

Proinflammatory cytokine levels in oral lichen planus, oral leukoplakia, and oral submucous fibrosis

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Abstract (J Korean Assoc Oral Maxillofac Surg 2015;41:171-175)

Objectives: The objective of this study was to identify salivary and serum concentrations of interleukin (IL)-8, IL-6, and tumor necrosis factor alpha (TNF- α) in patients with oral lichen planus, oral leukoplakia, oral submucous fibrosis, and healthy controls.

Materials and Methods: Patients selected included 54 oral lichen planus (41 to 65 years), 50 oral leukoplakia (42 to 65 years), 51 oral submucous fibrosis (41 to 65 years), and 50 healthy controls (42 to 65 years). Oral lichen planus, oral leukoplakia, and oral submucous fibrosis cases were diagnosed using histopathological analysis. Salivary and serum cytokine concentrations were measured using enzyme-linked immunoassay kits in all subjects.

Results: The levels of serum and salivary TNF- α , IL-6, and IL-8 were statistically significantly increased in oral leukoplakia, submucous fibrosis, and lichen planus in contrast to normal healthy subjects ($P < 0.05$). Serum and salivary correlation analysis revealed strong and highly significant correlations for TNF- α , IL-6, and IL-8 in all groups ($r = 0.72-0.82$, $P < 0.05$).

Conclusion: Salivary and serum cytokines were also elevated when analyzed in oral precancerous lesions. Thus, salivary and serum IL-8, IL-6, and TNF- α levels might act as diagnostic markers for detection of oral precancer.

Key words: Saliva, Oral lichen planus, Oral leukoplakia, Oral submucous fibrosis, Cytokines

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I. Introduction

Oral cancer is the eleventh most common cancer in the world¹. Its incidence is predominantly high in northern India, other Asian countries, and in certain places in the western hemisphere. It has been reported that 90% of oral cancers in India among men were attributable to chewing and smoking habits. In India, the age-standardized incidence rate of oral cancer is 12.6 per 100,000 people². Oral squamous cell carcinoma develops through a multi-step process of genetic, epigenetic, and metabolic changes resulting from exposure

to carcinogens³. The initial presence of a precursor cell subsequently developing into cancer is well established in oral cancer⁴. Oral leukoplakia, submucous fibrosis, and lichen planus are major known precursor lesions. The prevalence of malignant transformation of oral lichen planus is around 0.5% and approximately 1% for leukoplakia⁵. Ability to clinically predict malignant transformation is difficult and routine histopathological diagnosis has limited prognostic value. Despite advanced technologies for early detection of oral precancerous and cancerous lesions, there are limitations for its use, including that diagnosis is essentially subjective, all lesions exhibiting dysplasia do not eventually become malignant and may even regress, and carcinoma can develop from lesions in which epithelial dysplasia was not diagnosed on previous biopsies^{6,7}. Therefore, it is necessary to develop other methods for predicting the malignant potential of premalignant lesions and preventive measures⁷.

Recently, strong evidence has suggested that the nuclear factor- κ B (NF- κ B) signaling pathway plays a critical role in carcinogenesis, protection from apoptosis, and chemoresistance in a number of cancer types, including head and neck

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cancer, breast cancer, hepatocellular carcinoma, and gastric cancer⁸⁻¹². Recently, accumulating evidence has suggested that the NF-κB-dependent cytokine levels are elevated in saliva and tissue specimens of patients with oral premalignant lesions¹³. Furthermore, different cytokines can act as diagnostic tools for detecting oral cancerous and precancerous lesions and conditions. Interleukin (IL)-6 levels are significantly increased in oral cancer patients¹⁴⁻¹⁷. It has been evident that different cytokines are expressed by cancerous cells, the most common of which include tumor necrosis factor alpha (TNF-α), IL-1, IL-6, and IL-8¹⁵⁻¹⁷. Limited studies were conducted on roles of TNF-α, IL-6, and IL-8 in oral precancer and cancer¹⁵⁻¹⁷. The local and systemic nature of these responses suggests the hypothesis that cytokines with proinflammatory and proangiogenic activity are produced by precancers and cancers and could contribute to the pathogenesis of oral malignancy. In this study, we hypothesized that salivary and serum TNF-α, IL-6, and IL-8 levels could be elevated in oral precancerous lesions and conditions. To test this hypothesis, this study was conducted to analyze the concentrations of TNF-α, IL-6, and IL-8 in oral precancer using saliva and serum samples.

