Detection of interleukins-6 and 8 in saliva as potential biomarkers of oral pre-malignant lesion and oral carcinoma: A breakthrough in salivary diagnostics in Pakistan

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Abstract: Oral cancer is at rise in our population due to increasing use of areca nut (Betel nut) with or without tobacco. It is the second frequent malignant tumour for both the gender in Pakistan. This non-interventional case control study was carried out with the aim to explore saliva as diagnostic medium for detecting interleukins (IL) 6 and 8 as biomarkers of pre-malignant lesions (PML) and oral carcinoma. Total 105 subjects were recruited and were divided into three groups "A", "B" and "C" each comprising of 35 subjects. Group "A" comprised of cases with strong clinical evidence of oral PML. Group "B" constitute clinical and histologically proven OSCC and group "C" include disease free subjects as controls. Saliva from all the recruited subjects was procured by drooling method and stored at-20^{0}C before further process. All the collected samples were centrifuged at 4500 rpm for 15 minutes at 4^{o}C. Supernatant fluid was used in ELISA for detection and quantification of IL-6 & IL-8. Data was analysed by using Chi-square test and multivariate analysis was done by non-parametric test. P-value of 0.05 was taken as standard reference. Significant co-relation was found for qualitative salivary detection of IL-6 and IL-8 among the groups (P<0.001 and <0.0001 respectively). Regarding quantitative salivary concentration of leukotrienes, no significant co-relation was found in levels of IL-6 among the groups while there was significant association of IL-8 levels between the groups (P<0.0001).On post Hoc multiple comparison, significant co-relation was found among oral PML group and controls (P=0.001) and OSCC group and control (P=<0.0001). In conclusion salivary detection of IL-6 & IL-8 could be used as probable biomarker for early detection of oral PML & OSCC in etiologically distinct population of Pakistan.

Keywords: Oral pre-malignant lesions, oral squamous cell carcinoma, biomarkers, salivary diagnosis, interleukins.

INTRODUCTION

Carcinoma of oral cavity is the sixth most frequently occurring malignancy all over the globe. (Jemel A. 2010). It is a major health concerns in developing countries as it is the leading cause of death. (Shrime 2010, Ferlay, 2010, Kao 2009). It accounts for one-third of the world burden of oral cancer from Indian subcontinent and is one of the major concerns as it has a rising trend in younger population (Shah 2011, Subramanian 2009, Warna Kulassuriya 2009). Oral carcinoma is the second most frequently reported malignant tumour for both genders in Pakistan (Bhurgri 1998). It is at rise in our population resulted in highest reported incidence worldwide in 2005 (Bhurgri 2005). It constitutes 20-35% of all cancer seen in various public hospitals in Karachi and slightly less in other regions of Pakistan. Alarming cases are being consistently reported in younger age groups. Never the less it is a major cause of mortality in our population.

More than 95% of oral cancers are squamous cell carcinoma (SCC). Significant proportion of it developed or precedes from oral PML such as leukoplakia, erythroplakia, oral submucosal fibrosis (OSF) and Lichen Planus (Mehrotra 2006, Trivedi 2012).

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Cumulative effects of nutritional deficiency, dietary habits, bad oral hygiene, mal-directed sharp, gagged teeth and infection with human papilloma virus (HPV) has been implicated for contributing in oral carcinogenesis (Gillson 2001, Mark 2001). In Pakistan, the major risk factors for oral cancer are areca nut (betel nut, chalia, supari), betel quid (paan), tobacco chewing, naswar, paan masala (ghutka, mawa) and poor nutrition. Due to religious prohibition, alcohol consumption is not a prevalent habit in Karachi, therefore, not a major risk factor.

Variety of cytokines such as Tumor necrotic factor-alpha (TNF a), interleukin (IL-6 and IL-8) are found rose in OSCC and has important role in carcinogenesis. There are evidences that these cytokines are produced in a dysregulated manner in oral squamous cell carcinoma, having important roles in tumorogenesis, cell growth initiation, invasion, disturbance of tumour suppression, immunity and even survival (Rhodus 2005, Jamee 2008).

