Original Article

Evaluation of salivary MMP-9 in oral squamous cell carcinoma and oral leukoplakia using ELISA

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Abstract

Background: 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 (OSCCs). Oral cancer survival rates depend mainly upon the stage in which it is diagnosed. Successful early detection would eventually increase the survival rate. OSCCs may be preceded by potentially malignant disorders (PMDs) that are characterised by visible clinical changes in the oral mucosa. Correct diagnosis and timely treatment of PMDs may help prevent malignant transformation in oral lesions. Oral leukoplakia (OL) is the best known potentially malignant disorder of the oral mucosa with a malignant transformation rate of about 3% to 33%. Tumour markers in saliva have 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 tumourogenisis. The present study was done to evaluate the salivary levels of MMP-9 in OSCC and oral leukoplakia patients using enzyme-linked immunosorbent assay (ELISA).

Materials and Methods: The study was conducted among 102 subjects, which included 34 OSCC patients (group I), 34 OL patients (group II), and 34 healthy subjects (group III). Unstimulated saliva was collected by the passive drooling method from all the study subjects during the study period, centrifuged, and stored at -80°C. The salivary MMP-9 was estimated in mg/ml using the sandwich ELISA technique. The data were analysed using a statistical software package, EZR. One-way analysis of variance was used for the comparison of salivary MMP-9 levels in OSCC, OL, and normal oral mucosa. Scheffe's multiple comparison was carried out to compare salivary MMP-9 levels among the different histological grades of OSCC and oral epithelial dysplasia. For all statistical interpretations, $P \leq 0.0$ was considered the threshold for statistical significance.

Results and Conclusion: The mean salivary MMP-9 level in OSCC, OL, and normal oral mucosa was 50.9 ± 5.7 ng/ml, 31.6 ± 6 ng/ml, and 16.2 ± 4.8 ng/ml, respectively. Patients with OSCC had significantly higher levels of salivary MMP-9 when compared to OL and normal mucosa. Higher levels of salivary MMP-9 were observed in poorly differentiated OSCC when compared to well and moderately differentiated OSCCs. The salivary MMP-9 was higher in severe oral epithelial dysplasia when compared to mild and moderate oral

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epithelial dysplasias. As malignant transformation rates are higher in patients with severe oral epithelial dysplasia when compared to mild and moderate oral epithelial dysplasia, salivary MMP-9 could be considered as a surrogate marker of malignant transformation.

Keywords: ELISA, MMP-9, oral leukoplakia, oral squamous cell carcinoma

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 (OSCCs).[1] Oral cancer survival rates depend mainly upon the stage in which it is diagnosed. Successful early detection would eventually increase the survival rate.[2] OSCCs may be preceded by potentially malignant disorders (PMDs) that are characterised by visible clinical changes in the oral mucosa.[3] Correct diagnosis and timely treatment of PMDs may help prevent malignant transformation in oral lesions. Oral leukoplakia (OL) is the best known potentially malignant disorder of the oral mucosa with a malignant transformation rate of about 3% to 33%.[4] Tumour markers in saliva have emerged as a new diagnostic tool in the early detection of oral cancer. Saliva of cancer patients contains a higher amount of proteins, enzymes, and other chemicals which can be collected and analysed.[5] Salivary collection and sampling is a simpler procedure, and saliva is an easily accessible bio-fluid when compared to tissue biopsies and blood sampling.[5]

Human matrix metalloproteinases (MMPs) are a group of 23 structurally related end peptidase enzymes which cleave the internal peptide bond of proteins. Changes in MMPs are generally related to the ultimate clinical outcome in human diseases. They have the capacity to degrade the extra-cellular matrix, basement membrane matrix, and their components. MMP-9 is a gelatinase which plays an important role in tumourigenesis.[6] The present study was done to evaluate the salivary levels of MMP-9 in OSCC and OL patients.

MATERIALS AND METHODS

This comparative observational study was carried out in the patients visiting the Department of Oral pathology and Microbiology, Azeezia College of Dental Sciences and Research, Kerala. The study was started after obtaining clearance from the institutional ethic committee (AEC/REV/2019/31). Before commencing the study, a written informed consent in accordance with ethical codes adopted by the 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 102 participants (34 each in each group) were included in the study. Patients diagnosed clinically and histopathologically confirmed as OSCC (n = 34) were included in group 1. Patients diagnosed clinically with OL and showing various degrees of epithelial dysplasia histopathologically (n = 34) were included in group II. The control group (group III) included generally healthy individuals without any systemic illness (n = 34).