II. Materials and Methods

1. Eligibility

Consecutive patients clinically and histopathologically confirmed as having oral lichen planus lesions, oral leukoplakia, and oral submucous fibrosis were recruited for this study from Baba Nidhan Singh Hospital, Punjab, India, based on the definition of oral cancer and precancerous lesions by the World Health Organization⁹. Healthy normal patients served as the control group. Demographic characteristics of patient and controls characteristics were mentioned in Table 1. The mean and standard deviation of smoking and alcoholic sta-

tus was also ascertained. Patients and healthy subjects with matched periodontal status were selected.

All subjects had neither a smoking history nor any visible oral lesions under careful examination. Additionally, they had not received any treatments for the oral lesions within 90 days prior to the specimen collection, and had no history, symptoms, or signs of systemic infections or other diseases.

Histopathological diagnosis was made by a single oral pathologist. This study was approved by the ethical committee of Baba Nidhan Singh Hospital, Punjab, India and informed consents were obtained from all subjects as per the Declaration of Helsinki.

2. Evaluation during study

For each sample, 1.0 mL of supernatant was used for the enzyme-linked immunoassay (ELISA) cytokine assay by using the human ELISA kit for TNF-α, IL-6, and IL-8 (R&D Systems Inc., Minneapolis, MN, USA), according to the manufacturer's instructions.

3. Salivary and serum cytokine measurements

Blood and serum samples were drawn between 9:00 and 10:00 AM from all subjects. Serum was separated from blood cells by centrifugation at 1,000 g for 5 minutes. The whole unstimulated saliva was collected between 9:00 and 10:00 AM. The subjects abstained from eating and drinking for at least 2 hours prior to the sampling. All subjects were requested to swallow first, tilt their head forward at more than 45°, and then expectorate saliva (10 mL) into a sterile centrifuge tube without swallowing for 4 to 5 minutes. The saliva was then centrifuged for 25 minutes at 3,500 g, and the clarified supernatants were separated into 2.0 mL aliquots. All samples were immediately frozen at -60°C for future use. Prior to assays, the serum or saliva supernatants were allowed

Table 1. Demographic characteristics of patients and controls

Variable	Oral leukoplakia	Oral lichen planus	Oral submucous fibrosis	Control
Classification	50 (benign hyperkeratosis: 7, mild dysplasia: 27, moderate dysplasia: 8, severe dysplasia: 8)	54 (benign hyperkeratosis: 6, mild dysplasia: 16, moderate dysplasia: 14, severe dysplasia: 18)	51 (benign hyperkeratosis: 1, mild dysplasia: 23, moderate dysplasia: 12, severe dysplasia: 15)	50
Age (yr)	42-65	41-65	41-65	42-65
Smokers (cigarettes/day)	18±4.6	21±6.7	18±4.5	21±7.9
Alcoholic status (g of alcohol/day)	657.9±159.2	593.1±155.6	585.9±186.2	569.9±158.8

Values are presented as range or mean±standard deviation.

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to thaw completely at room temperature for two hours. For each sample, 1.0 mL of supernatant was used for the ELISA cytokine assays, using the human ELISA kit for TNF- α , IL-6, and IL-8, according to the manufacturer's instructions. The absorbances of the samples at 492 nm for TNF- α , IL-6, and IL-8 were measured with a spectrophotometer (Sirio; Seac, Florence, Italy). A standard curve was organized by plotting the absorbance value of the standards versus corresponding concentrations. The concentrations of the cytokines in the sample were then determined by extrapolating from the standard curve. Tests were run in duplicate to check reliability. Protein contents were expressed in pg/mL. The inter-assay coefficient of variation was 3.0% to 4.5% and the intra-assay coefficient of variation was 2.5% to 4.2%.

Data normality was evaluated by Smirnof Kolmogorof's

test. The Kruskal Wallis test was utilized for comparisons between the groups. Mann-Whitney test for comparisons performed to detect any differences between the two groups. A $P < 0.05$ was considered as statistically significant. All data was statistically analyzed using the SPSS version 11.5 (SPSS Inc., Chicago, IL, USA).

