Original Article

Salivary and serum tumor necrosis factor alpha, interleukin 1 alpha, interleukin 1 beta, interleukin 6 and interleukin 8 in patients with oral carcinoma and leukoplakia

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Abstract

Objectives:

The aim of study was to compare salivary and serum concentrations of tumor necrosis factor alpha (TNF-a), interleukin 1 alpha (IL-la), interleukin 1 beta (IL-1b), interleukin 6 (IL-6) and interleukin 8 (IL-8) in patients with oral squamous cell carcinoma (OSCC), oral leukoplakia and healthy controls.

Materials and methods:

A total of 150 participants between the age group of 26 and 60 years were included in the study. They were divided into groups of 50 each of OSCC, oral leukoplakia and healthy controls. TNF-a, IL- l a, IL-lb, IL-6, and IL-8 concentrations were determined by the quantitative sandwich ELISA technique.

Results:

Salivary concentrations of TNF-a, IL-lb, IL-6, and IL-8 were significantly higher in OSCC patients than in patients with oral leukoplakia and the healthy controls ($p<0.05$). Although salivary concentrations of IL-la in OSCC patients was higher than in patients with oral leukoplakia and the healthy controls, but was statistically not significant ($p=0.613$). IL-lb levels in oral leukoplakia group was significantly ($p=0.041$) lower than the control group unlike the other cytokines, which though were higher as compared to the control group but were statistically not significant.

Conclusion:

Elevated levels of inflammatory cytokines were seen in the saliva of the patients with OSCC. Hence, the possible role of these cytokines to monitor the malignant transformation of oral leukoplakia needs further follow up studies involving larger sample sizes.

Keywords: Oral leukoplakia, oral squamous cell carcinoma, TNF-a, IL-la, IL-1b, IL-6, IL-8

Introduction:

Oral leukoplakia is defined as "white lesion of the oral mucosa which cannot be classified clinically or histologically as any other lesion and which is not associated with any physical or chemical causative agent except the use of tobacco"[1]. The dysplastic or malignant alteration in oral leukoplakia ranges from 15.6-39.2%, while a five year cumulative malignant transformation ranges from 0.13-17.5%. Gupta PC et al., and Silverman S et al., reported annual malignant transformation rates of 0.3% and 0.06% respectively in India, which was low in comparison to the studies undertaken by Schepman KP et al., who reported an annual malignant transformation rate of 8.9%[2-4]. Oral cancer though prevalent worldwide is more common in some developing countries such as India, Pakistan and some parts of France.[5] 90% of all malignant neoplasms of the oral cavity are OSCC. It is more common in older males with a history of tobacco and alcohol consumption. Various other factors that contribute

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to its occurrence include diet related factors, genetic factors and certain viruses[6].

The process of carcinogenesis involves multiple alterations of the genome that are progressively accumulated during a protracted period. During this progression, cellular and tissue level changes occur. These precancerous changes seen in the oral mucosa have relevant diagnostic and prognostic value. Sensitive and specific biomarkers are likely to be important in screening most high-risk head and neck cancer patients owing to the absence of specific early alarming signs and hence prevent the metastasis of the target tumors. The identification of such molecular markers in various body fluids would prove to be an effective diagnostic tool to predict the early development of cancer.[7] Various molecular markers have been used with varying degrees of specificity and sensitivity. Though no reliable or clinically acceptable marker has been shown to identify tumor aggressiveness till date.[8,9] Previous in vitro studies of human cell lines as well as squamous cell tumors have demonstrated increased concentrations of certain pro-inflammatory, pro-angiogenic cytokines such as tumor necrosis factor alpha (TNF-a), interleukin (IL)-1 a, IL-lb, IL-6, and IL-8. It has been shown that these cytokines produced in a dysregulated fashion in oropharyngeal SCC have roles in cell growth, invasion, tumor suppression, immune condition and affect survival as well[10]. Saliva as a diagnostic biofluid is advantageous over blood-based testing. It is a reservoir of analytes from systemic sources that reach the oral cavity through various pathways and hence acts as a reflection of the health of an individual. Steroids, amines and peptides, enter the saliva by passive diffusion. Secretory IgA and enzymes such as amylase and lysozyme can be analysed in the saliva along with various drugs that can be measured as part of therapeutic drug monitoring and drug abuse. Thus, the composition of saliva reflects the levels of hormonal, immunological, toxicological and infectious disease markers.[11] Direct contact to the oral cavity, and the relationship between oral fluid and blood levels, make saliva a useful and promising diagnostic aid for identifying potential biomarkers for oral squamous cell carcinoma. As a biofluid, it provides a perfect medium to observe the course of a disease and treatment outcome through noninvasive means[12]. The aim of this study was to compare salivary concentrations of TNF-a, IL-la, IL-lb, IL-6, and IL-8 in patients with oral leukoplakia, OSCC and healthy controls. The role of these cytokines in OSCC if proved by future research may assist as an easy diagnostic test and a prognostic indicator for patients undergoing treatment and opening up new avenues in research of newer treatment modalities for OSCC.

