Salivary Marker IL-6 for Detection of Potentially Malignant Oral Disorders: A Pilot Study

Ramsheena Payambrot¹, Sahaya Shibu², Auswaf Ahsan³, Abdul Majeed Kummangal¹

ABSTRACT

Introduction: Cytokines play a pivotal role in modulating the immune response, and are either proinflammatory (IL-1, IL-8, IL-6, TNF-α and TGF-β), or anti-inflammatory (IL-2, IL-4, IL-10, IL-12, and IFN-γ). Interleukin-6 (IL-6) was selected as it is a multifunctional interleukin reported to be altered in potentially malignant oral disorders and in malignant lesions.

Aim: This study was designed to estimate the levels of salivary IL-6 in potentially malignant oral disorders (PMDs) viz leukoplakia, lichen planus, oral submucous fibrosis (OSF) and compare them with healthy controls. The aim was to evaluate the efficacy of IL-6 as a potential biomarker for these diseases.

Materials and Methods: The study sample comprised saliva collected from 12 subjects diagnosed with potentially malignant lesions and 12 healthy volunteers. The enzyme-linked immunosorbent assay (ELISA) test was used to measure concentrations of IL-6 levels in two groups and the concentration of IL-6 was compared among the PMD patients and healthy individuals.

Results: The levels of salivary IL-6 concentration were found to be significantly elevated in patients with PMDs as compared to the healthy control group (p<0.0001). Patients with OSF had significantly higher IL-6 concentrations in their saliva compared to patients with other premalignant lesions.

Conclusion: The result suggests that salivary IL-6 can be utilised as a potential biomarker to predict malignant transformation of PMDs. The present study was done with very few samples size. Hence, possible role of IL-6 to predict transition of premalignancy to malignant condition needs further research with larger sample sizes with patients having oral malignancy.

Key words: cytokines, interleukin-6, ELISA, potentially malignant disorders (PMDs), saliva.

INTRODUCTION

Oral mucosa represents the initial part of the digestive tract and is exposed to various exogenous toxins. In long term, such exposures can lead to changes that lead to precancerous lesions or cancers. Precancerous lesions of the oral mucosa, known as potentially malignant disorders (PMDs) in recent years consists of a group of diseases namely oral leukoplakia, oral submucous fibrosis (OSF) and oral lichen planus with a very high malignant transformation rate.

The etiology of precancerous lesions of oral mucosa is not well-known. Some risk factors such as tobacco chewing, tobacco smoking, and alcohol play a significant role in the development of potentially malignant oral conditions. Tobacco chewing is a major risk factor for oral leukoplakia, OSF, and lichen planus. Alcohol consumption may increase the risk of oral leukoplakia by 1.5 fold and for OSF by 2-fold.

Oral leukoplakia, the most common PMD of the oral cavity. Leukoplakia is a term describing “a white lesion of the oral mucosa that cannot be characterized clinically or microscopically as any other defined oral disease entity”⁵. Oral leukoplakia is classified into two main types: homogeneous type which appears as a flat white lesion and non-homogeneous type which includes speckled, nodular and verrucous leukoplakia. The homogeneous leukoplakia is a uniform, thin white area.

altering or not with normal mucosa. The speckled type is a white and red lesion, with a predominantly white area.

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Conflict of Interest: None

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The nodular type has small polypoid outgrowths and rounded predominantly white excrescences. Verrucous leukoplakia has an elevated, proliferative or corrugated surface appearance.

Oral lichen planus, clinically presented as a lesion with radiating whitish gray lines. It is more prevalent in middle-aged females. It appears frequently in all regions of the oral mucosa, predominantly on the buccal mucosa, gingiva and tongue. They are present bilaterally in most cases. OLP may be seen in six different subtypes including Reticular (web-like, fine white striae cross each other in the lesion – sometimes referred to as Whickham's Striae), Papular type, Bullous type, Plaque type, Atrophic (areas of erythematous lesion surrounded by reticular components) and Erosive or Ulcerative type (area in which erythematous areas are seen surrounded by reticular elements). The reticular type of oral lichen planus is often asymptomatic and only can be seen clinically. Atrophic and ulcerative subtypes have a greater increased malignant transformation risk compared to other subtypes.

Oral submucous fibrosis (OSF), is a chronic disease of the oral mucosa characterized by inflammation and a progressive fibrosis of the lamina propria and deeper connective tissues. Its etiology is multifactorial but arecoline in the areca nut is of utmost importance to develop newer, non-invasive and easy microscopically before progression into OSCC.