III. Results

The demographic characteristics of patient and controls are mentioned in Table 1. The levels of serum and salivary TNF- α , IL-6, and IL-8 were statistically significantly higher in advanced stages of precancerous lesions as compared to early stages ($P < 0.05$). (Tables 2, 3) The levels of serum and salivary TNF- α , IL-6, and IL-8 were also statistically signifi-

Table 2. Serum levels of NF- κ B mediators in leukoplakia, lichen, submucous fibrosis patients and healthy controls

		TNF- α	IL-6	IL-8
Control		1.31 \pm 0.40	5.13 \pm 2.11	13.45 \pm 2.71
Benign hyperkeratosis	Leukoplakia	1.18 \pm 0.30	11.02 \pm 3.79	16.77 \pm 6.03
	Lichen-planus	2.19 \pm 0.88	12.95 \pm 1.67	22.16 \pm 4.54
	Submucous fibrosis	-	-	-
Mild dysplasia	Leukoplakia	2.22 \pm 0.44	13.38 \pm 4.16	19.49 \pm 6.82
	Lichen-planus	3.20 \pm 0.79	17.10 \pm 4.78	23.05 \pm 7.48
	Submucous fibrosis	4.12 \pm 0.66	17.76 \pm 3.63	26.35 \pm 5.82
Moderate dysplasia	Leukoplakia	3.22 \pm 0.53	16.15 \pm 5.18	20.22 \pm 6.06
	Lichen-planus	3.74 \pm 0.82	17.01 \pm 4.05	21.96 \pm 6.11
	Submucous fibrosis	5.18 \pm 0.46	18.90 \pm 4.91	24.42 \pm 6.33
Severe dysplasia	Leukoplakia	3.94 \pm 0.34	16.07 \pm 3.63	20.05 \pm 7.05
	Lichen-planus	4.87 \pm 0.80	18.79 \pm 4.02	23.00 \pm 5.90
	Submucous fibrosis	6.27 \pm 0.53	21.70 \pm 4.04	28.25 \pm 6.47

(NF- κ B: nuclear factor- κ B, TNF- α : tumor necrosis factor alpha, IL: interleukin)

All data except 'Control' are $P < 0.05$ as compared to control.

Values are presented as mean \pm standard deviation.

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Table 3. Salivary levels of NF- κ B mediators in leukoplakia, lichen, submucous fibrosis patients and control healthy

		TNF- α	IL-6	IL-8
Control		8.51 \pm 1.74	16.6 \pm 1.88	738.5 \pm 98.5
Benign hyperkeratosis	Leukoplakia	12.10 \pm 3.96	30.53 \pm 6.80	770.74 \pm 106.26
	Lichen-planus	13.63 \pm 2.04	26.32 \pm 4.72	1,071.02 \pm 82.61
	Submucous fibrosis	-	-	-
Mild dysplasia	Leukoplakia	14.38 \pm 4.27	35.30 \pm 7.52	849.58 \pm 128.76
	Lichen-planus	18.08 \pm 5.27	32.71 \pm 8.13	1,074.95 \pm 99.17
	Submucous fibrosis	18.41 \pm 3.51	34.45 \pm 6.72	1,384.10 \pm 93.30
Moderate dysplasia	Leukoplakia	18.25 \pm 6.50	39.80 \pm 10.69	930.61 \pm 120.74
	Lichen-planus	18.30 \pm 4.94	34.04 \pm 8.07	1,071.09 \pm 91.65
	Submucous fibrosis	20.37 \pm 5.12	35.92 \pm 8.32	1,347.18 \pm 95.92
Severe dysplasia	Leukoplakia	18.39 \pm 3.58	39.61 \pm 6.53	919.87 \pm 107.65
	Lichen-planus	21.46 \pm 5.51	36.06 \pm 8.52	1,116.28 \pm 118.17
	Submucous fibrosis	24.33 \pm 5.16	42.26 \pm 9.03	1,437.82 \pm 114.69

(NF- κ B: nuclear factor- κ B, TNF- α : tumor necrosis factor alpha, IL: interleukin)

All data except 'Control' are $P < 0.05$ as compared to control.

Values are presented as mean \pm standard deviation.

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Table 4. Correlations between salivary and serum biomarker estimates

Parameter	R			
	Leukoplakia	Lichen-planus	Submucous fibrosis	Control
Serum and salivary TNF- α	0.72	0.74	0.72	0.78
Serum and salivary IL-6	0.78	0.78	0.82	0.81
Serum and salivary IL-8	0.81	0.78	0.79	0.82

(TNF- α : tumor necrosis factor alpha, IL: interleukin)

All data except 'Control' are $P < 0.05$ as compared to control.