Diagnosis and subsequent therapeutic intervention of oral PMLs & OSCC is currently based on clinical examination, location, stage of the disease and histopathological features. Although there is easy accessibility for the direct examination of oral cavity, even then these malignancies are often detected at late stage and the survival rate for oral cancers has remained

unchanged over the past couple of decades. Early detection can improve patient survival and diminishes the morbidity of treatment required for advance disease. Early diagnosis is perhaps a major factor contributing towards the outcome of treatment for this deadly disease.

Microscopic examination of the tissue remains the gold standard for diagnosis and identification of malignant oral lesions. Taking tissue for histopathology is an invasive procedure with many technique limitations for professionals and psychological discomfort for most patients. In recent decades there is paradigm shift from histopathology to molecular methods of diagnosis.

Recently saliva has been used as prospective source in molecular diagnostics by analysing microbiotomics, genomics, proteomics and salivary transcriptomes. Oral fluid (saliva) meets the demand for non-invasive, easily accessible bio-fluid of human body that nurtures a wide spectrum of biological analytes, informative for clinical diagnostic application and provides highly efficient diagnostic medium (Li 2004, Epstein 2002).

In Pakistan, saliva has never been explored as a diagnostic medium to detect biomarkers for oral PMLs and oral cancer. As genetic alteration can be successfully identified in bodily fluid draining the organ affected by the tumour, with this concept in mind, we attempted to investigate whether the ability to analyse saliva for potential biomarkers would be feasible to diagnose PMLs and OSCC in our etiologically distinct population.

We estimated cytokines IL-6 and IL-8 levels in saliva by ELISA technique. In this study, we analysed the application of saliva as a diagnostic fluid for the possibility of early detection of OSCC by these biomarkers.

MATERIALS AND METHODS

Subjects & methods

This non-interventional, case control study was carried out at department of Ear, Nose, Throat (ENT), Head and Neck Surgery, Dow Medical College of Dow University of Health Sciences and Civil Hospital Karachi, Pakistan from July 2011 to December 2012. Patients of any age, sex and ethnic group visiting out patients clinic having strong clinical evidence of oral PMLs, clinically and histologically proved, untreated OSCC at any stage and identical disease free subjects willing to participate as controls, were included in the study. Subjects with prior history of treatment for loco-regional or distant malignancy, those who did not turned up with histopathology report or having inconclusive biopsy report and subject with history of immune deficiency or autoimmune disorders were excluded.

All the participants were classified in groups 'A', 'B' & 'C' having 35 subjects in each. Group 'A' constitutes patients having strong clinical evidence of oral PML. Group 'B' includes clinical and histologically proven untreated OSCC and Group 'C' constitutes disease free subjects as controls. After taking informed consent, relevant clinical information was recorded on institutional ethical and review board approved performa (IRB-180/DUHS-10). Saliva from all recruits was procured as per standard 'draining (drooling) method'. Samples were stored at $+4^{o}C$ and later transferred to Dow Diagnostic, Research & Reference Laboratory to be stored at $-20^{o}C$ before further process. The saliva samples were centrifuged at 4500 rpm for 15 minutes at $4^{o}C$. Supernatant fluid phase was removed and used for ELISA for detection and quantification of IL-6 & IL-8.

ELISA for quantification of cytokines (IL-6 and IL-8) in saliva

Human, Enzyme linked Immuno Sorbent Assay (ELISA) was performed on supernatant fluid drawn off after centrifugation of saliva samples for detection and quantification of cytokines IL-6 and IL-8 by commercially available kits, Human S IL_6 & IL_8/NAP-1 Instant ELISA, manufactured by Bioscience, Bender-Med systems Vienna-Austria. Quantification of cytokines was carried out in picogram per milliliter (pg/ml).

