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Relation of salivary MMP-8 with oral submucous fibrosis and oral squamous cell carcinoma: a cross sectional analytical study

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ABSTRACT

Objectives We aim to evaluate salivary matrix metalloproteinases (MMP-8) levels in oral submucous fibrosis (OSF) and oral squamous cell carcinoma (OSCC) for the purpose of diagnosis at the early stage via non-invasive method.

Setting The study was multicentre, carried out at a tertiary care hospital in Karachi, Pakistan.

Participants A total 60 participants of any age, sex and ethnicity were randomly selected for the purpose of this study. Patients demonstrating clinical evidence of OSF and biopsy-proven cases of OSCC were included. Patients with indeterminate histopathological report, immunodeficiency, autoimmune disorder, chronic medical and periodontal disease (periodontal depth greater than 5 mm) and individuals with interincisal mouth opening greater than 35 mm were excluded from the study.

Interventions Salivary MMP-8 levels were observed in OSF, healthy and OSCC groups by using ELISA. One way analysis of variance was applied to establish whether MMP-8 levels of disease-free individuals and patients suffering from OSF and OSCC differed from each other.

Results Statistically significant difference in salivary MMP-8 expression in diseased and control group was observed. MMP-8 levels in OSCC (0.64 ng/mL) and OSF (0.66 ng/mL) were underexpressed as compared with healthy participants (7.9 ng/mL).

Conclusion MMP-8 levels were underexpressed in OSCC and OSF patients as compared with controls, which imply that MMP-8 level has an inverse relation with OSCC and OSF.

STRENGTHS AND LIMITATIONS OF THIS STUDY

⇒ Saliva samples are used to test the levels of matrix metalloproteinases (MMP-8) as an alternative to tissue samples taken through biopsy, which is time-consuming and expensive method.
⇒ Samples collected from major centres of OMFS in Karachi, which represents general population.
⇒ We could work on just one premalignant condition due to limited budget and time.
⇒ Could not compare salivary MMP-8 levels with tissue samples.
⇒ Could not compare the multiple pathological stages of oral submucous fibrosis and oral squamous cell carcinoma due to small sample size.

INTRODUCTION

The incidence of malignancy cases is expected to rise in the future due to direct relationship of developing cancers with increasing age of the population.[1] Among the malignancies of the head and neck region, oral squamous cell carcinoma (OSCC) accounts for 90% of all oral malignancies.[2] The highest prevalence of oral cancers is witnessed in South Asian region including countries like Bangladesh, India, Pakistan and Sri Lanka.[3] OSCC is considered risky due to its potential for metastasis and the probability of it being discovered at an advanced stage as it progresses to the cervical lymph nodes without pain.[4] OSCC emerges from oral mucosal disorders, which poses enhanced risk of developing oral malignancies. Among these oral potentially malignant disorders, oral submucous fibrous (OSF) has a higher tendency (2.3%–7.6%) of transforming to OSCC as compared with other premalignant disorders.[2] Therefore, detection of premalignant lesions or OSCC at an initial stage may prove to be significant in a lot of aspects, including early treatment, which may help cease the progress of disease to advanced stage. Therefore, early detection may subsequently contribute to better prognosis and increase in survival rate.

Identification of biomarkers, including proteins, DNA, peptides, RNA which aid in tumourigenesis or serve as tumour suppressors facilitate in accessing the risk of developing cancers, its progression and treatment responses.[1] In the past, biomarkers have been studied using blood, urine and tissue samples via immunohistochemistry and immunoassays.[5] Saliva has been recognised as a promising diagnostic medium for early

and accurate diagnosis and prognosis of a disease via biomarkers.[6] Due to the non-invasive method involved in obtaining saliva, it is considered the desirable biological fluid.[7] There are various advantages of using saliva for cancer detection including easily availability and minimal training required, positive correlation between plasma metabolites and saliva, easy collection, transportation and disposal nature.[8]

MMPs (matrix metalloproteinases) are endopeptidases which are dependent on zinc and are implicated in physiological degradation process of extra cellular matrix including, embryonic development, angiogenesis and wound healing.[9] MMPs have intermittently been considered as potential precancerous and cancerous biomarkers and have been related with tumour spread and metastases.[10] The balance that exists between MMPs and the tissue inhibitor of metalloproteinase (TIMPs) may be held accountable for the initiation and progression of various malignancies.[11] On the other hand, under expression of MMPs have been contemplated as a conceivable clinical intervention in cancer therapy.[1]

