Salivary Biomarker IL-8 Levels in Smokers and NonSmokers: A Comparative Study

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Abstract

Objective   It is estimated that the mortality rate from tobacco-related diseases will reach 10 million worldwide by 2030. It is validated that every three out of four oral cancers are caused by the use of tobacco in various forms especially smoking. Early detection is the only way to reduce this burden. Molecular-level analysis has currently become a valuable tool in the diagnosis and prognosis of diseases. Around 1000 different salivary protein biomarkers are being investigated in saliva for this purpose. Some of these markers are being investigated to evaluate the proportionality of cigarette smoking. The objective of this study was to evaluate the levels of salivary biomarker interleukin-8 (IL-8) among smokers and nonsmokers as a control group.

Materials and Methods   This is a comparative cross-sectional study conducted in Islamabad Dental Hospital, Bhara Kahu. A total of 60 patients were recruited and divided into two equal groups of smokers and nonsmokers. Unstimulated saliva samples were collected and analyzed using an enzyme-linked immunosorbent assay kit.

Statistical Analysis   The results were then analyzed by SPSS v25 using an independent sample t-test to evaluate the statistical difference and significance.

Results   A p-value (<0.001) was found to be significant for the IL-8 levels in smokers when compared with nonsmokers. The mean value for smokers was found to be 122.69 pg/mL and the mean for nonsmokers was evaluated to be 20.68 pg/mL.

Conclusion   As the levels of IL-8 were high, it was concluded to be an effective biomarker for the evaluation of smoking-based initial inflammatory changes detectable from saliva.

Keywords
► saliva
► smoking
► IL-8
► proteomic

Introduction

Molecular diagnostics have currently gained a lot of attention. Different proteomic tools have been designed to analyze the body fluids like blood, saliva, serum, and gingival crevicular fluids. These human fluids contain certain biomarkers that support in the diagnosis process. Among these biomarkers, interleukin-8 (IL-8) is the fittest as it is associated with numerous local and systemic illnesses, for example, periodontitis, diabetes, and heart disease.[1]

Human saliva can be used to obtain a wealth of information.[2] Saliva serves as a mirror, reflecting the healthy or diseased state of the human body. Oral and dental diseases, pharyngeal, esophageal, inflammatory, pre-neoplastic, neoplastic, autoimmune, and systemic diseases could be easily affiliated with a simple alteration of saliva pH, its flow rate or any other property. As it is a physiologic diagnostic medium, it has gained significant attention for its potential use as a biological fluid that can clinically be used for diagnosis.[3] Among the various diseases that can be diagnosed by salivary biomarkers, premalignant lesions and oral squamous cell carcinoma (OSCC) are being the most investigated.[4,5] Moreover, in the leu of ongoing coronavirus disease 2019 pandemic, saliva is also proving to be a promising noninvasive technique for its diagnosis and detection as salivary glands are hosting severe acute respiratory syndrome-coronavirus-2.[6,7]

Globally, nicotine has gained the fourth position among the psychostimulants. When smoked, tobacco may produce over 60 different types of carcinogens and free radicals that primarily result in mouth cancer.[8] In Pakistan in 2017, 163,360 people died due to tobacco use.[9] A steady but noticeable increase in smokers has been recorded in developing countries. The more the cigarettes consumed over increased number of years, the higher the risk of oral cancer. The human saliva contains certain biomolecules that maintain the homeostasis of the oral cavity.[10] According to a study, the levels of different proteins and biomarkers are increased in response to nicotinic exposure suggesting that the inflammatory process is responsible for the pathogenesis of premalignant lesions. This theory can be put into perspective knowing smoking adversely affects the oral cavity by altering free radicals and volatile aldehydes in saliva that further lead to the start of the damaging effects in the oral cavity.[11] Early detection and diagnosis of cancer are essential to decrease the rising burden of OSCC. In the past decade, saliva has become one of the popular diagnostic mediums because of its direct exposure to premalignant and carcinomatous lesions. This could prove not only to be a diagnostic medium but due to early detection, also a tool for prognosis of a lesion. Salivaomics is used to analyze the specific biomarkers (proteins, DNA, messenger RNA, microRNA, microbes, and metabolites) which is present in saliva through various tests conducted solely on saliva.[12] Among the group of cytokines, the following have been most studied: epidermal growth factor, IL-6, IL-8, vascular endothelial growth factor, IL-4, IL-10, endothelia and tumor necrosis factor-alpha.[13,14] They have been used to assess local diseases like dental caries, periodontitis, OSCC, in addition to systemic conditions like different carcinomas, Sjogren disease, and rheumatoid arthritis. In a study by Khan et al, differential expression of salivary proteins from 33 dental caries patients was compared with 10 control subjects and protein alterations were detectable making saliva a good indicative tool for dental caries.[15,16]