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 the supernatant was collected and frozen at −80°C until assayed. The salivary MMP-9 level was measured using the quantitative sandwich enzyme-linked immunosorbent assay (ELISA) technique MMP-9 ELISA KIT according to the manufacture instructions. The micro titre plate provided was pre-coated with an antibody specific to MMP-9. Standards or 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 450 nm. The concentration of MMP-9 in the samples was estimated in ng/ml by comparing the optical density values (OD) of the sample to the standard curve.

RESULTS

The mean age of the patients who were included in group I was 62.8 ± 12.9 years, and that of patients who were included in group II was 60.1 ± 11.5 years. The participants who were included in the group III (n = 34) showed a mean age range of 52.4 ± 9.7 years. Among the patients who were included in group I, 82.4% were males and 17.6% were females. In group II, 67.6% were males

and 32.4% were females, and in group III, 64.7% were females and 35.3% were males.

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. In group I (OSCC), 49% of the patients had the habit of chewing tobacco, 35% had the habit of smoking tobacco, and 16% of the patients had both chewing and smoking tobacco habits. Out of the patients who were included in group II, 82% of participants had a history of tobacco usage, while 18% of patients had no history of any habits. Of those having tobacco habit in group II, 50% of the patients had the habit of chewing tobacco, 29% of patients had the habit of smoking tobacco, and 21% of the patients had both habits of chewing and smoking tobacco.

In group I (OSCC), 52% of the lesions occurred on buccal mucosa, 23% on the tongue, 8% on the vestibule, 5% on the gingiva, 6% on the alveolar ridge, and 3% each on the lips and floor of the mouth. In group II (OL), 62% of the lesions occurred on the buccal mucosa, 22% on the tongue, 13% on the labial mucosa, and 3% on the gingiva.

All the tissue specimens in group I (OSCC) were histologically graded as poorly differentiated (A), moderately differentiated (B), and well differentiated (C) tumours, out of which 58.8% of the cases were well differentiated squamous cell carcinoma, 26.5% were moderately differentiated, and 14.7% were poorly differentiated squamous cell carcinoma. The subjects who were included in group II (OL) were histologically diagnosed as epithelial dysplasia and graded as mild (A), moderate (B), and severe (C), out of which 26.5% of cases were having mild dysplasia, 41.2% had moderate dysplasia, and 32.4% had severe dysplasia.

Evaluation of salivary MMP-9 levels among the study groups

The salivary MMP-9 levels were compared among group 1 (OSCC), group II (OL), and group III (healthy controls). The estimated mean salivary MMP-9 level in OSCC, OL, and normal oral mucosa was 50.9 ± 5.7 ng/ml, 31.6 ± 6.0 ng/ml, and 16.2 ± 4.8 ng/ml, respectively, and the difference was statistically significant ($P < 0.01$) [Table 1].

The mean salivary level of MMP-9 in poorly differentiated OSCC (A) was found to be 62.7 ± 6.4 ng/ml; in moderately differentiated OSCC (B), MMP-9 was 48.7 ± 0.9 ng/ml; and in well-differentiated OSCC (C), the level was 49.0 ± 2.2 ng/ml. A statistically significant difference was noted when comparing the mean salivary levels of MMP-9 in between poorly differentiated and moderately differentiated OSCCs and also between poorly differentiated and well-differentiated OSCCs. However, mean MMP-9 levels did not show a significant difference between moderately differentiated and well-differentiated OSCCs [Table 2].

The subjects who were included in group II (OL) were histologically diagnosed as epithelial dysplasia and graded as mild (A), moderate (B), and severe (C) dysplasias. The mean salivary level of MMP-9 in mild dysplasia (A) was 26.5 + 4.7 ng/ml; in moderate dysplasia (B), it was 30.4 + 3.3 ng/ml; and in severe dysplasia (C), it was 37.3 + 4.9 ng/ml. Cases showing mild dysplasia exhibited a significantly lower level of MMP-9 levels when compared to moderate and severe dysplasia ($P < 0.01$). However, no significant difference was noted between mild and moderate dysplasias [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 ECM 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.[7] 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 localisation of the MMP. Matrixmetalloproteinases-9 (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 carcinogenesis.[8] 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 the basic fibroblast growth factor (bFGF) by degradation of extra-cellular components, such as collagen type IV and XVIII and perlecan, respectively.[9] 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 transition.[10] The present study was conducted to evaluate the MMP-9 levels in saliva of patients with OSCC and OL.