Material and Methods

A total of 150 participants between the age group of 26 and 60 years, from the Department of Oral Medicine and Radiology, Jodhpur Dental College and General Hospital, Jodhpur, India were enrolled in this study. The study sample consisted of 3 groups: Group I-OSCC patients (n=50); Group II-oral leukoplakia patients (n=50); and Group III-age- and sex-matched healthy individuals enrolled as controls (n=50). Oral examination of all subjects was performed thoroughly in a well illuminated dental chair. Diagnosis of the patients in Group I and Group II was confirmed histopathologically as well to poorly differentiated SCC for Group I and mild to severe dysplasia for Group II respectively. The procedure of the study was explained to all participants and written informed consent was obtained from them. Approval was obtained from the Institutional Ethics Committee. Community Periodontal Index of Treatment Needs was measured in each patient after the collection of saliva to assess the impact of periodontal disease on levels of salivary cytokines[13].

Exclusion criteria

- Patients with acute and chronic inflammatory diseases
- Patients on medications that cause hyposalivation or hypersalivation
- Infectious diseases of the oral cavity
- History of any other systemic disorder that might influence the levels of salivary and/or serum cytokines
- Chemotherapy or radiotherapy patients

None of the individuals in the control group had any pathology in the oral cavity. Individuals were asked not to eat, drink, chew gum or mint, etc., for at least an hour prior to the evaluation. They were requested to swallow first, tilt the head forwards and expectorate their saliva into calibrated tubes. Samples were frozen and kept at -70 degree C until the biochemical analysis was done. TNF-a, IL-la, IL-lb, IL-6, and IL-8 concentrations were determined by the quantitative sandwich ELISA technique as previously described by Ondrey using commercially available kits (Bender MedSystems, Vienna, Austria) as per the manufacturer's recommendation[14].

Statistical analysis

The statistical package for social sciences (Version 13, SPSS INC) software was used to analyze the data. Normality of distribution was assessed by Smirnov Kolmogorof's test. Mann Whitney test for comparisons between two groups was performed. Values lower than 0.05 ($p<0.05$) were considered as statistically significant. The results were expressed as the mean±standard deviation.

Results

The demographic data of participants was as shown in Table 1. The mean age of OSCC group was 53.74±8.29 years which was slightly higher than oral leukoplakia (51.43±7.86 years) and control group (52.22±7.53 years). Concentrations of salivary TNF-a, IL-la, IL-lb, IL-6, and IL-8 were not affected by periodontal health. No significant differences in periodontal health, assessed by CPITN, were observed between the groups.

Salivary TNF-a levels

Salivary TNF-a concentration for the OSCC group was 112.68±42.63 pg/ml, whereas for oral leukoplakia group it was 28±6.37 pg/ml and 21.56±53.61 pg/ml for control subjects (Table 2). The difference between OSCC and oral leukoplakia was found to be statistically significant (p=0.027). Similarly the difference between OSCC and controls was also statistically significant (p=0.018). The mean salivary TNF-a concentration for the oral leukoplakia group was found to be 28±6.37pg/ml, though the levels where higher than that of the controls (21.56±53.61 pg/ml), this difference was statistically non-significant (p=0.243).

Salivary IL-la levels

Salivary IL-la concentration for the OSCC group was 221.4±154.6pg/ml, whereas for oral leukoplakia group it was 186.2±253.1 pg/ml and 162.6±156.4 pg/ml for control subjects (Table 2). The difference between OSCC and oral leukoplakia was found to be statistically.non-significant (p=0.613), though the values of OSCC group were higher when compared with oral leukoplakia group. Similarly the difference between OSCC and controls was also statistically non-significant (p=0.427). The mean salivary IL-la concentration for the oral leukoplakia group was found to be 186.2±253.1pg/ml, though the levels where higher than that of the controls (162.6±156.4 pg/ml), this difference was statistically non-significant (p=0.829).