The biomarkers associated with oral malignant or benign lesions have specific values in the saliva because of their close contact with the lesion. They may also assist in the prediction, prognosis, and reoccurrences of carcinomatous lesions.[17] The presence of IL-8 on cancer cells suggests its prime role in tumorigenesis.[18] In malignancies, IL-8 is elevated and causes tumor angiogenesis, tumor growth, cancer cell migration, and cell proliferation. In a normal healthy mucosal tissue, very low levels of IL-8 are detected as compared with its response to inflammatory, infectious, or cellular stresses. The level of IL-8 may raise from 10-fold to 100-folds.[19] Some researchers have gone a step ahead and declared 600 pg/mL to be the cutoff value in OSCC. Researchers have claimed that it is one of the biomarkers that holds great promise as a single biomarker for the detection of OSCC.[20] According to the research network, early detection has a five-phase validation process. Currently, IL-8 has surpassed the third phase with fruitful results.[21] But unfortunately, to date, no single marker has been universally accepted to identify OSCC.[22]

As it is an established fact that the use of tobacco is the prime agent involved in carcinogenesis, it is to be analyzed if the deranged values of the cytokines can be detected at earlier stages before the formation of premalignant or carcinomatous lesions. The toxins produced by smoking may take time for the formation of an established lesion in the oral cavity. If regular and thorough examinations are performed, the chances of diagnosing the condition can be increased at an earlier stage. For this, a thorough study involving only smokers without any disease was considered. If the levels of IL-8 cytokine are deranged by smoking, it proves to be an effective modality for warning the patient and the clinician for further adverse consequences to follow. Therefore, in this study, the prime aim was to evaluate the relationship between cigarette smoking and the levels of salivary biomarker IL-8, predicting the development of any suspicious lesion in this high-risk group.

Materials and Methods

This was a comparative cross-sectional study conducted in Islamabad Dental Hospital, Bhara Kahu over a period of 1 year, from January 2017 to January 2018. Ethical approval was taken from Ethical Review Board at Islamabad Dental Hospital before the initiation of data collection. A control group comprising of nonsmokers and an experimental group comprising of smokers was included. Each group containing 30 individuals. Nonprobability, convenience sampling technique was used. Both genders between the ages of 18 and 40 years were included. The criteria for inclusion were that patients should be smoking for a minimum of 5 years and

consuming up to 20 cigarettes or more per day. Age and gender match controls were also included with no history of smoking or use of tobacco in any form. Coexisting diseases including periodontal disease, diabetes, dermal, rheumatic, coronary heart disease, cancerous, dysplastic or any acute viral condition, immunodeficiency, patients with the insufficient salivary flow, history of consumption of alcohol or use of tobacco in any other form (snuff, paan, naswar), and previous and /or ongoing radiotherapy, chemotherapy were excluded from the study.