(a) Principle of assay

An anti-human IL-6 & IL-8 coated antibodies were already adsorbed onto micro wells of the kit. IL-6 & IL -8 present in the salivary samples or standards will binds to micro well antibodies and a biotin-conjugated is formed. Streptavidin-HRP is added which binds to this biotin conjugated. After incubation, unbound biotin conjugated anti-human IL-6 & IL-8 and Streptavidin-HRP was removed by washing. Substrate solution which is reactive to HRP was added to micro wells. A coloured product was formed in proportion to the amount of soluble human IL-6 & IL-8 present in the sample. The reaction was terminated by adding acid and absorbance was measured at 450 nm. A standard curve was prepared from seven human IL-6 & IL-8 standard dilution and concentration of interleukins was determined.

(b) Preparation of reagents

Wash buffer concentrate (25ml) vial provided with the kit was poured in clean graduated glass cylinder and 475ml of distilled water was added and mixed gently to form final 500 ml volume to make dilute wash buffer (Ix) (Phosphate-buffered saline with 1% Tween 20).Controls were prepared by adding 350µI distilled water to lyophilized control and mixed gently to ensure homogenous complete solubilisation.

(c) Test protocol

Total number of micro well strips needed to test the desire number of samples in addition to micro well strips for

blanks and standards (coloured) was determined. Standard strips were placed in position (A_1/A_2 to H_1/H_2) as per manufacturer's protocol. Distilled water was added into all standards, blank and control wells on the 96-wells plate of the kit as indicated on the label (A_1, A_2 to H_1, H_2). 100 µI of distilled water was added to designated sample wells. 50µI of sampled saliva aliquots were added to designated wells. Micro well strips were covered with adhesive film provided and incubated at room temperature for three hours. Adhesive film removed and wells emptied. Micro well strips washed six times with 400 µI of wash buffer solution prepared earlier. After last wash micro well strips tapped on paper towel to remove excess wash buffer and place upside down on a wet absorbent pad. 100µI of TMB substrate solution was added to all wells by micropipette including blank wells. Micro well strips dipped and incubated at room temperature for ten minutes. 100µI of stop solution (phosphoric acid 1M) was added to all wells including blank wells to stop enzyme reaction by quick pipetting, when highest standard dark blue colour developed (OD of 0.9-0.95). Absorbance of each micro well strip was read on a spectrophotometer using 450 nm as the primary reference wave length to measure the colour intensity. Absorbance was determined for both the samples and standards. Average absorbance values for each set of duplicate standards and samples were calculated in all salivary samples. Lower limits of detection of IL-6 and IL-8 were defined as the analyte concentration resulting in an absorbance significantly higher than that of the dilution medium. It was determined by mean plus standard deviation. Mean of six independent assays were calculated and lowest detectable value of IL-6 and IL-8 was determined to be 0.92pg/ml and 1.3pg/ml respectively. Below this level the concentration was designated as not detected (ND). Standard curve was plotted by mean absorbance of each standard concentration on the ordinate against human IL-6 and IL-8 concentration on the abscissa.

To determine the concentration of IL-6 and IL-8 in each saliva sample, the mean absorbance value was determined from ordinate and a horizontal line was extended to standard curve. At the point of intersection a vertical line was extended to abscissa and the corresponding value of interleukins concentration was read. As the samples were diluted in ratio of 1:2 thus the concentration read from standard curve was multiplied by dilution factor (X2).

STATISTICAL ANALYSIS

Data was entered and analysed using computer SPSS version 16 (SPSS Inc. Chicago, IL, USA). Statistical analysis of the data was computed to see relationship among the groups by applying Pearson Chi-Square test. Quantitative data was analysed to look for co-relation amongst the groups by applying one-way ANOVA test. For multiple comparison among the groups, Post Hoc. Dunnet t test was applied. P-value of 0.05 was used as reference value.