The association of MMP-2 and MMP-9 with malignancy has been depicted considerably in the past studies, owing to the part they play in degradation of basement membrane.[12] Significantly higher levels of several other MMPs involving MMP-1, MMP-3,MMP-3 to MMP-10, MMP-11 and MMP-13 were also observed in tumour samples in comparison with healthy mucosa.[13] Enhanced values of MMP-12 were also observed in OSF and OSCC, demonstrating that it may play a significant role in early detection of the disease.[2]

MMP-8 has been originally recognised as neutrophil collagenase. In addition to its expression in neutrophils, MMP-8 is also expressed in endothelial cells, epithelial cells, macrophages and fibroblasts. Recent literature claims that it plays a substantial part in tumour and metastasis suppressive behaviours.[11] One such study reported expression of MMP-8 along with MMP-9 in pericancerous region (inflammatory cells) instead of cells affected by OSCC.[14] Positive association of MMP-8 with survival of patients has also been seen.[15] Furthermore, high MMP-8 expression was observed to play a protective role in tongue carcinoma and in a carcinogen-induced mice model.[15]

In this study, we aim to evaluate salivary MMP-8 levels in OSF and OSCC for the purpose of diagnosis at the early stage via non-invasive method.

MATERIALS AND METHODS

This research study is a cross-sectional analytical study which was carried out at three oral and maxillofacial surgery centres of Dow University of Health Sciences (DUHS) over a period of 3 months. Power of the study was computed to justify the sample size of 20 participants in each group by using NCSS PASS V.15 software, employing the mean of MMP-8 levels in diseased and control groups, as reported in table 1,[16] 95% CI, 1.8 mean square error and power of the test found more than 99%.

Table 1 MMP-8 comparison among groups, gender and oral habits

Categories	MMP-8 level (ng/mL)	P value
Group*		
Healthy (n=20)	7.9[a,b] (2.9)	<0.001†
OSF (n=20)	0.66[a] (0.8)	
OSCC (n=20)	0.64[b] (0.4)	
Gender‡		
Male	0.61 (1.9)	0.277§
Female	2.7 (6.6)	
Oral habits‡		
None	7.0 (3.8)	<0.001**
Betel nut/ tobacco smoking/ smokeless tobacco	0.5¶ (0.1)	
Betel nut	0.8¶ (0.9)	
Betel nut and betel leaf	0.5¶ (0.4)	
Combination of all	0.84¶ (0.9)	

[a,b] Significant on comparison at 5% using post hoc (Tukey's test).
*Values are represented as mean (SD).
†One-way ANOVA.
‡Values are represented as median (IQR).
§Mann-Whitney test.
¶Significant on pairwise comparison with none.
**Kruskal-Wallis test.
ANOVA, analysis of variance; MMP, matrix metalloproteinases; OSCC, oral squamous cell carcinoma; OSF, oral submucous fibrosis.

Study participants

A total of 60 patients were recruited randomly on the basis of inclusion and exclusion criteria. Subjects of any age, sex and ethnicity were included in the study. Cases with clinical evidence of OSF as per classification of OSF: Passi *et al*[17] and biopsy-proven cases of OSCC who were not previously treated were included in the study. Those with indeterminate histopathological report, immunodeficiency, autoimmune disorder, chronic medical and periodontal disease (periodontal depth greater than 5 mm) and individuals with interincisal mouth opening greater than 35 mm were excluded from the study.

After selecting the patient, questionnaire was filled out to maintain record. The questionnaire that was used for this study was our department's questionnaire (OMFS department, DUHS) comprising 53 questions overall, which was specifically designed for OSF and OSCC patients and has been in use for the past few years. The first section of the questionnaire comprised of sociodemographic variables and medical history of the participant and the second section contained questions related to the disease. Every participant was thoroughly explained the purpose of the study and signature was requested on the consent form, which was drafted in accordance with Declaration of Helsinki. Results were disseminated to the patient personally via phone call and all queries were

answered appropriately. Patients were not involved in the recruitment to and conduct of the study.

For saliva collection, patients were instructed to come early in the morning and to avoid eating and smoking 1 hour prior to their arrival. Participants were requested to droll the unstimulated saliva in a falcon tube after rinsing the oral cavity with tap water and then resting for 10–15 min. Unstimulated saliva in the quantity of 2–5 mL was gathered and then transported to the lab in an icebox for further processing. Before centrifugation, the sample was weighed and balanced. Centrifugation machine was precooled at 4°C before processing the samples at 8000 rpm for 15 min to evacuate insoluble materials. Pellet was discarded and Supernatant obtained was transferred to 2.0 mL Eppendorf tubes. The samples were then stored at - 80°C until they were used for ELISA investigation. The technique was performed according to the instructions mentioned in the manual of the kit and the absorbance was noted immediately at 450 nm wavelength. We analysed each sample in duplicate for the purpose of statistical analysis. Concentration of MMP-8 was recorded by generating a Standard curve against optical densities.