After taking informed consent, a detailed medical history and an essential periodontal examination were conducted to remove any cofounding factors. Periodontal assessment was conducted by using Community Periodontal Index of Treatment Needs (CPITN) probe and individuals with no pocketing and no bleeding on probing were included in the study to remove the bias of compromised periodontal heath. Since patients with good oral hygiene were included in the study their plaque index (PI), Gingival index (GI), pocket depth (PD), bleeding on probing (BoP) and clinical attachment level (CAL) were in normal range. A thorough examination of the oral cavity was also performed to rule out any existing disease state. IL-8 is found to be higher in saliva making its detection more effective, easy and without cumbersome invasive tests.[23] Hence a morning sample of unstimulated whole saliva was collected by the saliva collecting device according to the manufacturer's instructions (Pure•SAL, Oasis Diagnostics Corporation, Vancouver, United States).[10] Collected samples were tested for levels of IL-8 cytokine by enzyme-linked immunosorbent assay (ELISA) procedure according to the manufacturer's instructions (PicoKine, Boster Biological Technology, Pleasanton, United States).

The data collected was entered into Statistical Package for Social Sciences (SPSS) version 25 and was analyzed accordingly. The bio-data was used to analyze the mean and standard from the numerical data of age. The salivary concentration of IL-8 was presented as a mean and standard error. The values of IL-8 were then compared with the control group by using an independent t-test. The value of p-value less than or equal to 0.05 was considered to be statistically significant at a 95% confidence interval.

Results

Sixty subjects were recruited for this study. Out of these, 30 were smokers ($n = 30$) and 30 subjects who were age and periodontally healthy were recruited for controls ($n = 30$). Only males were willing to participate in the study. Because of cultural limitations females did not reveal their actual smoking status or they refused to take part in the study. The mean age of smokers noted was 29.17 ± 6.50, while that of nonsmokers was 29.20 ± 6.27 years. Statistics of age have been shown in ►**Table 1**.

The mean value of IL-8 in smokers noted was 122.69 ± 52.39 pg/mL. However, the mean value of nonsmokers of IL-8 levels noted was 20.68 ± 8.80 pg/mL as shown in ►**Table 2**. An independent t-test was then applied to compare the IL-8 levels between smokers and nonsmokers.

Table 1 Age statistics of both smokers and nonsmokers

Age (y)	
Mean	29.18
Standard deviation	6.33
Range	22
Minimum	18
Maximum	40

Table 2 Interleukin-8 levels statistics for both smokers and nonsmokers

Subjects	Smokers $n = 30$ (pg/mL)	Nonsmokers $n = 30$ (pg/mL)	p-Value (independent t-test)
Mean	122.6980	20.68	0.001
Standard deviation	52.3953	8.80	
Range	208.62	39.01	
Minimum	20.74	0.90	
Maximum	229.36	39.91	

A statistical difference was noted in the levels of IL-8 with a p-value less than 0.0001 ($p < 0.0001$). There was an increase in salivary IL-8 levels in smokers when the values were compared with the nonsmokers. The correlation for the IL-8 levels in smokers and nonsmokers has been shown in ►**Fig. 1**.

Discussion

IL-8 is regarded as the prototype of the cytokine family. Its production is induced by environmental stresses, chemicals, steroids, and inflammatory signals. The relationship between IL-8 and cancer has been much investigated with statistically positive values.[18] The proinflammatory nature of IL-8 leads to growth inhibition in normal cells but in cancerous cells, it promotes cell division, invasion and alter tumor suppression by interfering in the nuclear factor- κB pathway.[24]

