Original Article - Comparative Study

Evaluation of Salivary Interleukin-6 in Patients with Oral Squamous Cell Carcinoma, Oral Potentially Malignant Disorders, Chronic Periodontitis and in Healthy Controls - A Cross-Sectional Comparative Study

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Abstract

Introduction: Oral squamous cell carcinoma (OSCC) accounts for 95% among all head and neck cancers. Literature reveals saliva as a predictive, diagnostic and prognostic tool in carcinoma, inflammatory and genetic disorders. Expression of salivary interleukin-6 (IL-6) has been reported in patients with OSCC and in oral potentially malignant disorders (OPMDs). This study aims at the following objectives: • To evaluate the changes in the salivary levels of IL-6 in healthy individuals and those with chronic periodontitis (CP), OPMD and OSCC. • To compare the estimated levels of salivary IL-6 individually in healthy individuals and those with CP, OPMD and OSCC. • To assess the estimated levels of salivary IL-6 individually within histopathologically differentiated OSCC. • To analyse salivary IL-6 as a reliable biomarker in the diagnosis of OSCC. **Materials and Methods:** Totally, 60 patients were divided into four groups consisting of 15 patients in each group. Salivary samples were collected by simple drooling method. The concentration of IL-6 is to be determined by using Quantitative sandwich ELISA technique. All analyses were carried out using Statistical Package for Social Sciences (SPSS). **Results:** The concentration values of IL-6 were found to be more in OSCC group in comparison with the other three groups and the concentration values of OPMD were found to be more than in the CP and control group and was statistically significant. **Discussion:** We attempted a study to evaluate the salivary IL-6 in patients with OSCC, OPMDs and CP in comparison with the healthy controls. We achieved a pragmatic result showed that salivary IL-6 can be a reliable biomarker in the detection of OSCC. Saliva, due its wide array of functional characteristics, is an upcoming diagnostic fluid in the field of medicine and salivary IL-6 can be one such biomarker in the diagnosis of OSCC.

Keywords: ELISA, interleukin-6, oral potentially malignant disorders, oral squamous cell carcinoma, saliva

Introduction

Oral squamous cell carcinoma (OSCC) accounts for 95% among all oral and oropharyngeal cancers.[1] It is the sixth most prevalent cancer worldwide which occurs predominantly due to the influence of carcinogens. India has the highest incidence of oral cancer because of the increased usage of tobacco.[2] Oral potentially malignant disorders (OPMDs) are the conditions with a clinical presentation that carry an increased risk to develop into OSCC.[3] Periodontitis is a multifactorial inflammatory chronic disease[4] and is known as the sixth most common chronic inflammatory disease in the world that occurs in more than 50% of adults worldwide.[3] Virchow's hypothesis stated that the origin of cancer was chronic inflammation.[5] Early diagnosis improves the rate of survival and thus OPMDs play a definitive role when

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diagnosed earlier and thereby decreasing the rate of incidence of OSCC.[6] The ultimate gold standard in diagnosis of oral cancer is biopsy. Various body fluids have been reported in literature in diagnosis of systemic disease and conditions because of its structural composition.[7] Numerous tumour markers identified in serum, oral tissue and saliva have been reported for early diagnosis and a predictive prognostic factor in OSCC patients.[2]

Literature reveals saliva as a predictive, diagnostic and prognostic tool in carcinoma, inflammatory and genetic disorders and saliva is not only confined to the diseases of oral cavity but also mirrors the systemic diseases.[7] Amongst the cytokines, interleukin-6 (IL-6), IL-8 and tumour necrosis factor (TNF)-alpha have been invested in various conditions.[8] Therefore, present study aims at evaluating the reliability of IL-6, as a diagnostic biomarker in OPMDs and OSCC.

To evaluate the reliability of salivary expression of IL-6 as a diagnostic marker in OSCC and OPMDs.

Objectives

- To evaluate the changes in the salivary levels of IL-6 in healthy individuals, individuals with chronic periodontitis (CP), individuals with OSCC and OPMD
- To compare the estimated levels of salivary IL-6 individually in healthy individuals, individuals with CP, OPMD and OSCC
- To assess the estimated levels of salivary IL-6 individually within histopathologically differentiated OSCC.