For statistical analysis, data were entered and analysed on SPSS (V.23.0). Descriptive statistics were reported as per appropriate distribution. Kruskal-Wallis was run to determine the difference in MMP-8 expression among patients with different oral habits while Mann-Whitney test was run to detect MMP-8 levels difference between male and female. One way analysis of variance (ANOVA) was applied to determine if MMP-8 levels of disease-free individuals and patients suffering from OSF and OSCC differ from each other. This was preceded by post hoc Tukey's test to make multiple comparisons between groups. A $p \leq 0.05$ was considered statistically significant. Levels of MMP-8 were recorded in ng/mL.

Patient and public involvement

Patients were involved in the study after explaining to them the purpose and design of the study. The participation was entirely voluntary, and patients were encouraged to reach out to us in case of further elaboration. The results of the study will be disseminated to the participants via email and phone call.

RESULTS

The study comprised 60 participants. Each group consisted of 20 individuals, comprising 60% males and 40% females. The mean age value was 37.7±9.7 years (table 2). The peak incidence of cases reporting with OSF and OSCC was found to be in age group of 38 and 45 years.

The participants of study were inquired about their oral habits. The most common oral habit identified among diseased individuals was intake of betel nut (50.5%) for an average duration of 15.19±6.99 years and second intake of eating betel leaf (25.4%) for about 14.77±7.93 years.

Table 2 Sociodemographic data

Category	Healthy N=20 (%)	OSCC N=20 (%)	OSF N=20 (%)
Age (mean±SD)	33.1±4.8	46.8±8.2	32.5±9.7
Sex			
Male	9 (45.0)	13 (65.0)	14 (70.0)
Female	11 (55.0)	7 (35.0)	6 (30.0)
Oral habits			
None	20 (100.0)	3 (15.0)	0 (0)
Betel quid/tobacco smoking/smokeless tobacco	0 (0)	5 (25.0)	3 (15.0)
Betel nut	0 (0)	6 (30.0)	7 (35.0)
Betel nut and betel leaf	0 (0)	4 (20.0)	7 (35.0)
Combination of all	0 (0)	2 (10.0)	3 (15.0)

OSCC, oral squamous cell carcinoma; OSF, oral submucous fibrosis.

The table below (table 3) demonstrates tumour-related variables. The data include histological grading as per Broder's classification which classifies OSCC based on tumour cell's degree of keratinisation and differentiation.[18] The table also displays nature of the lesion, site of primary tumour and cervical lymph nodes involvement.

Patients presenting with OSF were staged in accordance with clinical classification.[19] Seventy-five per cent of participants presented with stage IV, while 20% with stage III and 5% with stage II. However, none presented with stage I.

Data related to OSF and OSCC signs and symptoms demonstrated that 50% of the patients presented with limited mouth opening whereas 30% of the diseased group participants presented with lesion on buccal mucosa and 20% presented with other signs and symptoms including burning sensation, xerostomia, etc. Mouth opening of each participant was also recorded. Healthy group demonstrated mean mouth opening of 39.95±3.41 mm while OSF group demonstrated 14.7±6.85 mm and OSCC patients demonstrated a mean of 17.78±8.68 mm. Greater mouth opening measurements were found in participants belonging to healthy group as compared with participants belonging to OSF and OSCC group.

Table 1 depicts the descriptive statistics of MMP-8 levels in the saliva samples of disease-free participants as well as participants belonging to OSF group and OSCC group. The maximum MMP-8 expression in study participants was found to be 12.63 ng/mL and minimum was found to be 0.18 ng/mL. Higher values were observed in healthy group (mean=7.9 ng/mL) as compared with diseased groups. The table also demonstrates the MMP-8 expression among gender and patients with various oral habits. The results reveal higher MMP-8 levels among females as compared with males. Among patients with various

Table 3 Tumour-related variables

Variables	Category	N=60 (%)
Histological grading	Well differentiated	2 (10)
	Moderately differentiated	16 (80)
	Poorly differentiated	2 (10)
	Anaplastic	0
Nature of the lesion	Verrucous	3 (14.3)
	Exophytic	8 (38.1)
	Endophytic	1 (4.8)
	Ulcerated	9 (42.9)
Tumour site	Buccal mucosa	13 (61.9)
	Retromolar region	2 (9.5)
	Maxilla	2 (9.5)
	Tongue	3 (14.3)
	Palate	1 (4.8)
Cervical lymph node status	Ipsilateral (level 1)	9 (28.1)
	Ipsilateral (level II)	14 (43.8)
	Bilateral (level I)	4 (12.5)
	Bilateral (level II)	5 (15.6)

oral habits, patients consuming a combination of betel nut, betel leaf, smokeless tobacco and tobacco smoking demonstrate higher MMP-8 levels compared with patients consuming either of these products.