Table 1. Age and Gender distribution

<table>
<thead>
<tr>
<th></th>
<th>Age (Mean ± SD)</th>
<th>Gender</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Leukoplakia</td>
<td>58.75 ± 5.56</td>
<td>4</td>
</tr>
<tr>
<td>Lichen-planus</td>
<td>54.75 ± 4.78</td>
<td>0</td>
</tr>
<tr>
<td>OSF</td>
<td>54.00 ± 6.37</td>
<td>4</td>
</tr>
<tr>
<td>Control</td>
<td>46.36 ± 7.51</td>
<td>8</td>
</tr>
</tbody>
</table>

Table 2. Association of habits in different groups

<table>
<thead>
<tr>
<th></th>
<th>Smoking n (%)</th>
<th>Smoking and alcohol</th>
<th>Smoking and betel quid</th>
<th>Smoking, Alcohol and betel quid</th>
<th>None</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukoplakia</td>
<td>1(25)</td>
<td>1(25)</td>
<td>1(25)</td>
<td>1(25)</td>
<td>0</td>
<td>0.09</td>
</tr>
<tr>
<td>Lichen-planus</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4(100)</td>
<td></td>
</tr>
<tr>
<td>OSF</td>
<td>0</td>
<td>1(25)</td>
<td>2(50)</td>
<td>1(25)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>12(100)</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Site of lesion

<table>
<thead>
<tr>
<th></th>
<th>Buccal mucosa n (%)</th>
<th>FOM</th>
<th>Gingiva</th>
<th>Mandibular mucosa</th>
<th>Tongue</th>
<th>None</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukoplakia</td>
<td>2(50)</td>
<td>1(25)</td>
<td>0</td>
<td>1(25)</td>
<td>0</td>
<td>0</td>
<td>0.09</td>
</tr>
<tr>
<td>Lichen-planus</td>
<td>3(75)</td>
<td>0</td>
<td>1(25)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>OSF</td>
<td>3(75)</td>
<td>0</td>
<td>2(50)</td>
<td>1(25)</td>
<td>0</td>
<td>1(25)</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>12(100)</td>
<td></td>
</tr>
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</table>
to use diagnostic medium and tools for the detection of PMDs. The detection of discriminatory biomarkers in saliva samples are the most promising answer at this stage[15].

**Materials and Methods**

A case control study was conducted on PMD patients who enrolled at the outpatient department of KMCT Dental College during January 2021 to May 2021. Study subjects were recruited by professionally qualified, well trained and experienced Oral Pathologists. The demographic details and information on previous history were collected. This study was approved by Institution Ethical Committee (IEC) of KMCT Dental College (IEC No: KMCTDC/IEC/2020/24). According to the ethical principles, a written and informed consent was obtained from all the study participants to confirm that they are fit to participate in the study before collecting saliva. A total of 24 cases were included and the cases were selected from the Department of Oral Medicine and Radiology, KMCT Dental College and Hospital, Calicut, India. All the patients were in the age group of 21–80 years. They were divided into two groups consisting of 12 cases each: Group I (study group): patients with clinically diagnosed and histopathological proven PMDs like oral leukoplakia, oral lichen planus and oral sub mucous fibrosis. Group II (control group): Healthy subjects free of any habits and systemic diseases. Patients with oral leukoplakia, lichen planus, oral submucous fibrosis were included and the patients from the following categories: distant metastasis, secondary malignancies, cardiac insufficiency or myocardial infarction within the last 6 months, any systemic or topical treatment suppressing the immune system such as steroids or other immunosuppressive drugs, Pregnant and lactating subjects, Patients who did not display any alcohol consumption, tobacco use, prosthetic use and the primary site of the carcinoma were excluded.

**Sample collection**

The unstimulated saliva was collected from patient between 8.00 am and 10.00 am. The patients were asked to rinse their mouth thoroughly with water 10 min before saliva collection, and they were asked to spit out or swallow saliva already present in the mouth. After the individuals were comfortably seated and after a few minutes of relaxation, they were trained to avoid swallowing saliva and asked to lean forward and drool all the saliva they produced into a vial using a custom-made saliva collecting funnel for 5–10 mins. A sufficient amount of saliva was collected. Once collected, the vials were stored in a portable ice carrier box and immediately transferred to the laboratory. Stored saliva was melted and transferred to test tubes. They were subjected to centrifugation for 20 minutes at the speed of 2000-3000 r.p.m to remove debris. The supernatants were carefully drawn using micropipettes and transferred to Eppendorf tubes which were stored at –20°C until analysis.