Salivary IL-lb levels

Salivary IL-lb concentration for the OSCC group was 898±41.43 pg/ml, whereas for oral leukoplakia group it was 131±22.41 pg/ml and 325±52.12 pg/ml for control subjects (Table 2). The difference between OSCC and oral leukoplakia was found to be statistically significant (p=0.012). Similarly the difference between OSCC and controls was also statistically significant (p=0.027). The mean salivary IL-lb concentration for the oral leukoplakia group was found to be 131±22.41pg/ml, though the levels were lower than that of the controls (325±52.12 pg/ml), this difference was also statistically significant (p=0.041).

Salivary IL-6 levels

Salivary IL-6 levels for the OSCC group was 79.6±53.31pg/ml, whereas for oral leukoplakia group it was 39±12.26 pg/ml and 14±2.61pg/m1 for control subjects (Table 2). The difference between OSCC and oral leukoplakia was found to be statistically significant (p=0.035). Similarly the difference between OSCC and controls was also statistically significant (p=0.013). The mean salivary IL-6 concentration for the oral leukoplakia group was found to be 39±12.26 pg/ml, though the levels were lower than that of the controls (14±2.61 pg/ml), this difference was statistically non-significant (p=0.728).

Salivary IL-8 levels

Salivary IL-8 concentration for the OSCC group was 1,278.31±451.16 pg/ml, whereas for oral leukoplakia group it was 268.31±158.32 pg/ml and 236.05±133.21 pg/ml for control subjects (Table 2). The difference between OSCC and oral leukoplakia was found to be statistically significant (p<0.0001). Similarly the difference between OSCC and controls was also statistically significant (p<0.0001). The mean salivary IL-8 concentration for the oral leukoplakia group was found to be 268.31±158.32 pg/ml, though the levels where higher than that of the controls (236.05±133.21 pg/ml), this difference was statistically non-significant (p=0.689).

Discussion

Chronic inflammation and carcinogenesis are closely related, and it has been shown that chemokines play a variety of roles in the development and progression of human cancer. A vast array of these molecular markers has been associated with the complex events of carcinogenesis and cancer progression. Some of the proposed markers are frequently debated and results often seem to contradict each other, due to the small

number of individuals included in each study or the heterogeneity of selected individuals, which may differ in various features, notably tumor location[15]. The development of oral cancer has been shown to be closely associated with the altered cytokine response. In oral cancer, cell stimulation with pro-inflammatory cytokines leads to up regulation of positive cell cycle regulators such as nuclear factor kappa B (NF-kB), signal transducer and activator of transcription (STAT) and mitogen-activated protein kinase/extracellular signal-regulated (ERK) pathway. Upregulation of these factors in place of growth inhibition, promotes cell survival and proliferation[16].

Results of this study show that patients with oral cancer have highly significant salivary TNF-a, IL-lb, IL-6, and IL-8 concentrations compared to patients with leukoplakia and healthy controls, with the exception for IL- 1 a concentrations, which though higher in OSCC patients was statistically not significant (p=0.613). Increased concentration of salivary cytokines in OSCC patients has also been reported by various authors. Significantly a higher salivary IL-lb concentration was reported by Arellano-Garcia et al., in patients with oral cancer when compared to healthy controls[17]. Rhodus et al., also reported similar results of higher significant concentrations of salivary IL-6 and TNF-a in oral cancer patients and in patients with oral precancerous lesions[10,18].

They showed that TNF-a, IL-la, IL-6, and IL-8 were elevated in the whole unstimulated saliva of subjects with OSCC (n=13) compared with oral premalignant lesions (n=13) and controls (n=13). It may be argued that higher concentrations of salivary cytokines be a result of a lesion with epithelial discontinuity and surrounding inflammation, and not directly related to cancer.

The findings of Saheb Jamee et al., reported significantly higher salivary IL-6 concentrations in oral cancer patients compared to healthy controls[19]. They reported no significant differences in salivary TNF-a between the two groups. These findings were not in accordance with the present study. The present study however reports a highly significant difference in TNF-a concentrations (p=0.027) in oral cancer patients and healthy controls. Brailo et al., reported lower salivary TNF-a concentration as in oral cancer patients which were insignificant, as opposed to the findings of the present study[20]. The serum concentrations reported were however significant. These conflicting results may be attributable to the differences in ELISA technique, the population being studied and the fact that these studies involved a smaller sample size compared with the present study.