RESULTS

Total of 105 subjects were recruited for the study, 70 (66.7%) were males and 35 (33.3%) were females with male/females ratio of 2:1. As regards to age, overall minimum age was 20 years and maximum was 80 years with a mean of 43.2 years (table 1). Age range is depicted in fig. 1.

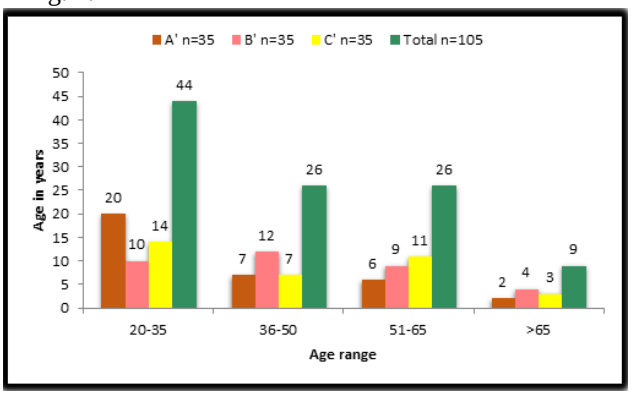

Fig. 1: Age Range

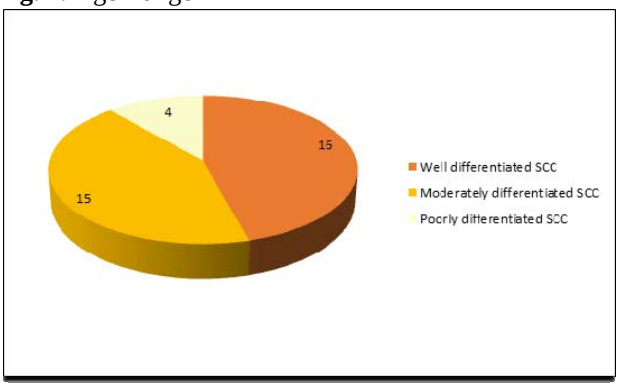

Fig. 2: Differentiation of squamous cell carcinoma Group 'B' (n-35).

In group 'A', oral submucosal fibrosis (OSF) was found in majority of the cases. OSF was observed in 27 (77.1%). Leukoplakia was detected in 10(28.6%) cases. Erythroplakia was found in 06 (17.1%) cases. All these PMLs were found alone or in combination with other pre-neoplastic lesions. 02(5.7%) cases were diagnosed as lichen planus alone on examination and on histopathology (table 2).

The subjects recruited in group 'B', were classified as per American Joint Committee on Cancer (AJCC), tumour-node-metastasis (TNM) classification (table 3). De-differentiation of OSCC on histopathology is mentioned in fig. 2.

In group 'A', IL-6 was not detected in almost all the salivary samples except one case. IL-8 was detected in

26/35 (74.3%) subjects and not detected in 09 (25.7%) cases. In group 'B', IL-6 was detected in the salivary samples of 13 (37.1%) cases and in 22 (62.9%) cases, it cannot be detected. IL-8 was detected in 33 (94.3%) and it was not detected in 02 (5.7%) subjects. It is observed that IL-8 is consistently found raised in majority of the cases of group 'A' & 'B'. In group 'C', IL -6 was not detected in any of the subject while IL -8 was detected in 10 (28.6%) cases. Although these subjects were disease free with no persistent history of risk factor exposure, the raised level of cytokines was attributed to some other dento-gingival implications.

On statistical analysis, significant co-relation was found amongst the groups on Pearson's Chi-Square analysis (P-<0.0001) for salivary detection of IL-6. Salivary detection of IL-8 was also found to have significant relation among the groups by Pearson's Chi-Square analysis (P-<0.0001) (table 4).

