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Salivary Diagnosis of Oral Pre-Neoplastic and Oral Squamous Cell Carcinoma in Etiologically Distinct Population of Pakistan

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Abstract: Objective: To evaluate salivary detection of HPV-16 & 18 and interleukin 6 & 8 could serve as informative biomarker for Oral Pre-neoplastic Lesions (PNL) and oral Squamous cell Carcinoma (OSCC) in our population.
Study Design: Non-interventional, case control study.
Setting: Department of ENT, Head & Neck Surgery, DMC of DUHS & CHK-Pakistan.
Duration: July 2011 to December 2012.
Subjects and Methods:
The participants were divided in three groups 'A', 'B' & 'C' having 35 subjects in each. Group'A' constitutes patients having strong clinical evidence of oral pre-neoplastic lesions. Group 'B' includes clinical and histologically proven OSCC and Group 'C' constitutes disease free subjects as controls. After taking informed consent, relevant clinical details were recorded on IRB approved performa. Saliva was procured by 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. Samples were centrifuged at 4500 rpm for 15 minutes. Cell pellets were used for identification of high risk HPV-16 & 18 by real-time PCR method. Supernatant fluid phase was removed and used for detection and quantification of IL_6 and IL_8 by ELISA. Data was entered and analyzed 04 SPSS. P-value of 0.05 was taken as standard reference.
Results:
Significant relationship was observed between the groups for salivary HPV-18 detection by Real-time PCR (P= 0.002) while for salivary detection of HPV-16, no significant association was found (P= 0.245).
Significant association was found for qualitative salivary detection of IL_6 and IL_8 between the groups (P= < 0.0001 and < 0.0001 respectively). Regarding quantitative salivary concentration, no significant co-relation was found in salivary levels of IL_6 between the groups while there was significant association of salivary IL_8 levels between the groups (P= <0.0001). On post Hoc multiple comparison, significant co-relation was found between oral PNL group and controls (P=0.001) and OSCC group and controls (P= <0.0001).
Conclusion:
Detection of HPV infection for the causation of oral cancer cannot be fully established. Salivary concentration of IL_6 and IL_8 in oral pre-neoplastic lesions and oral cancer are useful biomarkers in etiologically distinct population of Pakistan.

Keywords: Pre-neoplastic lesions, Oral squamous cell carcinoma, Salivary diagnosis, Human papilloma virus, Interleukin**s.**

1. Introduction

Oral cancer is reported sixth frequently occurring malignancy all over the globe[1]. It is the most common cancer and possess as a major health problem in developing countries, where it is the leading cause of death. It constitutes 2-4% of the malignancy in the west but, this carcinoma accounts for almost 40% of all cancers in the Indian subcontinent, where it contributes one-third of the world burden of oral cancer. It is one of the major health concerns as it has a rising trend in younger population [2][3][4][5].

Carcinoma of oral cavity is the second most frequent malignant tumor for both the gender in Pakistan. The incidence is reported highest worldwide between 1995-2002.[6][7]. It constitutes 20-35% of all cancers seen in various public hospitals in Karachi and slightly less in other regions of Pakistan. Increasing cases are being consistently reported in younger age groups. Never the less it is a major killer in our population [6].

Cumulative effects of nutritional deficiency, chewing habits, bad oral hygiene, mal-directed sharp, teeth and infection with human papilloma virus (HPV) has been implicated for contributing in oral carcinogenesis[8][9]. In Pakistan, the major

risk factors for oral cancer are areca nut (betel nut, chalia, supari), betel quid (paan), tobacco chewing, (ghutka, mawa) and poor nutrition. Due to religious prohibition, alcohol consumption and sexual promiscuity are not a prevalent habits in Karachi, therefore, not a major risk factor.

Link between human papilloma virus and squamous cell carcinoma of the head and neck was suggested[9][10]. Recently, the association is more established as HPV-16 & 18 was the most detectable virus in salivary samples, serum and biopsy cell blocks of the patients with pre-neoplastic lesions & OSCC[10] [11] [12] [13] [14].

Pro-inflammatory, pro-angiogenic cytokines such as Tumor Necrosis Factor-Alpha (TNF $\propto$), interleukin (IL_6 & IL_8) are raised in PML and OSCC. There are evidences that these cytokines are produced in a dysregulated fashion and that they have roles in tumourogenesis, cell growth, invasion, interruption of tumor suppression, immune status and even survival [15] [16].