Table 1: Comparison of salivary MMP-9 levels among the study groups

Groups	Mean (ng/ml)	SD	*F*	*P*	Scheffe multiple comparisons		
					Pair	*F*	*P*
OSCC (Group I)	50.9	5.7	330.28	*P*<0.01	I and II	104	*P*<0.01
OL (Group II)	31.6	6.0			I and III	328.3	*P*<0.01
Normal oral mucosa (Group III)	16.2	4.8			II and III	64	*P*<0.01

Table 2: Comparison of salivary MMP-9 levels in different histological grades of OSCC

Grade	Mean (ng/ml)	SD	*n*	*P*	Scheffe multiple comparisons		
					Pair	*F*	*P*
Poorly differentiated (A)	62.7	6.4	5	*P*<0.01	A and B	37.1	*P*<0.01
Moderately differentiated (B)	48.7	0.9	9		A and C	44.4	*P*<0.01
Well differentiated (C)	49.0	2.2	20		B and C	0	0.973

Table 3: Comparison of salivary MMP-9 levels in different histological grades of oral epithelial dysplasia

Grade of Dysplasia	Mean (ng/ml)	SD	*n*	*F*	*P*	Scheffe multiple comparisons		
						Pair	*F*	*P*
Mild (A)	26.5	4.7	9	16.84	*P*<0.01	A and B	2.2	0.124
Moderate (B)	30.4	3.3	14			A and C	15.9	<0.01
Severe (C)	37.3	4.9	11			B and C	8.2	<0.01

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, painless, and stress-free approach to disease detection.[11] The advantages of saliva sampling over serum and tissues are non-invasive collection of samples, smaller sample aliquots, good cooperation with patients, cost-effectiveness, easy storage and transportation, and greater sensitivity and correlation with levels in blood.[12] In salivary diagnostics, unstimulated saliva is preferred over stimulated saliva since the latter contains diluted concentration of biomarkers, which may be difficult to detect.[13] In the present study, unstimulated saliva collection by the passive drooling technique proposed by Mahvash Navazesh[14] was adopted.

The mean age group of patients with OSCC in our study was 62.8 ± 12.9. Out of 34 patients who were included in group I, 82.4% were males and 17.6% were females. This was in accordance with the study by Tandon *et al*., who reported that the highest peak of prevalence of OSCC (39.50%) was above 50 years.[15] Out of 1020 patients in their study, 602 (59.01%) were males and 418 (40.98%) were females. The authors suggested that the sex differences in oral cancer may largely reflect different cultural behavior and lifestyle factors among the population.

The mean age of participants with OL in our study was 60.1 ± 11.5 years, out of which 67.6% were males and 32.4% were females. In a study conducted by Chaturvedi *et al.,* the most affected age groups in leukoplakia were between 60 and 69 years, which is in accordance to other published data.[16] The onset is usually observed after the age of 40 years, and gender distribution ranges from a strong male predominance of 6:1 to almost 1:1. Srivastava *et al.* have reported that in India, the prevalence of leukoplakia varies from 0.2% to 5.2% and the majority occurs in the age range of 35–45 years.[17] People who use both alcohol and tobacco are at high risk of developing oral cancer due to synergistic effects because the dehydrating effect of alcohol on cell membranes enhances the ability of tobacco-associated carcinogens to permeate the mouth tissues.[18] Out of the patients with OSCC, 91% of participants had a history of tobacco usage, while 9% of the participants had no history of any deleterious habits. Out of the patients with OL, 82% of participants had a history of tobacco usage, while 18% of patients had no history of any habits.

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 the tongue and floor of the mouth, which is more common in the Western world.[19] 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.

In our study in group II (OL), 62% of the lesion occurred on the buccal mucosa, 22% on the tongue, 13% on the labial mucosa, and 3% of lesions occurred on the gingiva. This is in accordance with the study by Gopinath *et al*., who reported that 46% of OL in men were located on buccal mucosa, whereas in women, the lateral border of the tongue and buccal mucosa had similar prevalence (36.5% and 34.8%, respectively). The authors suggested that it may

be due to the tobacco-related habits, especially in India, where the betel quid is in intimate contact with the buccal mucosa and the lateral border of tongue.[20]