Materials and Methods

This was a cross-sectional comparative study on patients with OSCC and OPMD. Study subject recruitment was done from the outpatient department of private dental college from a South-Indian population in Chennai. Sample size calculation was done using G Power software. Totally, 60 patients divided into four groups consisting of 15 patients in each group were included under the study. The study was conducted for a period of six months from May to October, 2022. Random selection procedure was used to choose participants. This study was approved by the Ethical Committee (MADC/IEC-1/058/2021).

Inclusion criteria

Individuals with willingness to participate in the study:

- Group I: Patients with clinically diagnosed and histopathologically proven OSCC
- Group II: Patients with clinically diagnosed and histopathologically proven OPMD
- Group III: Individuals with CP matched with the scores of CPI probe as per the WHO guidelines with a probing depth of more than 5 mm
- Group IV: Healthy individuals with matched periodontal condition and free of any habits and systemic disease.

Exclusion criteria

- Patients unwilling to consent for the study
- Individuals with serious systemic illness
- Active infectious disease
- Individuals with collagen vascular disease, diabetes mellitus, hypertension
- Pregnant and lactating women
- Group I: OSCC
  - Currently undergoing or having undergone any form of definitive therapy for OSCC in the form of radiation, chemotherapy or any other adjunctive treatments
  - Any other concurrent or recurrent oral cancer.
- Group II: OPMD
  - Patients with underlying systemic illnesses
  - Oral ulcers.
- Group III: CP
  - Mild periodontitis status.
- Group IV: Healthy individuals (control group).
  - No current use of prescribed or non-prescribed medication
  - No chronic/acute illnesses
  - No oral lesions
  - No acute or subacute inflammation or infection
  - No pathological dry mouth syndrome
  - Inability to collect sufficient saliva samples on a reliable basis.

Individuals were clinically diagnosed by the principal investigator and two senior professors to confirm the diagnosis and clinically diagnosed. A detailed history of the habits and disease was elicited. Patient education and counselling regarding the habits was done. Periodontal status of all patients was matched using CPI as per the WHO guidelines. Patients with Community Periodontal Index of Treatment Needs (CPITN) 3 and 4 scores were identified as having periodontitis. No previous calibrations were conducted before the study. Clinical and histopathological analysis were performed by experienced professionals with more than 20 years of experience, who were not calibrated for the purpose of this study. Interrater reliability was not carried out by the examiners for this study.

Samples from all the patients were collected by simple drooling method. Then, the samples were centrifuged at 3000 rpm for 10 min. The supernatants were collected carefully using micropipette, transferred to Eppendorf tubes and stored at a temperature of −20° centigrade until unit analysis.

The concentration of IL-6 was determined by using quantitative sandwich ELISA technique. The analysis was done using KET6017 EliKine™ Human IL-6 ELISA Kit going with the manufacturer's i.e., ABBKINE's recommendations. The kit was based on sandwich Elisa to the precoated IL-6 antibody microplate. Standards and samples were added, incubated (for 2 h) and washed with buffer. Detection antibody with horse radish peroxidase conjugate was added to the wells of the washed plate, which resulted in the progressive development of a blue coloured complex with the conjugate. The colour

development was then stopped by the addition of stop solution turning the resultant final product yellow. The intensity of colour developed was proportional to the IL-6 present which was measured in a microplate reader (Robonik ELISA plate reader) at a wavelength of 450 nm. The results were expressed as pg/mL of saliva.

Statistical analyses

The data obtained was entered in Microsoft Excel Spreadsheet and was subjected to statistical analysis. All analyses were carried out using Statistical Package for Social Sciences (version 19, IBM, Chicago, USA). Categorical variables were expressed as numbers (*n*) and percentage (%). Continuous variables were expressed as mean and standard deviations. The data was subjected to normality test using Shapiro–Wilk test. Parametric tests of significance were used for normally distributed quantitative data. Non-parametric tests of significance were used for non-normally distributed qualitative data. One-Way ANOVA was used to find the statistical difference of optical density (OD) values and concentration values between the four groups. *Post-hoc* Tukey test was used for multiple comparisons. Kruskal–Wallis test was used for intergroup comparisons of various categories present in OD and concentration values of OSCC patients. The level of significance was set at 0.05.

Results

Table 1 shows the habit history, age and sex of the patients, anatomical site of OSCC included in the study in group I.