In carcinomatous lesions, the values of IL-8 are well beyond 10-fold as compared with the control group that outweigh the assumptive inflammatory condition, if present.[25] A study, in which a comparison between oral lichen planus, oral leukoplakia, OSCC and controls was done, demonstrated statistically higher levels of IL-8 among the OSCC group as compared with others.[26] In another study, the researchers concluded that tobacco in any form would cause oxidative stresses within the cells. This would cause the proinflammatory cycles to produce raised levels of cytokines. Malondialdehyde (MDA) is an indicator of oxidative stress within a cellular structure. MDA and the number of cigarettes consumed per day were found in correlation. Hence, smoking induces oxidative stresses within the cells that further increase the levels of MDA.[27] These results were in accordance with the results obtained from our study as in our
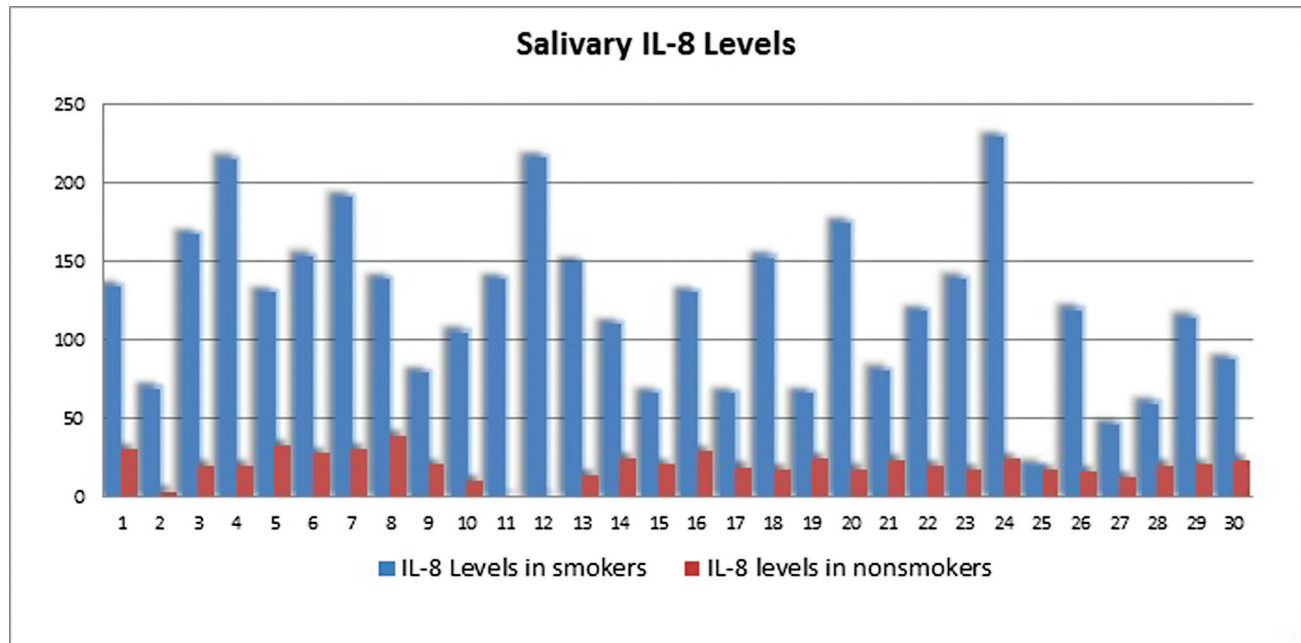
Fig. 1 Correlation of interleukin-8 (IL-8) levels in smokers and non-smokers.

study the levels of IL-8 were raised in smokers by an oxidative stress induced along with proinflammatory changes, indicating a significant increase in the levels of IL-8 among smokers as compared with nonsmokers (smokers= 122.69 pg/mL and nonsmokers= 20.68 pg/mL).

IL-8 is released from the alveolar macrophages in smokers and nonsmokers. An old study assessed the concentrations of IL-8 from the bronchial lavage and found the levels to be much lower in smokers as compared with nonsmokers (46.8 ± 12.7 pg/mL vs. 124.1 ± 24 pg/ml) with the *p*-value being significantly indicating that cigarette smoking decreases IL-8 secretion in human alveolar macrophages.[28] These results were in contrast to the results obtained in our study. However, a few years later Koch et al worked on the release of IL-8 in human alveolar macrophages. The results found were in accordance with our study as a significant raise in IL-8 levels was noted in smokers' bronchial lavage fluid.[29]