Quantitative analysis of interleukins was performed between the groups. Over all, the minimum concentration of salivary IL-6 detected was 7.9pg/ml and maximum was 330.3pg/ml with a median value of 77.4pg/ml. Minimum salivary concentration of IL-8 was 2.7pg/ml and maximum was 1368.2pg/ml with a median value of 472pg/ml. IL-6 was quantified in only one case of group 'A' while it was not detected in any of group 'C' individuals, so further quantitative analysis was not performed. In group 'B' minimum salivary level of IL-6 detected was 7.9 pg /ml and maximum was 330.3 pg/ml with a median value of 61.2pg/ml.

In group 'A' the minimum salivary concentration of IL-8 was 2.7pg/ml, and the maximum was 1143.2pg/ml with a median value of 305pg/ml. In group 'B' the minimum concentration of IL-8 was 26.9pg /ml and maximum was 1368.2pg/ml with a median value of 873.6pg/ml. In group 'C' the minimum salivary concentration was 23.5pg/ml and maximum was 108.6pg/ml with a median value of 52.2pg/ml.

Lots of variations were observed in the values of salivary concentration of IL-8 quantified. As the numeric data was skewed and unevenly distributed, non-parametric analysis was performed to see the co-relation among the groups by applying one-way ANOVA test, which shows significant relationship of concentration of IL-8 between the groups (P-<0.0001). Post Hoc analysis for multiple comparisons by two sided, Dunnet. t-test, shows significant co-relation of salivary concentration of IL-8 among oral PML group and controls(P-0.001) and OSCC group and controls (P-<0.0001) (table 5).

DISCUSSION

Multifunctional cytokines such as tumour necrosis factor-alpha (TNF-a) interleukin (IL-6 and IL-8) are raised in OSCC and has important role in carcinogenesis. There are evidences that these cytokines are produced in an irregular manner in OSCC and that they have contribution in cell growth initiation, invasion, interruption of tumour suppression, tumour immunity and even survival (Rhodus 2005, Jamee 2008).

Salivary detection of IL-6 and IL-8 levels may serves as potential biomarkers for screening and early detection. This may provide prognostic benefits in terms of survival, monitoring treatment outcomes and can serve as a guide for planning strategies for future novel treatment plan of targeted anti-leukotrein therapy (Gasche 2011, Shinriki 2011. Kiethubthew 2010). One could argue on this higher levels of salivary cytokines may be due to an innocent or benign epithelial lesion and inflammation, not directly related to PML or OSCC. It is said that periodontal and gingival disease and smoking can also affect concentration of salivary cytokines but this is not proved. There is remote possibility of altered cytokines levels to be impaired by these conditions. Although gingival or periodontal conditions were not standardized in context of present study, it over-weighs any potential contribution of these cytokines from other sources in the presence of frank pre-neoplastic and malignant oral lesions as these cytokines are predominantly produced by the tumour cells and lymphocytes infiltrating the area.

Results obtained from the present study reflects that local production and resulting increased salivary concentration of cytokines are chiefly contributed by oral PML & OSCC rather than influenced by local inflammatory, gingival or periodontal disease and smoking. Our findings are in conjunction with the studies reported (St; John 2004 and Brailo, 2012

Studies demonstrated that IL-6 levels play a pivotal role in suppression of anti-neoplastic host immune response. It promotes angiogenesis and lymph-angiogenesis by regulation of vascular endothelial growth factor C synthesis. It also suppresses the IL-8 response, thus promoting tumour growth and progression. Basic and translational research on role of IL-6 in OSCC revealed its function as promotion and progression of carcinogenesis. Significantly higher salivary concentration of IL-6 was reported in OSCC cases compared to normal control (Vucicevic Boras 2005, Katakura 2007, Duffy, 2008, Sato, 2010, Brailo 2012, Gasche, 2011, Culig 2013). They believed that this high level of salivary IL-6 is considered to be bad prognostic factor as it is pro-carcinogenic cytokine.