Diagnosis and subseqrent therapentic intervention of PNLs and OSCC is currently based on clinical examination, histopathological features and staging. Histological examination of the tissue remains the gold standard for diagnosis and identification of malignant oral lesions. Biopsy is an invasive technique with surgical implications, technique limitations for professionals and psychological implications for most patients. There is switch from histopathology to molecular methods of disease diagnosis in recent decade and exfoliative cytology has gained importance as a rapid and simple method for obtaining DNA samples.

Recently saliva has been used as prospective source in molecular diagnostics by analyzing 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. It provides highly efficient diagnostic medium.

In Pakistan, Saliva has never been explored as a diagnostic medium to detect biomarkers for PNLs and oral cancer. We attempted to investigate whether the ability to analyze saliva for potential biomarkers would be feasible to diagnose pre-neoplastic lesions and OSCC in our etiologically distinct population.

2. Subjects & Methods:

This non-interventional, case control study was carried out at department of ENT, Head and Neck Surgery, Dow Medical College of Dow University of Health Sciences and Civil Hospital Karachi, Pakistan between July 2011 to December 2012. Patients of any age, sex or ethinic group visiting out patients clinic having strong clinical evidence of Pre-neoplastic lesions, 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-regianal or distant malignancy, those who did not turned up with histopathology report or having inconclussive biopsy report and subject with history of immune deficiency or autoimmune disorders were excluded.

The participants were divided in three groups ‘A’, ‘B’ & ‘C’ having 35 subjects in each. Group‘A’ constitutes patients having strong clinical evidence of pre-neoplastic lesion of oral cavity. Group ‘B’ includes clinical and histologically proven untreated OSCC and Group ‘C’ constitutes disease free subjects as controls. After taking informed consent, personal information’s, demographic data, relevant clinical information was recorded on institutional ethical and review board approved performa. Saliva from all cases was procured as per standard ‘draining (drooling) method’. Samples were stored at $+4^oC$ and later transferred to Dow Diagnostic, Research & Reference Laboratory to be stored at -20^oC before further process. The saliva samples were subjected to centrifugation at 4500 rpm for 15 minutes at 4^oC. Cell pellets sediments were used for identification of high risk HPV-16 & 18 by Real-time PCR method. The supernatant fluid phase was removed and used for ELISA for detection and quantification of IL_6 & IL_8.

PCR for identification of high risk HPV-16 & 18 in saliva:

(a) DNA Extraction:

Total DNA was extracted from all the samples using PureLink® Viral RNA/DNA Kits (Invitrogen Life Technologies, Carlsbad USA) for rapid, efficient purification of viral nucleic acids according to the manufacturer's protocol. Briefly; the collection tubes having saliva samples were centrifuged at 15,000 rpm. Pellets were re-suspended in 200 µl lysis buffer .Negative control was prepared by adding 100 µl of HPV-Neg control provided with amplification kit to the tube labelled C*neg*. The volume of the lysate was estimated to be about 200 µl. Proteinase K 25 µl and 200 µl Buffer AL per 200 µl lysate was added. The solution was mixed immediately by pulse-vertexing for 15 seconds and incubated at 56°C for 10 minutes. The solution was briefly centrifuged to remove drops from inside the lid. The pH of the lysate was checked. This must be acidic (<7.0) to obtain maximum binding of DNA to QIAamp membrane. Ethanol (96–100%) per 200 µl lysate was added and mixed again by pulse-vertexing for 15 seconds and briefly centrifuged to remove drops from inside the lid.620 µl of the lysate was applied to the QIAamp Spin Column (in a 2 ml collection tubes) without wetting the rim. The cap closed and centrifuged at 8000 rpm for 1 minute. The QIA amp spin column was placed in a clean 2 ml collection tube and the tube was discarded containing the filtrate. The above two steps were repeated until the whole lysate was loaded. A maximum of 700 µl can be loaded onto the QIA amp spin column. The column was centrifuged at 8000 rpm for 1 minute. The collection tubes were discarded and the spin column was placed in new wash tubes. The column was washed with 500 µl Wash Buffer (W5) with ethanol .Centrifuged again at 8000 rpm for 1 minute and flow through was discarded. The wash step was repeated with 500 of µl wash buffer (W5) once. The collection tubes were discarded and the spin column was placed in other, clean wash tubes. The spin column was centrifuged at maximum speed for 1 minute to remove any residual wash buffer (W5).The spin column was placed in a clean 1.7 ml recovery tubes. Eluted with 50 µl sterile RNase-free water (E3) supplied with the kit (water was added to the centre of the cartridge).Incubated at room temperature for 1 minute. The spin column was again centrifuged at maximum speed for 1 minute to elute nucleic acids. The spin column was discarded from the recovery tube contained purified viral nucleic acids. (DNA).12.5 µl of extracted DNA was used in the HPV genotype analysis using Real-time PCR. The remaining DNA was stored at -70°C for future use.