No significant differences in salivary IL-6 were found in the studies by St. John et al., in oral cancer patients when compared to healthy controls.[8] There was a statistically significant difference in salivary IL-8 levels for OSCC patients compared with both precancer patients and controls (p<0.0001) in a study carried out by Punyani et a1[15]. Vucicevi Boras et al., reported significantly higher salivary IL-6 concentrations in oral cancer patients[22]. In contrast to the heterogeneity of the results suggested by different studies, it can be concluded that altered cytokine production is seen in patients with OSCC. Altered levels of various cytokines have been reported in patients with oral premalignant lesions, such as oral lichen planus, oral submucous fibrosis and oral leukoplakia[20,22,23] The role of salivary cytokines in leukoplakia has not been investigated previously, and there has been only one published report, by Rhodus et al., regarding its role in leukoplakia[10]. Though the levels were higher compared with the control group except for IL-lb, this difference was found to be statistically non-significant (p<0.005) in this study. The levels of IL-lb were significantly lower in the patients with oral leukoplakia (131±22.41 pg/ml) as compared to the

Table 1: Demographic characteristics of the participants

	Age (mean)	Males	Females	CPITN
Oral Cancer	53.74	40	10	2.36
Leukoplakia	51.43	40	10	1.97
Controls	52.22	40	10	2.12

CPITN- Community Periodontal Index of Treatment Needs

Table 2: Salivary TNF-a, IL-la, IL-lb, IL-6, and IL-8 levels in patients with OSCC, oral leukoplakia and healthy controls (pg/ml)

	TNF- α (pg/ml) (mean ± SD)	IL-1α (pg/ml) (mean ± SD)	IL-1β (pg/ml) (mean ± SD)	IL-6 (pg/ml) (mean ± SD)	IL-8(pg/ml) (mean + SD)
OSCC	112.68±42.63	221.4±154.6	898±41.43	79.6±53.31	1,278.31±451.16
Leukoplakia	28±6.37	186.2±253.1	131±22.41	39±12.26	268.31±158.32
Controls	21.56±53.61	162.6±156.4	325±52.12	14±2.61	236.05±133.21

control group (325±52.12 pg/ml) in the present study. This finding was not in accordance with the findings of Rhodus et al., where statistically significant difference was observed in the concentrations of TNF-a, IL-la, IL-6, and IL-8. This difference amongst the two studies could be due to the difference in the histopathology pattern of the leukoplakia tissue. All the precancerous lesions studied by Rhodus et al., showed moderate or severe dysplasia. They analyzed and compared the levels of TNF-a, IL-1 a, IL-6, and IL-8 in whole unstimulated saliva among oral lichen planus patients, often seen with epithelial discontinuity; individuals of oral squamous cell carcinoma and healthy controls. The periodontal health of the groups in this study showed no differences.

Saliva as a diagnostic tool has been in use since ancient cultures, where it was considered as a part of the circulation, and changes in certain aspects of patients' health could be assessed by changes in the saliva[12]. This ability to use saliva to diagnose and assess a patient's health and disease states is considered to be a highly attainable goal in the field of health care research and health promotion. Measurement of the concentration of drugs, hormones, antibodies and other molecules can be done. Therapeutic drugs like theophylline, lithium, methadone and cyclosporine as well as abusive drugs such as alcohol, cocaine, opiates and methamphetamines can also be monitored. All natural steroid hormones of significance such as estrogens, testosterone, progesterone, cortisol, DHEA and melatonin can also be monitored in saliva[15]. Informative analytes generally are present in lower amounts in saliva than in serum. However, this is no longer a limitation with the advent of new and highly sensitive techniques[24]. The diagnostic methods for saliva are inexpensive, non-invasive and easy-to-use, thus making it a preferred clinical tool over serum. For patients, the non-invasive collection techniques reduces anxiety and discomfort and simplifies the procurement of repeated samples for monitoring over a period of time. It does not clot, thus lessening the manipulations required while handling[25]. The present study showed that TNF-a, IL-la, IL-lb, IL-6, and IL-8 were increased in concentrations in the saliva of subjects with oral cancer, suggesting a possibility towards their use as potential biomarkers for OSCC. Though the levels were higher in the leukoplakia group, further studies with larger sample size are needed to accept or reject the utility of these cytokines in predicting precancerous lesions.

Conclusion

Increase in the salivary levels of pro-inflammatory cytokines might reflect the transition of OSCC from oral leukoplakia. Does this increase in the concentrations of salivary cytokines takes place before carcinoma? This however becomes clinically evident and its application for monitoring the malignant transformation to OSCC remains to be answered by further follow up studies, as cross-sectional studies fail to provide the answer. Similar sample sizes with a broader representation of disease sites and stage, as well as prospective studies of treated patient populations, will be needed to confirm the results of this study with respect to direct protein analysis using ELISA.

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