The results of salivary IL-6 levels in our study are at odds with the findings of the majority of the published reports. In present study salivary IL-6 levels were not found raised significantly in oral PML and OSCC cases. It was detected in only one case of pre-malignant lesion and in 13/35(37.1%) cases of OSCC. These finding may

Table 1: Gender distribution and Descriptive analysis of age

Group	Numbers of cases	Gender		Age in Years			
		Male	Female	Minimum	Maximum	Mean	Std. Deviation
A	35	25 (71.4%)	10 (28.6%)	20.00	75.00	39.3	15.9
B	35	24 (68.6%)	21 (60%)	26.00	80.00	46.7	14.4
C	35	21 (60%)	14 (40%)	23.00	70.00	43.5	13.7
Total	105	70 (66.7%)	35 (33.3%)	-	-	-	-

Table 2: Types and sites of pre-malignant lesions Group 'A' (n-35)

S. No	Pre-malignant Lesions	Number of Cases	Sites	Number of Cases
1	Oral Sub-mucosal fibrosis	27(77.1%)	Cheek, Palate& Pillars	27 (100%)
2	Leukoplakia	10(28.6%)	Cheek/Lip Tongue	09 (90%) 01 (10%)
3	Erythroplakia	06(17.1%)	Cheek Tongue	04 (66.7%) 02 (33.3%)
4	Lichen Planus	02(5.7%)	Cheek and RMT	02 (100%)

N.B: The number of cases outnumbered the total as many subjects were having more than one lesion.

Table 3: TNM staging of OSCC Group 'B' (n-35)

Tumour size 'T' (cms)					Cervical nodes 'N' (Clinical)					Distant Metastasis 'M'	
T_1	T_2	T_3	T_4	Total	N_0	N_1	N_2	N_3	Total	M_0	M_1
02 (5.7%)	12 (34.3%)	18 (51.4%)	03 (8.6%)	35 (100%)	20 (57.1%)	09 (25.7%)	05 (14.3%)	01 (2.9%)	35 (100%)	35 (100%)	00 (-)

Table 4: Qualitative analysis; salivary detection of cytokines IL_6 and IL_8 by ELISA

Groups	Number of Samples evaluated	IL-6		IL-8	
		Detected	Not Detected	Detected	Not Detected
A	35	01 (2.9%)	34 (97.1%)	26 (74.3%)	09 (25.7%)
B	35	13 (37.1%)	22 (62.9%)	33 (94.3%)	02 (5.7%)
C	35	00	35 (100%)	10 (28.6%)	25 (71.4%)
Total	105	14 (13.3%)	91 (86.7%)	69 (65.7%)	36 (34.3%)

NB: Non-detectable; Samples measured below the lowest standard point are considered to be non-detectable. Pearson Chi-Square analysis between the groups, P-value IL-6<0.0001 -Significant, P-value IL-8<0.0001 – Significant

Table 5: Quantitative analysis; salivary detection of cytokines IL_6 & IL_8 by ELISA

Group	No. of Samples	IL-6 (Pg/ml)			IL-8 (Pg/ml)		
		Minimum	Maximum	Median	Minimum	Maximum	Median
A	35	217.8	217.8	217.8	2.7	1143.2	305.0
B	35	7.9	330.3	61.2	26.9	1368.2	873.6
C	35	ND	ND	ND	23.5	108.6	52.1

Kruskal Wallis, One-way ANOVA analysis between the groups. P-value for IL-8- <0.0001-Significant, Post Hoc; Dunnett-T test for IL-8 Group A - C, P-value 0.001 – Significant, B - C, P-value <0.0001 - Significant

probably attributed to low incidence of cervical nodal and distant metastasis in our study in context of the pro-angiogenetic and lymph-angiogenetic functions of IL-6. This is a probable reason that we did not found clinical evidence of distant metastasis in any of the subject in oral cancer group and found no clinically palpable cervical nodal metastasis (N_0) in significant number of cases of OSCC, even at advanced staged disease. Blockade of lymphatic and blood channels due to associated submcosal fibrosis may be another reason contributing to these finding.