**IL-6 Estimation**

The concentration of salivary IL-6 was quantified by a commercially available ELISA kit (Wuhan Fine Biotech, China). The assay was carried out according to the manufacture’s instruction. Briefly, the kit was based on sandwich enzyme-linked immune-sorbent assay (ELISA) technology. The capture antibody was pre-coated onto 96-well plates. Biotin-conjugated antibody was used as the detection antibodies. The standards, test samples and biotin-conjugated detection antibody were added to the wells subsequently, and washed with wash buffer. HRP-Streptavidin was added and unbound conjugates were washed away with awash buffer. TMB substrates were used to visualize HRP enzymatic reaction. TMB was catalyzed by HRP to produce a blue color product that changed into yellow after adding an acidic stop solution. The density of yellow is proportional to the target amount of sample captured in plate. Optical density (OD) absorbance at 450nm in a microplate reader (Rayto ELISA plate reader) was added, the OD obtained was then used for calculation of IL-6 present in each sample. The detection range of the kit was from a minimum of 4.688-300pg/ml. The results were expressed as pg/ml of saliva and then the concentration used for calculation of IL-6 present in each sample

**Statistical methods**

All statistical procedures were performed using Statistical Package for Social Sciences (SPSS) 20.0. Calculations for power (80%) of study was performed before the commencement of the study. All quantitative variables expressed in mean and standard Deviation. Qualitative variables expressed in percentages. Shapiro-Wilk test was used for testing the normality assumption of the quantitative data. Chi square test for qualitative and One way ANOVA for quantitative variables was used for association between variables. Probability value (p <0.05) was considered statistically significant.

**Results**

The samples of 24 cases were divided into two groups, 12 premalignant lesions which consisted of 4 each of leukoplakia, lichen planus and OSF out of 24, and 12 healthy subjects were included. The age of patients ranged from 52-65 years (group 1 four cases) (Mean±SD) (58.75± 5.56), from 49-60 years (group 2 four cases) (54.75 ± 4.78), from 48-63 years (group 3 four cases) (54.00 ± 6.37), from 31-58 (group 4 twelve cases) (46.36± 7.51), (Table 1).

When comparing gender predilection for premalignant lesions, leukoplakia and OSF showed in female predominance and lichen planus showed in female predominance (Table 1).For leukoplakia, 25% of cases show an association with smoking, 25%of cases show an association with smoking and alcohol, 25%of cases show an association with smoking and betel quid, 25%of cases show an association with smoking, alcohol and betel quid. For lichen planus, there is no association with habits. For OSF, 25% of cases show an association with smoking and alcohol, 50% of cases show smoking and betel quid and 25% of cases shows smoking, alcohol and betel quid.

<table>
<thead>
<tr>
<th>Table 4. IL-6 (pg/ml) levels in different groups</th>
<th>(Mean ± SD)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukoplakia</td>
<td>15.32±3.88</td>
<td>0.001**</td>
</tr>
<tr>
<td>Lichen-planus</td>
<td>15.00 ±2.86</td>
<td></td>
</tr>
<tr>
<td>OSF</td>
<td>24.15±5.50</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5.01±3.06</td>
<td></td>
</tr>
</tbody>
</table>

*p value <0.05 is statistically significant; ** <0.001 is statistically highly significant*
The P-value for the association is shown in table 2, it was not statistically significant.

8 cases showed the premalignant lesions were from buccal mucosa, for leukoplakia (50%), for lichen planus (75%) and for OSF (75%), one cases from the floor of the mouth (FOM) for leukoplakia (25%), one cases from gingiva for lichen planus (25%), one cases from mandibular mucosa for leukoplakia (25%) and one cases from tongue (25%). The P-value for the association between the site of the lesion is shown in table 3, it was also not statistically significant.

The patient with leukoplakia showed 15.32±3.88 pg/ml (mean and SD) for the concentration of salivary IL-6, for lichen planus 15.00±2.86 pg/ml, for OSF 24.15±5.50 pg/ml, and 5.01±3.06 pg/ml for control subjects respectively. One way ANOVA for IL-6 levels among the four groups was compared, and the results were statistically highly significant (P<0.001). The levels of IL-6 are significantly higher in in PMDs when compared to control group. Salivary IL-6 also showed a significantly higher level in OSF compared to other PMDs patient. The exact P-value for the IL-6 levels in different groups is shown in Table 4.

