

Evaluation of salivary MMP-9 in Oral Squamous Cell Carcinoma using Enzyme Linked Immunosorbent Assay - A Pilot Study

Krishnasree R.J¹, Jayanthi P², Varun BR³, Prathiba Ramani⁴, Rathy R¹

ABSTRACT

Introduction: Oral squamous cell carcinoma (OSCC) is the predominant histological subtype of Oral cancer, which is the sixth most common malignancy worldwide. Despite advances in therapy, the overall survival of oral cancer ranges between 45-55%. Successful early detection would eventually increase the survival rate. Tumour markers in saliva has emerged as a new diagnostic tool in the early detection of oral cancer. Matrix metalloproteinase-9 (MMP-9) is a gelatinase which plays an important role in tumorigenesis. The present study was done to evaluate the salivary levels of MMP-9 in OSCC patients using Enzyme Linked Immunosorbent Assay (ELISA).

Materials and Methods: The study was conducted among 30 subjects which included 15 OSCC patients (Group 1) and 15 healthy subjects (Group 2). Unstimulated saliva was collected by passive drooling method from all the study subjects, centrifuged and salivary MMP-9 level was estimated using sandwich ELISA technique. The mean salivary MMP-9 levels were compared between the study groups using Student-t test and $p < 0.05$ was considered to be statistically significant.

Results and Conclusion: A statistically significant difference was noted in the mean salivary MMP-9 level between OSCC and healthy controls. Higher levels of salivary MMP-9 were observed in poorly differentiated OSCC when compared to well and moderately differentiated OSCCs. Higher levels of salivary MMP-9 in poorly differentiated OSCC suggests the potential role of MMP-9 as a prognostic marker in OSCC.

Keywords: Oral squamous cell carcinoma, matrix metalloproteinase, saliva

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INTRODUCTION

Cancer of the lip and the oral cavity is collectively the sixth most common malignancy worldwide, out of which 90% are oral squamous cell carcinomas (OSCC).¹ Oral cancer survival rates depends mainly upon the stage in which it is diagnosed. Successful early detection would eventually increase the survival rate.² Tumour markers in saliva has emerged as a new diagnostic tool in the early detection of oral cancer. Saliva of cancer patients contains higher number of proteins, enzymes and other chemicals which can be collected and analyzed.³ Salivary collection and sampling are a simple procedure and saliva is an easily accessible bio fluid when compared to tissue biopsies and blood sampling.⁴

Human Matrix Metalloproteinase (MMPs) are a group of 23 structurally related end peptidase enzymes which cleaves the internal peptide bond of proteins. Changes in MMPs are generally related to the ultimate clinical outcome in human diseases.⁵ They have the capacity to degrade extracellular matrix, basement membrane matrix and their components. MMP-9 is a gelatinase which plays an important role in tumorigenesis.³ This pilot study was done to evaluate the salivary levels of MMP-9 in OSCC.

MATERIALS AND METHODS

This comparative observational study was carried out during the period of January 2020 to June 2021 in the patients

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visiting the Department of Oral Pathology and Microbiology, Azeezia College of Dental Sciences and Research, Kollam. The study was started after obtaining clearance from the Institutional Ethics Committee. Before commencing the

study, a written informed consent in accordance with ethical codes adopted by national committee for Medical Research Ethics was duly filled by all the participants. The study samples were selected by convenience sampling according to specific inclusion and exclusion criteria. A total number of 30 participants (15 each in one group) were included in the study.

Patients diagnosed clinically and histopathologically confirmed as OSCC (n=15) were included in Group 1. Control group (Group 2) included generally healthy individuals without any systemic illness and tobacco habit. Salivary samples were obtained in the morning and subjects were asked not to eat, brush their teeth, or use mouth rinse at least 2 hours prior to salivary sample collection on that day. The participants were asked to slightly open the mouth and allow saliva to drain into the container. 1.5 ml of unstimulated whole saliva was collected into a sterile centrifuge tube. After collection, the saliva was immediately centrifuged and supernatant was collected and frozen at -80 °C until assayed.

The salivary MMP-9 level was measured using quantitative sandwich ELISA technique and MMP-9 ELISA KIT according to the manufacture instructions. The micro titre plate provided has

been pre-coated with an antibody specific to MMP-9. Standards and samples were added to appropriate micro titre plate wells followed by biotin conjugated antibody specific to MMP-9. The biotin conjugated antibody and enzyme conjugated avidin exhibit a change in colour. The enzyme substrate reaction was terminated by the addition of sulphuric acid solution and the colour change was measured spectrophotometrically at a wavelength of 450nm. The concentration of MMP-9 in the samples in ng/ml was determined by comparing the optical density values (OD) of the sample to the standard curve.

The data was entered into excel sheet and statistical analysis was done using SPSS software version 28.0.1.1. Mean MMP-9 was compared between the study groups using Student-t test and p<0.05 was considered the threshold for statistical significance.

RESULTS

The mean age of patients who were included in group 1 was 64.0±4 years and those who were included in group 2 was 60 ±3.5years (Table 1).

