls = 38, P < 0.0024 two-tailed)

By applying Mann-Whitney U-tests, significant differences were observed in serum MMP levels between cases and controls in MMP 1, 2. In all these MMPs, with the exception of MMP 2, cases were recorded to have higher values, as evidenced by mean and median levels, given in table 1 - 3. For MMP 2, control group had higher serum levels than cases.

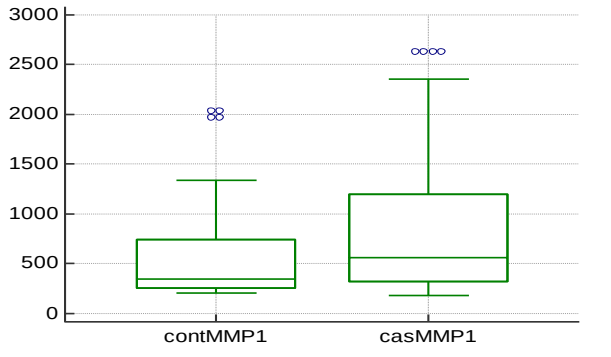
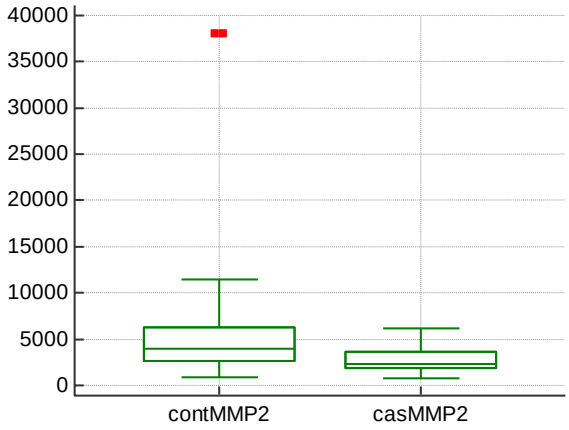



Figure 1: Boxplot of cases of OSCC (casMMP 1) and healthy controls (contMMP1) on levels of serum MMP1 (Boxes stand for 25th /75th percentiles; vertical bars show 10th /90th percentiles. Solid line passing horizontally in the Centre of the box shows median. Circular points represent possible outliers and filled boxes show probable outliers.




Circle is an outlier, being lesser than the [lower quartile - 1.5 times the interquartile range], or higher than the [upper quartile + 1.5 times the interquartile range]. Filled box is a far out quantity that is [less than the lower quartile - 3 times the interquartile range], or more than the [upper quartile + 3 times the interquartile range]

Figure 2: Boxplot of cases of OSCC (casMMP 2) and healthy controls (contMMP2) on levels of serum MMP2

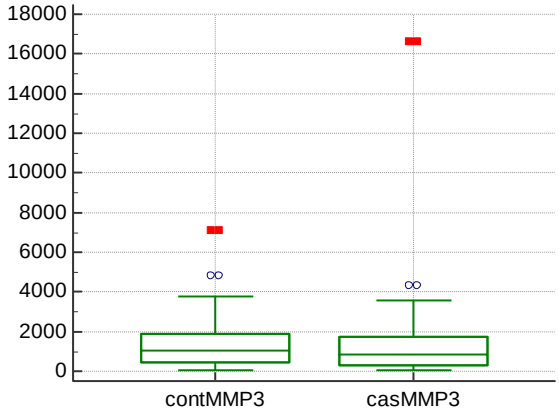



Figure 3: Boxplot of cases of OSCC (casMMP 3) and healthy controls (contMMP3) on levels of serum MMP3

Table 1 : Median level of MMP 1 in cases and controls

| Levels | N | Controls Median (pg/ml) | Cases Median (pg/ml) | P-value |
|---|---|---|---|---|
| MMP 1 | 38 | 350.5 | 565.8 | 0.0024 |
| MMP 2 | 38 | 4006.4 | 2345.9 | 0.0022 |
| MMP 3 | 38 | 1038.7 | 858.3 | 0.550 |

We used ROC curve analysis to further assess the diagnostic efficacy of the markers for binary categorization. To compute AUC, ROC curves were generated to find the optimal cut-off value that yielded an optimal sensitivity level. The AUC indicates the likelihood that the MMP value for a randomly selected positive patient will be greater than the result for a randomly selected negative case; an AUC of 0.5 indicates that probability is not better than random. The ROC curves were only plotted for the MMPs which showed statistical significance on Mann- Whitney U test i.e. MMP 1, 2

A cut-off value is used to establish a positive assay-based test outcome, i.e., positive, with the marker value exceeding a cut-off threshold, if higher levels of a biomarker are linked with an adverse outcome by convention. A ROC curve is a depiction of true positive value vs false positive value for a continuous scale marker, assessed for all potential cut-off point values. The ROC curve measures a marker's discriminating capacity to distinguish cases from controls in a binary outcome, such as cancer diagnosis.