IL-8 causes tumour suppression and have anti-tumour activity through various extra cellular and intracellular pathways. In our study the salivary levels of IL-8 was found consistently raised in oral PMLs and OSCC. A

previous study reported significantly raised IL-8 concentration at protein levels in OSCC at a cut off value of 600 pg/ml. IL-8 concentration at mRNA level was also found raised in the same study. This study inferred that elevation of IL-8 concentration both at protein and mRNA levels can discriminate inflammatory oro-dental disease like gingivitis and periodontitis from OSCC (Wong, 2006).

High expression of salivary IL-8 and IL-1 beta levels in OSCC cases compared to healthy control was reported (Arellano-Garcia, 2008, Brinkmann, 2011). It was found that multiplexed assay of both IL-8 at protein and mRNA levels measured by electro-chemical sensor have significant difference between OSCC and controls. These results were closely matching the data measured by traditional assay, (ELISA and PCR) using saliva samples (Shah, 2011). It was reported that four genes from normal salivary transcriptomes core (NSTC) which encode for IL-8 and other markers are higher in saliva of the patients with OSCC (Lee, 2009).

There are many possible reasons for these relative discrepancies in these reported studies. Variability observed in these biochemical parameters may be attributed to different life styles, distinct geographic regions, genetic differences, ethnicity and indulgence in peculiar habits. Our findings of salivary IL-8 levels are in close concordance with the reported studies. In spite of contradicting heterogenecity of the results, one can elute that there is a definite alteration in cytokine production in cases of OSCC which is reflected as altered concentration of at least one pro-inflammatory cytokines in saliva as are the finding in present study.

In presence of these results in various studies, the predictive value of solitary salivary biomarker is doubtful for early detection of oral PML and OSCC. The present study is an effort to initiate the salivary molecular diagnosis in our region. Detection and qualification of salivary cytokine levels in this study is considered to act as roadmap for initiation and further research for saliva based diagnosis of oral PML and oral cancer. Growing body of evidences suggesting that panel of combination of multiple biomarkers exhibit improved accuracy for detection of oral PML & OSCC compared to single marker. Thus identification of common set of salivary biomarkers is important for diagnostic precision (Silva, 2010, Arellano-Garcia 2008, Jiang 2009), Consequent upon a recent visionary investment by National Institute of Dental and Craniofacial Research (NIDCR) of United States for the programme of development and validation technologies for saliva based diagnostics, engineers and scientist are now able to develop portable point-of-care diagnostic platform based on nanotechnology and micro fluidic techniques for rapid detection and analysis of salivary biomarkers (Zoiber, 2008).

Oral Fluid Nano Sensor Test (OFNASET) is a stat of art hand held automated saliva based device which has revolutionaried the field of salivary diagnostics. This point-of-care electrochemical saliva based biosensor platform with mini-Lab-on-chip technique, developed recently, is giving promising results for multiplexed detection of salivary biomarkers. It inherently enables simultaneous and rapid detection and amplification of multiple protein and nucleic acid biomarkers (Gan, 2007, Greenberg, 2010).

The present study is believed to be the first of its kind in Pakistan that investigated biomarkers of oral PML & OSCC in saliva. This unprecedented study would spark new avenues for research in saliva based diagnosis. Considerable excitement exists to envision saliva based diagnosis of oral cancer as a clinical reality in etiologically distinct population of Pakistan.

CONCLUSION

Absence of significantly raised levels of salivary IL-6 in cases of OSCC, in our etiologically distinct population may possibly attributed to lower incidence of cervical nodal metastasis and absence of distant metastasis even at advance stage in context of its pro-angiogenetic and lymph-angiogenic function. This study highlighted that salivary expression of IL-8 levels were significantly found rose in oral PML & OSCC. The higher levels of salivary IL-8 were more pronounced in cases of OSCC. These findings are suggestive of diagnostic role of salivary IL-8 levels as potential biomarker for early detection of oral carcinogenesis.

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