A noninterventional study assessed three different groups: group A including patients with precancerous conditions, group B including the biopsy-proven but untreated cases of oral carcinoma, and group C including the age-matched healthy controls. The saliva of each participant was collected using the drool technique. ELISA was used to detect the levels of IL-8 and IL-6. In group A, IL-8 was detected nearly 75% of the cases; in group B it was detected in 95% of the cases, whereas in the control group only 10% of the patients were detected with IL-8. The levels of IL-8 were consistently raised in both groups A and B with significant *p*-value.[30] Our study is kind of a prequal study to the above-mentioned study indicating that the levels of IL-8 in patients who smoked more than 20 cigarettes a day for 5 years or more were found to be elevated compared with healthy individuals with no history of smoking. Smoking is a risk factor for developing precancerous and cancerous lesions.

However, the subjects of our study may or may not develop any suspicious lesions in future. Levels of IL-8 may play a role in predicting the development of any suspicious lesion in such high-risk subjects, but a longitudinal study would provide a better verdict on the role of IL-8 in this kind of health prediction.

A local study was performed to evaluate the levels of IL-8 and IL-6. A total of 105 subjects were recruited and divided into three groups: group A: potentially malignant lesion, group B: histologically proven OSCC, and group C being healthy controls. The salivary sample was collected by drooling method. ELISA was used to assess the levels of IL-8 and IL-6. Statistically raised levels of IL-8 were recorded in the patients diagnosed with OSCC when compared with other groups. However, the levels were raised (873.6 pg/mL) beyond the levels noted in our study (20.74–229.36 pg/mL).[31] The increased levels of IL-8 in our study can be linked to the harmful habits of the selected participants as smoking induces inflammatory changes that are proven to be detectable via biomarkers.

St John et al of researchers worked on finding a biomarker namely IL-6 and IL-8 in the oral cavity and oropharyngeal squamous cell carcinoma.[32] Both salivary and serum samples were taken from patients newly diagnosed with T1 or T2 stage cancer. Age and sex-matched controls were also included. ELISA and polymerase chain reaction (PCR) both were used for analysis. IL-8 was detected at higher levels in saliva as compared with the serum that was further confirmed by PCR of mRNA and protein levels (sensitivity 99% and specificity 90%). The highlight of this research was that they correlated the levels of IL-8 with age, sex, alcohol, and tobacco use. To their astonishment, the levels found were not related to any of these variables as concluded by the researchers. These results are not in accordance with the present study's results.

Similar to the present study, another local study demonstrated the increase in levels of salivary IL-8 in the naswar users to be 173.48 ± 46.52 pg/mL, while the nonnaswar users showed the IL-8 levels to be 33.39 ± 22.44 pg/mL, thus predicting the development of any suspicious lesion in high-risk population.[33] For a biomarker to be accepted in terms of identifying a diseased state, it needs to pass through a validation process. IL-8 although has achieved good results, but unfortunately hasn't reached the end stage due to fewer studies conducted.[21]

Certain limitations of the study exist. First, this study was performed on a small scale with a limited number of patients. For a definitive conclusion, larger-scale analysis nationally and internationally should be conducted. Second, this is a cross-sectional study with the participants being involved only once; hence, the aftermath of smoking at regular intervals was not assessed. For better results, a longitudinal study should be considered. Moreover, details regarding smoking habit were not recorded except for the inclusion criteria devised stating that the subjects should be smoking for a minimum of 5 years and consuming up to 20 cigarettes or more per day.

Conclusion

The levels of IL-8 between smokers and nonsmokers were found to have a statistically significant difference ($p = 0.001$) with higher levels noted for smoking. This research validated IL-8 to be a valuable tool as not only a biomarker for precancerous and cancerous lesions but also indicative of adverse changes within the oral mucosa caused by excessive smoking that are not yet visible to the naked eye.

Conflict of Interest
None declared.

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