Table 2 shows the age, sex and habit history of the patients and the various type of OPMDs diagnosed.

Table 3 shows intergroup comparison of concentration values between the groups. The mean OD values for OSCC, OPMDs CP and control group were 0.79 ± 0.09, 0.38 ± 0.1, 0.19 ± 0.02 and 0.13 ± 0.01, respectively. The concentration values were found to be very highly statistically significant between the four groups ($P < 0.001$).

Figure 1 shows concentration values of the study participants. From the graph, it is evident that mean concentration values were higher for OSCC followed by OPMD, CP and controls.

Table 4 shows *post hoc* Tukey's test with multiple internal comparison of dependent variables. From the results, it was found out that there was a very high statistical significance observed for OD values of OSCC in comparison with other three groups. Thus, it was inferred that OD values were higher for OSCC group than other three groups and this was found to be highly statistically significant ($P < 0.001$). The OD values of OPMD were found to be higher than CP and control groups and this was also found to be highly statistically significant ($P < 0.001$). It must be noted that though OD values of OPMD is more than CP and control group, it was lesser than OSCC group.

Discussion

Cancer has become one of the leading diseases in the world. Various malignant lesions presenting in the oral cavity are termed as oral cancer.[9] The major threat factors of OSCC involves tobacco smoking, betel chewing, excessive

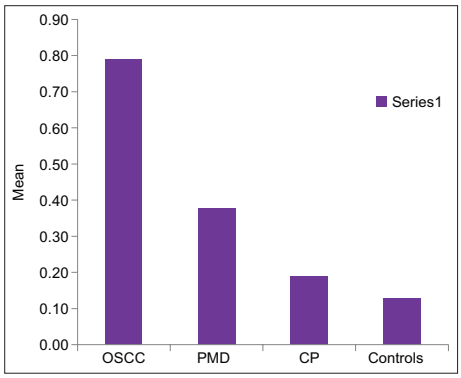

Figure 1: Concentration value

Table 1: Age, sex, anatomic site of oral squamous cell carcinoma with type of habits of the patients

Age/sex	Diagnosis	Type of habit
52/male	OSCC of left buccal mucosa	Pouching smokeless tobacco
76/male	OSCC of right alveolus	Pouching smokeless tobacco
45/male	OSCC of the right buccal mucosa	Pouching smokeless tobacco
47/male	OSCC of the tongue	Smoking tobacco
70/male	OSCC of the left alveolus	Pouching smokeless tobacco
55/male	OSCC of left buccal mucosa	Pouching smokeless tobacco
55/male	OSCC of left buccal mucosa	Chewing smokeless tobacco
65/male	OSCC of right alveolus	Pouching smokeless tobacco
36/male	OSCC of left alveolus	Pouching smokeless tobacco
58/female	OSCC of the tongue	Sharp cusps
55/female	OSCC of left lateral border of tongue	Pouching nasal snuff
56/female	OSCC on right buccal mucosa	Pouching smokeless tobacco
76/male	OSCC in the left buccal mucosa	Pouching smokeless tobacco
44/female	OSCC in the right lateral border of the tongue	Pouching smokeless tobacco
63/male	OSCC of left attached gingiva in relation to 36, 37, 38	Smoking tobacco

OSCC: Oral squamous cell carcinoma

Table 2: Age, sex, type of oral potentially malignant disorders with type of habits of the patients

Age/sex	Diagnosis	Type of habit
28/male	Grade 4 OSMF	Pouching smokeless tobacco
40/male	Grade 4 OSMF	Pouching smokeless tobacco
36/female	Oral lichenoid dysplasia	Idiopathic
30/male	Homogenous leucoplakia	Smoking tobacco
47/male	Homogenous leucoplakia	Smoking tobacco
39/male	Grade 4 OSMF	Pouching smokeless tobacco and smoking tobacco
54/male	Non-homogenous leucoplakia	Smoking tobacco
66/female	Homogenous leucoplakia	Smoking tobacco
54/male	Grade 4 OSMF	Pouching smokeless tobacco
47/male	Grade 4 OSMF	Pouching smokeless tobacco
74/male	Grade 4 OSMF	Pouching smokeless tobacco
30/male	Grade 4 OSMF	Pouching smokeless tobacco and smoking tobacco
48/male	Grade 4 OSMF	Pouching smokeless tobacco
45/male	Grade 4 OSMF	Pouching smokeless tobacco
34/male	Grade 4 OSMF	Pouching smokeless tobacco and smoking tobacco