The difference observed in salivary MMP-8 levels in healthy, OSF and OSCC participants was significant ($F_{(2,31.039)}$=57.1, p<0.001). Due to violation of assumptions, Welch ANOVA was run simultaneously with basic ANOVA to authenticate the findings. A significant difference was witnessed between mean differences in salivary MMP 8 levels of individual between groups (p<0.001).

post hoc (Tukey's test) was run to for multiple comparisons between groups. Healthy participants demonstrated significant difference in MMP-8 levels in comparison with individuals belonging to OSF (p<0.001) and OSCC groups (p<0.001) respectively. However, difference in MMP-8 levels observed between the two diseased groups was non-significant (OSF and OSCC).

DISCUSSION

MMP-8, also known as neutrophil collagenase or collagenase-2, performs a significant part in mediating inflammatory process.[1] It breaks down triple helical collagen (type 1) and various ECM and non- ECM substrate.[20] In gingivitis and periodontitis, expression of MMP-8 is enhanced. It has also been reported that MMP-8 suppresses neuroinflammation[21] and osteoarthritis.[22] Raised levels of MMP-8 appears in OSCC but it does not associate with clinical and pathological features, nor it upsurges the survival of OSCC patients.[14 23] However, higher levels of MMP-8 in tongue tumours favoured lower mortality rate and better prognosis.[15] A meta-analysis indicated moderate diagnostic accuracy of blood and salivary miRNA for OSCC and disclosed less invasive and reliable diagnostic characteristics.[24]

It was observed that adhesion between cells enhanced but adhesion between cell and matrix remained unaffected by MMP-8 in tongue cancer cells.[25] Shen *et al* compared presence of type of different proteins in saliva of healthy and OSCC patients. He revealed 52 proteins which were present only in OSCC patients and 29 proteins which were expressed only in healthy individuals. The first MMP that was reported to have tumour- suppressive characteristics was MMP-8.[26] The oncosuppressive properties possessed by MMP-8 have been delineated in various malignancies which may include melanoma,[27] skin,[26] breast,[28] lung[27] and carcinomas of tongue.[15]

The current study estimated salivary MMP-8 levels in three groups of patients. Due to small sample size, we were not able to take all the pathological types of OSF and OSCC. We aim to do it in the near future on larger sample size. We observed lower quantities of MMP-8 in diseased groups as compared with healthy group. In addition, OSCC group exhibited the lowest level of MMP-8 as compared with premalignant group. Lesser expression of MMP-8 in diseased groups and least in OSCC patients may support the explanation that increased levels of MMP-8 demonstrates lower mortality and better prognosis.[15] The protective role of MMP-8 in cancer is justified in a study conducted on mice which reported that absence of collagenase-2 (MMP-8) increased the incidence of skin tumours in male $Mmp8^{-/-}$mice. The study elaborates that MMP-8 may target substrates other than collages/matrix components. Owing to its proteolytic activity on

inflammatory mediators, it contributes to the anti-tumour defence system.[26] The results of current study coincide with a Taiwanese study which assessed the function of MMP-8 promotor in susceptibility of oral cancer. The study suggested that polymorphism at promotor region and two non-synonymous polymorphism of MMP-8 may not be significant in mediating risk of developing oral cancer in Taiwanese population.[11] Pradhan-Palikhe *et al* implied that patient's survival is improved via MMP-8 expression by tumour cells as well as carcinogenesis is protected by MMP-8 from OTSCC.[29] No significant association between MMP-8 and lymph nodes metastasis has been reported.[30]

Contrary to the results of this study, Kuropkat *et al* found elevated levels of serum MMP-8 of head and neck cancer patients compared with healthy participants. He further elaborated those levels of MMP-8 correlated with T and N status of tumour as well as overall TNM staging.[31 32]