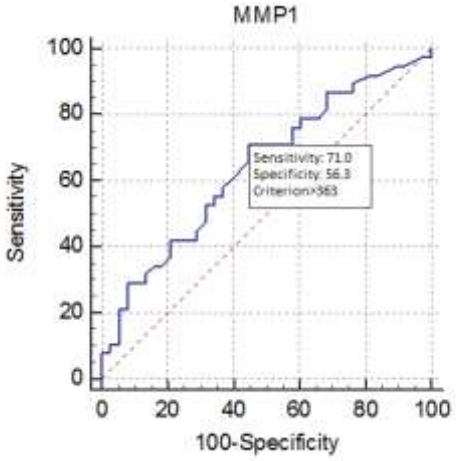



Figure 4: MMP 1 values in the serum of 38 cancer patients and 38 healthy controls are compared using a ROC curve. For various cut-off points, this ROC curve plots the sensitivity (true positive rate) as a function of the 100 - Specificity (false positive rate). A sensitivity/specificity pair pertaining to a specific decision threshold is represented by each point on the ROC curve. The AUC, which shows the chance that a cancer patient would have an MMP 1 level elevated than healthy person, is also calculated using the ROC curve. The AUC of the biomarker in this figure is 0.642, i.e. statistically significant (p0.05).

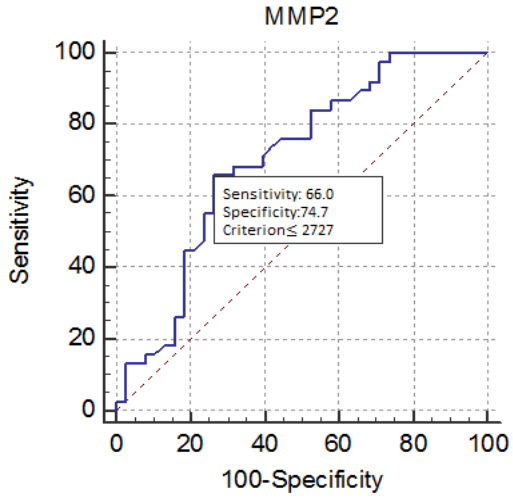



Figure 5 : ROC curve for MMP 2 in serum of 38 cancer patients to 38 healthy controls.

The AUC, which runs from 0.5 (for a non-informative marker) to 1 (for an ideal marker) and refers to the likelihood that a case randomly selected has a higher marker value than a control, similarly chosen, was determined for each ROC curve. The confidence intervals for AUC were calculated by binomial exact test.

Table 4: ROC analysis for biomarkers

| | ROC parameters | | | | |
|---|---|---|---|---|---|
| Marker | AUC | 95% CI | Sensitivity (%) | Specificity (%) | Cut- off value (pg/ml) |
| MMP 1 | 0.652 | 0.514 to 0.739 | 71.0 | 56.3 | > 363 |
| MMP 2 | 0.700 | 0.578 to 0.813 | 66.0 | 74.7 | ≤ 2727 |

ROC curve analysis (Figure 4-5) was used to determine the diagnostic measure of the two statistically significant markers (MMP1, 2).The AUC of both markers was substantially higher than 0.5.

## DISCUSSION

As it is with most cancers, it is evident that OSCC has multi-factorial etiology. Keeping this in view, a compilation of various markers will lend sensitivity and more specificity to effectually detect and diagnose cancer at an early stage. Hence, we used a Multiplex ELISA technique to quantify MMP levels in serum acquired from OSCC cases (n=38) and healthy controls (n=38). Via our analysis of MMP 1,2,3 panel via multiplexing (Table 1 - 3) , 2 MMPs were identified with confidence, such that each of them showed significant change in levels between cancer cases and healthy subjects. However by AUC analysis of these 2 MMPs, MMP 1 was discovered to be the sole marker with sufficient sensitivity and specificity to detect OSCC, as will be discussed below. The other MMPs included in this panel, (MMP 3) showed case- control variability that was statistically insignificant.

It was found that the mean level of MMP 1in patients (n=38) was significantly higher than controls which confirms our knowledge that the raise in MMPs not only highlights cancer growth but aids cancer to spread as well. However for MMP 2, the pattern was reversed, being higher in controls as compared to cases.The mean MMP 3 level did not differ significantly between controls (n=38) and patients (n=38). The reasons for this discrepancy have not been looked at in this study, and additional research is needed. Some of these findings were negated whereas others were corroborated by earlier studies. For example, study by When comparing OSCC linked fibroblasts to normal fibroblasts, Zhang et al. found that MMP 1 levels were greater in OSCC associated fibroblasts[21]. Similarly, Ha et al. connected OSCC invasiveness to elevated MMP 1 levels.[22].

However, our findings on MMP 2 appear to diverge from those of earlier studies. A study on MMP 2 by Shrestha et al. concluded that in OSCC patients, increased MMP 2 expression was connected to a worse prognosis.[23].Another research by Kamata et al. found that increased MMP 2 expression significantly promotes cell invasion.[24]. These results are not strictly contradictory to the present study. Even if MMP 2 may not have diagnostic potential, it can be further researched regarding its prognostic potential, or as a drug target.

Stott-Miller et al. detected considerably higher, but not statistically significant, levels of MMP 1 and 3 in saliva of OSCC patients compared to healthy controls, which is consistent with our findings.[25]. Yan et al. argue that MMP 1 yielded significant results for oral cancer detection via ROC curve analysis[26].

There was a substantial difference in expression between cancer patients and healthy controls for two proteinases (MMP 1, 2). Further analysis of these MMPs, and the panel in general, in patients with a range of disease types and severities and therapies should be performed as it was beyond the scope of this study.

## CONCLUSIONS

MMP 1 expression was found significantly elevated in cases with 71.0% sensitivity and 56.3% specificity so it can be further evaluated not only as a diagnostic and prognostic tumour marker but a drug target as well.

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