Matrix metalloproteinase 9 (MMP-9), also known as 92 kDa type IV collagenase, 92 kDa gelatinase, or gelatinase B (GELB), is a matrixin, a class of enzymes that belong to the zinc-metalloproteinase family involved in the degradation of the extra-cellular matrix. In humans, the MMP-9 gene encodes for a signal peptide, a propeptide, a catalytic domain with inserted three repeats of fibronectin type II domain, followed by a C-terminal hemopexin-like domain. MMP-9 functions as a tumour promoter in the process of carcinogenesis as well as an anticancer enzyme at later stages of the disease in some specific situations. This dual role is based on the findings in animal models, where it was observed that MMP-9 knockdown mouse models exhibited decreased incidence of carcinogenesis, whereas tumours formed in MMP-9-deficient mice were significantly more aggressive.[8]

The proteolytic activity of MMPs is required for a cancer cell to degrade physical barriers during local expansion and intravasation at nearby blood vessels and extravasation and invasion at a distant location. During invasion, the localisation of MMPs to specialised cell surface structures, called invadopodia, is requisite for their ability to promote invasion.[8,9]

MMP-9 participates in the angiogenic switch because it increases the biovailability of important factors in this process, such as VEGF, which is the most potent mediator of tumour vasculature, and bFGF, by degradation of extracellular components, such as collagen types IV and XVIII and perlecan, respectively. The angiogenic balance is tightly regulated by MMPs because they can also down-regulate blood vessel formation through the generation of degradation fragments that inhibit angiogenesis. Such molecules include tumstatin, endostatin, angiostatin, and endorepellin, which are generated via cleavage of types IV and XVII collagen, plasminogen, an inactive precursor of a serine proteinase plasmin, and perlecan. Over-expression of several MMPs, including MMP-9, has been associated with epithelial to mesenchymal transition (EMT), a highly conserved and fundamental process of morphological transition.[10]

In our study, the salivary MMP-9 levels were evaluated and compared among OSCC, OL, and healthy controls. The mean salivary MMP-9 level in OSCC (50.9 ± 5.7 ng/ml) was found to be higher than those in OL (31.6 ± 6.0 ng/ml) and healthy subjects (16.2 ± 4.8 ng/ml). The mean salivary levels in OSCC were also estimated among different grades of OSCC. The mean salivary level of MMP-9 in poorly differentiated OSCC was found to be 62.7 ± 6.4 ng/ml, whereas in moderately differentiated and well-differentiated OSCCs, the salivary MMP-9 were 48.7 ± 0.9 ng/ml and 49.0 ± 2.2 ng/ml, respectively. The levels were significantly higher in poorly differentiated OSCC, suggesting the potential role of MMP-9 as a prognostic marker in OSCC.

Dalirsani *et al.* reported that the salivary level of MMP-9 in OSCC patients (49.27 ± 44.5 ng/ml) was found to be significantly higher when compared to the control group (44.68 ± 40.95 ng/ml).[21] Peisker *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 cancer.[22]

To date, the knowledge about specific molecules involved in malignant transformation of OPML and early detection of OSCC has not been satisfying. Only a few studies have examined salivary tumour markers in patients with OL. In the present study, the mean salivary level of MMP-9 was estimated in OL (31.6 ± 6 ng/ml). The salivary levels of MMP-9 were also estimated in different grades of epithelial dysplasia. The mean salivary level of MMP-9 in mild epithelial dysplasia was 26.5 + 4.7 ng/ml; in moderate epithelial dysplasia, it was 30.4 + 3.3 ng/ml; and in severe epithelial dysplasia, it was 37.3 + 4.9 ng/ml. A statistically significant difference was observed when comparing the levels of salivary MMP-9 between mild/moderate dysplasia and severe dysplasia. However, the difference between mild and moderate epithelial dysplasias was not statistically significant. A significantly higher level of salivary MMP-9 levels in severe epithelial dysplasia suggests that MMP-9 may be used as a surrogate marker for malignant transformation.

CONCLUSION

In the current study, there were increased levels of salivary MMP-9 in OSCC and OL compared to normal healthy controls. Higher levels of salivary MMP-9 were observed in poorly differentiated OSCC compared to well and moderately differentiated OSCC. 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 carcinoma implies that MMP-9 may possibly be involved in tumour infiltration

metastasis. The mean salivary MMP-9 was higher in severe dysplasia compared to mild and moderate dysplasias in the current study. As malignant transformation rates are higher in patients with severe dysplasia as compared to mild and moderate dysplasias, salivary MMP-9 could be considered as a surrogate marker of malignant transformation. Further long-term follow-up studies on a larger sample would ascertain the exact role of MMP-9 in OSCC and OL.

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Conflicts of interest

There are no conflicts of interest.

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