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cantly increased in oral leukoplakia, submucous fibrosis, and lichen-planus in contrast to normal healthy subjects. Serum and salivary correlation analysis revealed a strong and highly significant correlation for TNF- α , IL-6, and IL-8 levels in all groups as shown in Table 4.

IV. Discussion

Serum and salivary TNF- α , IL-6, and IL-8 were higher in advanced stages as compared to early stages of precancerous lesions and conditions, data which supports previous studies^{14,15-24}. TNF- α , IL-6, and IL-8 are potent angiogenic mediators with significant effects on tumor growth, and are associated with increased tumor vessel density and a worsened outcome¹⁵⁻¹⁸. Thus, these cytokines might act as surrogate biomarkers of angiogenesis and prognosis. It has been previously reported that excessive cell proliferation and activation of cellular actions can be instigated by chronic inflammation, which leads to the induction of irreversible DNA damage²⁵. TNF- α , IL-6, and IL-8 released through the inflammatory response would promote tumor growth, while tumor growth further stimulates the inflammatory response, resulting in cyclic progression²⁶. In the present study, serum and salivary TNF- α , IL-6, and IL-8 levels were upregulated in precancerous lesions and conditions. This finding is in line with most previous conducted work¹⁴⁻¹⁷, although one study has shown that salivary TNF- α and IL-6 were downregulated^{18,19}. This could be due to a small sample size and not using age- or gender-matched samples. Proinflammatory cytokine levels are elevated in submucous fibrosis due to immunoregulatory activity. Increased levels of NF- κ B mediators might be associated with the development of oral precancerous and cancerous lesions. In the normal cell, stimulation of cytokines causes growth inhibition, while in oral cancer cells, stimulation of cytokines leads to upregulation of positive cell cycle regulators including NF- κ B, signal transducers, and activators of transcription and the mitogen-activated protein kinase/extracellular signal-regulated pathway¹⁶. Upregulation

of TNF- α , IL-6, and IL-8 might be protective in action.

Smoking is a risk factor of oral precancerous and cancerous lesions². However, while smoking and chronic alcohol usage might be risk factors for cancer, this study showed no significant difference between controls and patients. TNF- α might act as an endogenous tumor promoter as well as an inducer of tissue remodeling required for tumor growth and spread²². IL-6 signaling has also been implicated in tumorigenesis²³. TNF- α , IL-6, and IL-8 were also elevated in periodontitis patients²¹, but in the present study, subjects with periodontitis were excluded. So, the present study shows that the elevation of TNF- α , IL-6, and IL-8 is correlated with precancerous and cancerous lesions. Indeed, elevation of these cytokines in saliva might indicate the potential precancerous development into oral cancer²². Alternatively, these cytokines may be improperly produced in precancerous lesions, which could lead to induced growth, invasion, disruption of tumor suppression, and immune status. NF- κ B activation leads to the upregulation of anti-apoptotic genes as a cell survival mechanism, by inducing physiological stress, which triggers an inflammatory response. In addition, NF- κ B induces cytokines that regulate TNF- α , IL-6, and IL-8, which leads to the recruitment of leukocytes to the sites of inflammation²⁷. There was good correlation between salivary and serum TNF- α , IL-6, and IL-8 in all groups. Since saliva can be easily collected, measurement of these biomarkers of diseases may prove useful in early detection of oral cancer risks. The salivary analysis for oral diagnosis may prove a cost effective method for screening large populations²⁴.

Hence, salivary levels of NF- κ B mediators were significantly elevated and may have diagnostic and prognostic utility as useful biomarkers for detection of precancerous and cancerous lesions. This study did not take into account factors such as diet, alcoholic history, and environmental factors, so further studies are required on large samples including these variables to determine the relationship between salivary biomarkers and oral cancer, and to further clarify their mechanism of action. It can only be concluded that patients with

oral precancerous lesions have elevated salivary and serum cytokines compared to healthy controls and that the levels of salivary cytokines increase with the severity of dysplasia.

V. Conclusion

Salivary and serum cytokines were also elevated when analyzed in oral precancerous lesions. Thus, salivary and serum IL-8, IL-6, and TNF- α levels might act as diagnostic markers for detection of oral precancer.

Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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