**Discussion**

Saliva offers some distinctive advantages when used for diagnosis of disease. The whole saliva can be collected non-invasively and by individuals with limited training, including the patient. No special equipment is needed for collection of the fluid. Further, analysis of saliva may provide a cost-effective approach for the screening of large populations. Advances in the use of saliva as a diagnostic fluid have been affected by current technological developments: enzyme–linked fluorescence technique, Western blot assays, polymerase chain reaction (PCR)17.

Cytokines are a group of small, mainly secreted proteins that affect the behaviour of cells in a diverse number of ways. The binding of cytokines to specific cell membrane cytokine receptors can induce several activities within the cell, such as growth, differentiation, or death18. Most cytokines have pleiotropic effects; however, some are generally considered as pro-inflammatory, such as interleukin-1beta (IL-1β), IL-6, IL-8, tumour necrosis factor-alpha (TNF-α), and transforming growth factor-beta-1 (TGF-β1), whereas others are associated with anti-inflammatory effects, such as IL-2, IL-4, IL-10, IL-12, IL-13, interferon-gamma (IFN-γ). Among the cytokines, interleukins have a crucial function and are implicated in oral PMDs.

Molecular markers which are identified and characterized in saliva have distinct advantages in pathological diagnosis as they reflect the overall health and disease states in an individual. Because of the anatomical proximity of saliva to the oral cavity, salivary testing would be ideal for evaluating potentially malignant and malignant oral lesions19. A review mentioned that markers can be used for risk assessment of malignant transformation in patients with OPMDs as well as for prophylactic conciliation and fair management of the high-risk OPMD patient group20.

IL-6 is a multifunctional cytokine with growth-promoting and anti-apoptotic activity. There is evidence that IL-6 regulates activation of the Janus kinases (JAK) and signal transducers and activators of transcription (STATs), which then stimulate pathways involving mitogen-activated protein kinase (MAPK), which in turn supports PMDs development21. Salivary IL-6 mRNA was quantified by realtime quantitative PCR. Salivary IL-6 protein concentration was measured by enzyme linked immune-sorbert assay. IL-6 protein expression in tumor samples was investigated by immunohistochemistry22. Higher levels of IL-6 in saliva compared with serum suggest that measurement of this marker in saliva may be more useful than serum for diagnostic and therapeutic aims23. Immunohistochemical analysis significantly higher expression of IL-8 in OSCC specimens and TNF-alpha in OSCCs and OPMDs with dysplasia as compared to NOM24. Cytokines directly involved in inflammation and immune response, the role of salivary cytokines in tumor growth and progression linked them to the incidence of oral malignant lesions25. A similar study reported that the evaluated 3 protein markers, IL-1β, IL-8 and LGALS3BP using ELISA, from unstimulated saliva samples. Among these LGALS3BP was significantly elevated specifically in early stage OSCCs and PMDs26.

In our study, among the premalignant lesions, we found that leukoplakia and OSF which were associated with tobacco habits contributed to an elevated IL-6 level. It also showed an increase in IL-6 level in OSF when compared to the other two premalignant lesions and control. Our observation, however, none of our study samples have yet transformed to the malignancy, thus, confirming and extending the previous findings that IL-6 expression could be used as a specific marker for lesions that are at high risk of malignant transformation. Precisely, the increased levels related to the clinical stage of the disease seemed to be the most sensitive parameter, having a better chance of identifying transformation to cancer particularly in the early stages.

**Conclusion**

Pro-inflammatory cytokines are elevated in the saliva of individuals with PMDs compared to controls, thus suggesting that Salivary IL-6 can be utilised as a potential biomarker for precancer that have higher risk of malignant transformation and patients with high levels of IL-6 levels may benefit from follow-up to have a better chance of identifying recurrences at an early stage. Validated diagnostic and/or prognostic significance which needs to be further confirmed by large population size at multicenter level.

**Author Contribution**

Dr. Ramsheena was involved with the conceptualization, methodology and paper review. She wrote the original manuscript and is accountable for all aspects of the work. Dr. Abdul Majeed was involved with the conceptualization of the paper. He critically reviewed and edited the manuscript. Dr. Sahaya Shibu critically revised the article. Dr. Auswaf Ahsan provided all support, guidance, insight and approving the final version of the manuscript. All authors read and approved the final manuscript.

**References**