OSMF: Oral submucous fibrosis

Table 3: Intergroup comparison of concentration values between the groups

Parameter	Group	Mean±SD	*P*
Concentration values	Group I OSCC	0.79±0.09	<0.001***
	Group II OPMDs	0.38±0.1	
	Group III CP	0.19±0.02	
	Group IV control	0.13±0.01	

****P*<0.001 - highly statistically significant. OSCC: Oral squamous cell carcinoma, SD: Standard deviation, OPMDs: Oral potentially malignant disorders, CP: Chronic periodontitis

alcohol consumption, exposure to ultraviolet and ionising radiation, infection with human papillomavirus and human immunodeficiency virus.[10] Salivary biomarkers could be excellent and convenient tools for achieving public screening of oral cancer at early stage.[11] Histopathology remains the gold standard in the diagnosis of cancer. Various gradings and staging are followed in differentiating various cancers and yet AJCC staging is universally followed across the globe.[12] Apart from various histopathological diagnosis, various biomarkers are unravelled in the field of medicine, out of which saliva, due to its multifunctional activity, is an upcoming source for diagnosis. IL-6 is a known glycoprotein and a pleiotropic cytokine possessing various functional characteristics.[13] The literature reveals various studies on the diagnostic role of IL-6 in malignancy.[14] Saliva is regarded as a potential alternative to blood serum and urine and to be a non-invasive technique.[15] In our study, we have attempted to enlighten the characteristic feature of salivary IL-6 in OSCC, OPMDs and CP as compared with healthy controls.

In our study, the levels of salivary IL-6 were highly elevated in patients with OSCC and moderately elevated in patients with OPMDs. There was also a difference in the levels of Group I and II patients with the Group III patients. Though IL-6 is an inflammatory cytokine, its elevated levels are notable in OSCC and OPMDs.

In recent times, various studies are being done in highlighting IL-6 in OSCC and OPMDs like that of the study done by Park *et al*., in 2019,[16] and Huang *et al.* in 2023.[17] These studies reveal the increased levels of IL-6 in OSCC and henceforth IL-6 can be a reliable biomarker which is in correlation with our study with increased levels of salivary IL-6 in OSCC and OPMDs.

Research by Panneer Selvam *et al.* (2015),[6] to estimate salivary IL-6 as a molecular marker to diagnose leucoplakia and OSCC, enumerated the increase in salivary IL-6 in patients with leucoplakia and OSCC, compared with healthy controls. The results highlighted the reason for elevated salivary IL-6 as local production by the tumour cells and the difference in its levels between these two lesions might indicate the progression of precancer to cancer. The changes in the oral cavity due to OSCC, and OPMDs could be a contributing factor for the raised levels in our study.

Our study among the Chennai sub-population had a statistical significance while correlating salivary IL-6 among the OSCC and OPMDs which was corresponding to the study, performed by Dineshkumar *et al*.,[2] and Khyani *et al*.,[18] to explore salivary IL-6 as a biomarker for OPMDs and OSCC concluded that salivary IL-6 could be used as a probable biomarker for early detection of OPMDs and OSCC in an aetiologically distinct population in Pakistan.

In a study done by Lee *et al.* in 2018,[19] in view of evaluating saliva and plasma cytokine biomarkers (IL-1β, IL-6, IL-8 and TNF-α) in patients with OSCC in comparison with healthy controls, the results were in accordance with our study which revealed that along with the other salivary biomarkers, IL-6 also had statistically significant results.

Various systematic reviews and meta-analyses done by various authors regarding the efficacy of salivary IL-6 in OSCC adds a notable remark to our study in evaluating salivary IL-6 in OSCC, OPMDs and CP with healthy controls. Piyarathne *et al*. in 2021,[3] Uz and Eskiizmir in 2021,[9] Mozaffari *et al*.