Additional research on MMP-8 revealed that MMP-8 may not play a significant role in degradation of collagen in head and neck squamous cell carcinoma (HNSCC). No correlation was witnessed between MMP-8 plasma levels and serum levels of ICTP and IIINTP, which are collagen degradation products. MMP-8 appeared to be associated with favourable outcome.[33] In addition, another study concluded that level of MMP-8 in plasma did not show any relation with cervical nodes involvement or patient's survival.[29]

A systemic review and meta-analysis were carried out to observe the relation between MMPs and OSCC metastasis. The study observed positive relationship between MMP-1,MMP-2, MMP-3 to MMP-7, MMP9 and metastasis in cervical lymph nodes. However, MMP-8,MMP-25 and MMP-26 did not appear to have any relationship with lymph node metastasis.[12] Serum levels of MMPs among OSCC patients were estimated in a Pakistani research study. Levels of MMP-1, MMP-8, MMP-10 to MMP-12 and MMP-13 demonstrated significant increase as compared with controls. In contrast, our study demonstrated significantly lower levels of MMP-8 in OSCC patients in comparison with OSF and controls.[34]

A study published recently represented the substrate of MMP-8 (FXYD5) to interpret the protective part that MMP-8 plays in OSCC of tongue. The study implied that cell adhesion is increased by reducing FXYD5 levels, which leads to reduced motility. FXYD5 is labelled as a novel substrate of MMP-8 and considered significant for therapeutic target.[25] Åström *et al* demonstrated that obstruction of TGF-b1 and VEGF-C function and transformed proteinase expression mediates the suppression of MMP-8 in OTSCC.[35]

We also studied the levels of MMP-8 among participants with various oral habits. The results determined higher expression of salivary MMP-8 among participants consuming a combination of betel nut, betel leaf, smokeless tobacco and tobacco smoking. In contrast to this, a study conducted in Taiwan observed no significant interaction among the genotype of MMP-8 and oral habits.[11]

The current study also estimated the salivary MMP-8 concentrations in OSF. The inclusion of OSF group was considered in this study as OSF is a premalignant disorder of the oral cavity which may advance to oral malignancy. In addition, it is considered a community health concern in Pakistan as oral malignancies are one of the commonly reported malignancies.[2] Its detection via levels of salivary MMP-8 may aid to cease its progression to OSCC. In our study, lower concentration of MMP-8 was reported in OSF group in comparison with healthy individuals. Since OSF is a premalignant condition and most of the cases are presented in the advanced clinical stage with the high chances of progression to OSCC, therefore, we have identified no such difference in the levels of OSF and OSCC. In contrast, increased concentration of MMP-13 in OSF as compared with controls has been demonstrated in a study conducted previously. However, the levels in OSF were lower as compared with OSCC.[36] Significant difference on 5A allele in gene promoter region of MMP-3 was observed in OSF group compared with control.[37]

MMP-2 contributes to pathogenesis and progression of OSF; hence it is considered a significant mediator. Increased concentration of MMP-2 was observed in OSF and the levels corelated with disease severity. It was observed that this protease helps in identification of disease advancement and malignancy.[38] In contrast to this, the effect that arecoline (areca nut) has on MMP-2 was studied and it was observed that arecoline has an inhibitory role on MMP-2 and opposite role on TIMP-1. The effect was observed in buccal mucosal fibroblast.[39]

Interpretation

Significant difference in salivary MMP-8 levels was observed in all three study groups. MMP-8 levels were under expressed in OSF and OSCC patients as compared with controls which implies that MMP-8 level has an inverse relation with OSCC and OSF.

Limitation

Due to limited sample size, budget and time, we could not work on gene polymorphism and were not able to compare the different pathological types of OSF and OSCC with different clinical stages. We aim to focus on gene polymorphism in MMP-8 on larger sample size in the future.

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Contributors All authors were involved in the study design and conceptualisation. AK is the principal investigator and the guarantor. She drafted the manuscript and performed ELISA. ZA supervised the study. He also collected and validated the data. ZS revised the manuscript and was involved in sample collection and processing. SH collected the data and did literature review. WAF did statistical analysis, results writeup and interpretation. SA did the final review, editing of the manuscript and supervision of the project.

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Competing interests None declared.

Patient and public involvement Patients and/or the public were involved in the design, or conduct, or reporting, or dissemination plans of this research. Refer to the Methods section for further details.

Patient consent for publication Consent obtained directly from patient(s).

Ethics approval This study involves human participants and was approved by Ethical Review board and Committee of Dow University of Health Sciences issued the ethical approval (Ref: IRB- 1016/DUHS/Approval/2018/66) after synopsis approval to commence sample collection. Participants gave informed consent to participate in the study before taking part.

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