Out of the patients who were included in Group I, 91% of participants had a history of tobacco usage, while 9% of the participants had no history of any deleterious habits (Graph 1).

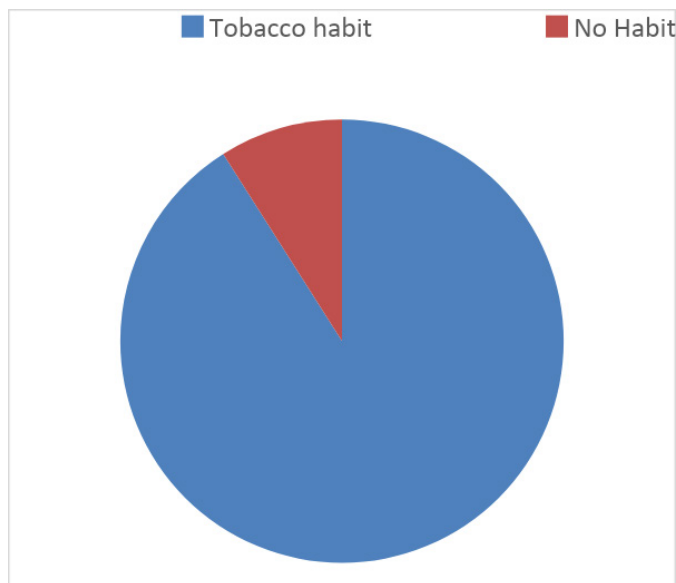
In Group 1 (OSCC), 52% of the lesions occurred on buccal mucosa, 23% on the tongue, 8% on the vestibule, 6% on the gingiva, 3% on the alveolar ridge and 3% each cases on the lips and floor of the mouth (Graph 2).

The subjects who were included in Group 1 (OSCC) were histologically graded as poorly differentiated (A), moderately differentiated (B) and well differentiated (C) tumours, out of which 58.8% of cases were well differentiated squamous cell carcinoma, 26.5 % were moderately differentiated and 14.7 % were poorly differentiated squamous cell carcinoma.

The salivary MMP-9 levels were compared among Group 1 (OSCC) and Group 2 (healthy controls). The estimated mean

Table 1: Age distribution of participants in the study group

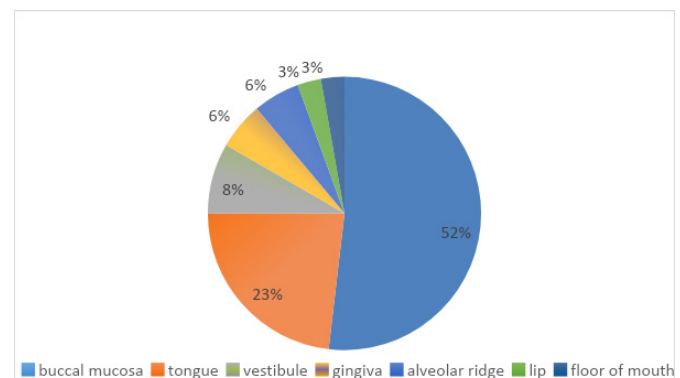
Age (in yrs.)	OSCC (Group I)		Normal (Group II)	
	Count	Percent	Count	Percent
<=30	0	0.0	0	0.0
31 - 40	1	6.6	0	0.0
41 - 50	0	0.0	0	0.0
51 - 60	5	33.3	6	40.0
61 - 70	4	26.6	8	53.3
>70	5	33.3	1	6.6
Mean ± SD	64.0±4 years		60±3.5years	



Graph 1: Habit Distribution among Participants of Group1

Table 2: Mean salivary MMP-9 levels in the study group

Groups	Mean (ng/ml)	SD	N	p
OSCC	48.8	5.7	15	p<0.01
Normal	17.1	4.8	15	



Graph 2: Site Distribution of lesion in oral squamous cell carcinoma

salivary MMP-9 level in Group 1 was 48.8 ± 5.7 ng/ml and in Group 2 was 17.1 ± 4.8 ng/dl. (Table 2, Graph 3). When the levels of salivary MMP-9 were compared between the groups using Student t test, a statistically significant difference was obtained ($p < 0.01$).

The mean salivary MMP-9 in poorly differentiated OSCC was higher when compared to moderately differentiated and well differentiated OSCC, but the difference was not statistically significant ($p = 0.06$) (Table 3).

DISCUSSION

Matrix Metalloproteinases (MMPs) are a major group of enzymes that regulate cell–matrix composition. The MMPs are zinc-dependent endopeptidases known for their ability to cleave one or several extra cellular matrix constituents, as well as non-matrix proteins. They comprise a large family of proteases that share common structural and functional elements and are products of different genes. They are grouped as, the collagenases (MMP-1, 8, 13), the gelatinases (MMP-2, 9), the stromelysins (MMP-3,10,11), the membrane-type MMPs (MMP-14,15,16,17,24,25) and others (MMP-7,26,20,19,21,23,27,28) based partly on historical assessment of the substrate specificity and cellular localization of the MMP. MMP-9 is also known as 92 kDa type IV collagenase, 92 kDa gelatinase or gelatinase B (GELB). It functions as a tumour promoter in the process of carcinogenesis⁵.