Table 4: *Post-hoc* Tukey's test with multiple comparison of dependent variables

Dependent variable	Mean difference (95% CI)	*P*
OD value		
Group I OSCC		
OPMD	48.933 (38.949–58.91)	<0.001***
CP	67.82 (57.842–77.81)	<0.001***
Controls	70.28 (60.29–80.26)	<0.001***
Group II OPMD		
CP	18.89 (8.909–28.87)	<0.001***
Controls	21.34 (11.36–31.33)	<0.001***
Group III CP		
Controls	2.45 (−7.53–12.43)	0.91
Concentration value		
Group I OSCC		
OPMD	0.411 (0.34–0.47)	<0.001***
CP	0.599 (0.53–0.66)	<0.001***
Controls	0.66 (0.59–0.72)	<0.001***
Group II OPMD		
CP	0.18 (0.12–0.25)	<0.001***
Controls	0.24 (0.18–0.31)	<0.001***
Group III CP		
Controls	0.06 (−0.005–0.12)	0.08

***P<0.001 - very highly statistically significant. OSCC: Oral squamous cell carcinoma, OPMD: Oral potentially malignant disorder, CP: Chronic periodontitis, CI: Confidence interval, OD: Optical density

in 2018,[20] and Rezaei *et al*. in 2019,[21] in their systematic reviews and meta-analyses found that salivary IL-6 and IL-8 were highly elevated than the serum. Their result concluded that salivary IL-6 and IL-8 could be potential biomarkers in OSCC which was in correlation with our study in evaluating salivary IL-6 in patients with OSCC.

Analogous with our study, a recent study done by Harshy *et al*. in 2022,[22] in comparing the levels of salivary IL-6 between 20 cases of OPMDs and OSCCs suggested that salivary IL-6 was statistically significant. In our study, there was statistical significance when comparing the salivary IL-6 between OSCC, OPMDs and CP and healthy controls which was not in accordance with the study done by St John *et al*.,[23] that indicated that IL-8 in saliva and IL-6 in serum hold the most promise as biomarkers for OSCC. The reason for the contrasting results with our study could be due to the improper distribution of samples and the different study groups and the different methodology that was followed between the two studies. Furthermore, we attempted to compare the levels of salivary IL-6 within well, moderately and poorly differentiated OSCC; it was statistically insignificant due to the unequal distribution of cases with histologically proven OSCC. Most of the past studies in different populations revealed a favourable result which disclosed the efficacy of salivary IL-6 in OSCC, being a non-invasive technique.

Summary and Conclusion

Medical science constantly innovates to introduce new investigative modalities and modes of treatment plan. The treatment of cancers is a major challenge facing the field of medicine. OSCC with its poor prognostic nature still remains disputed on the basis of early diagnosis and prevention. Serum biomarkers have become the reliable investigative modality for decades. In recent years, the literature enshrines the use of salivary biomarkers as a reliable diagnostics source in the field of cancer. On this basis, we attempted a study to evaluate the salivary IL-6 in patients with OSCC, OPMDs and CP in comparison with healthy controls. Cytokines present in tumour micro environment are low-molecular weight proteins that control cell proliferation, survival, migration, as well as immune cell activation. They also modulate the anti-tumoural immune response; although, in chronic inflammation, they induce tumour transformation.[24] That could be the reason for the increased values of salivary IL-6 in different groups of our study. CP is a continuous inflammatory disease in which microorganisms can stimulate mononuclear phagocytes to release inflammatory factors, resulting in an increase in inflammatory cytokines such as IL-6, TNFα, IL-5 and IL-8.[25] To determine the differences in the levels of IL-6, an inflammatory cytokine, we included another group of CP. We achieved a pragmatic result that showed that salivary IL-6 can be a reliable biomarker in the detection of OSCC. Our results also highlighted the varying levels of IL-6 in OPMDs and CP. While comparing all the three groups with the healthy controls, there was a drastic difference between the groups according to its severity. Our efforts to enumerate the differences between the groups of OSCC, OPMDs and CP and the healthy controls were statistically significant, but while comparing the IL-6 among the well-differentiated, moderately differentiated and poorly differentiated OSCC, the results were insignificant due to failure in case distribution within the group.

Future perspectives

Equal distribution of samples within the groups of OSCC and OPMDs might succour the prompt and significant statistical outcome.

Larger samples are required to standardise salivary IL-6 as a predictable biomarker because it would aid in more predictable results.

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Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form, the patient(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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Conflicts of interest

There are no conflicts of interest.

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