MMP-9 participates in the angiogenic switch because it increases the bioavailability of important factors in this process, such as the vascular endothelial growth factor (VEGF), which is the most potent mediator of tumour vasculature, and basic

fibroblast growth factor (bFGF), by degradation of extracellular components, such as collagen type IV, XVIII and perlecan, respectively. Over expression of several MMPs (MMP-2, -3, -9, -13, and 14) has been associated with epithelial-mesenchymal transition (EMT), a highly conserved and fundamental process of morphological transition⁵. The present study was conducted to evaluate the MMP-9 levels in saliva of patients with OSCC and healthy controls.

Saliva has been viewed as an important diagnostic fluid for a very long time because unlike blood and other body fluids, salivary diagnostics offers an easy, inexpensive, and painless and stress free approach to disease detection⁶. The advantages of saliva sampling over serum and tissue are non-invasive collection of sample, smaller sample aliquots, good cooperation with patients, cost effectiveness, easy storage and transportation, greater sensitivity and correlation with levels in blood. In salivary diagnostics, unstimulated saliva is preferred over stimulated saliva since the latter contains diluted concentration of biomarkers, which may be difficult to detect. In the present study, unstimulated saliva collection by passive drooling technique proposed by Mahvash Navazesh was adopted⁴.

In our study, the most common site affected for patients with OSCC was the buccal mucosa (52%), followed by the tongue (23%), vestibule (8%), gingiva (5%), alveolar ridge (6%) and 3% cases each on the lips and floor of the mouth. This is in accordance with the study of Mehrotra et al, where the authors reported that in India, the gingivo-buccal complex (alveolar ridge, gingival-buccal sulcus, buccal mucosa) forms the most common site for oral cancer followed by tongue and floor of the mouth which is more common in the Western world⁶. Traditionally, the paan is placed in the gingival-buccal sulcus and often retained for a prolonged duration, which is responsible for the high prevalence of gingivo-buccal cancer.

Darzilani et al (2019) reported that the salivary level of MMP-9 in OSCC patients (49.27 ± 44.5 ng/ml) were found to be significantly higher when compared to the control group (44.68 ± 40.95 ng/ml)⁷. Peskier et al (2016) demonstrated that salivary MMP-9 was significantly increased in OSCC patients by +19.2% compared to healthy controls. The ROC curve was created to demonstrate the predictive power of MMP-9 (sensitivity 100%; specificity 26.7%) for OSCC patients. Thus, the authors concluded that MMP-9 could be used as a diagnostic adjunct for early detection of oral cancer⁸.

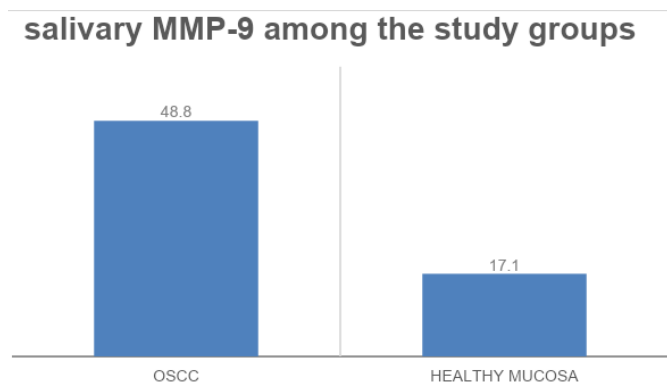
In our study, the salivary MMP-9 levels was evaluated and compared among OSCC and healthy controls. The mean salivary MMP-9 level in OSCC (48.8 ± 5.7 ng/ml) was found to be higher than in healthy subjects (17.1 ± 4.8 ng/ml) and this difference was statistically significant ($p < 0.01$). The mean salivary levels in OSCC was also compared among different grades of OSCC. Although the levels were higher in poorly differentiated OSCC compared to moderately and well differentiated OSCC, the difference was not statistically significant ($p = 0.06$).

CONCLUSION

In the current pilot study there was increased levels of salivary MMP-9 in OSCC when compared to normal healthy controls. As MMP-9 is thought to cause type IV collagen degradation, a main component of basement membranes, the increased level of salivary MMP-9 in poorly differentiated

Table 3: Evaluation of Salivary MMP-9 levels in different grades of OSCC

Grades of OSCC	Mean (ng/ml)	SD	N	p
Poorly differentiated	52.7	6.4	5	p=0.06
Moderately differentiated	48.7	0.9	5	
Well differentiated	46.0	2.2	5	



Graph 3: Comparison of salivary MMP-9 levels between the study groups



carcinoma implies that MMP-9 may possibly be involved in tumour infiltration metastasis. Further long term follow up studies on a larger sample would ascertain the exact role of MMP-9 in OSCC.

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