(b) Real-time PCR:

Genotyping for HPV-16 and HPV-18 in isolated DNA from patient’s samples were performed using Real-time PCR

machine (Smart Cycler II, Cepheid, USA) using the Real-time PCR Kit HPV-16/18 Real-TM Quant (Sacace Biotechnologies, Italy). This is an *in vitro* Real-time amplification test for detection and genotyping of Human Papillomavirus 16 & 18 in the urogenital swabs, saliva, biopsies and paraffin embedded tissues.

(c) Principle of Assay:

Kit HPV 16/18 Real-TM Quant is based on two major processes: isolation of DNA from specimens and multiplex Real-time amplification. Amplification results of HPV-16 DNA were detected on the FAM/Green channel, amplification results of HPV-18 DNA were detected on the ROX/Orange channel and β-globin gene used as internal control was detected on the JOE/HEX/Yellow channel.

(d) Protocol:

Real- time PCR reaction volume was 25 µl. The required numbers of tubes were prepared (Number of tests + 3 standards and 1 negative control). Mixture prepared for the samples were added into the tubes with PCR- buffer-FRT 20 µl of Hot Start DNA Polymerase and the tubes vertexed carefully. This mix is stable for 3 months at +4°C. The reaction mixture was carefully shifted to new sterile tubes and 15 µl of reaction mixture was added into each tube with samples and controls. 10 µl of extracted DNA sample was added to appropriate tube and for each panel, 4 controls were prepared. 10 µl of Quantitative Standards HPV (QS1 HPV, QS2 HPV, and QS3 HPV) was added into 3 labeled tubes and 10 µl of DNA-buffer was added to the tube labeled Negative Control.

(e) Real Time Amplification:

All the tubes were capped and transferred into Real Time Thermal Smart Cycler. The Smart Cycler (Cepheid, USA) instrument was programmed as per manual of instruction. The positions of the tubes were programmed and the concentrations of the Quantitative Standards entered (reported on the HPV 16/18 Quant Data Card) in the JOE (Human DNA), FAM (HPV-16) and ROX (HPV-18) channels in order to generate standard curves.

(f) Data Analysis and Interpretations:

The software of Smart Cycler Real-time PCR was utilized for the interpretation of results all the way through the presence of crossing of fluorescence curve with the threshold line generated by the system in Real-time. As per the software, internal control (Human DNA) is detected on the JOE/HEX/Yellow channel, HPV 16 on the FAM/Green channel and HPV 18 on ROX/Orange channel. (Figure-1)

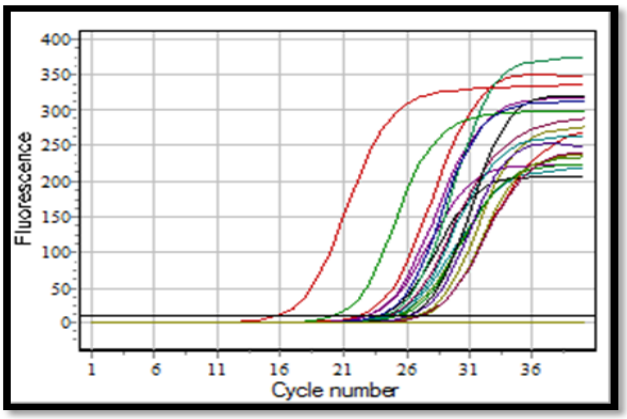

Figure-01: Crossing of fluorescence curves detected in one go; internal control (Human DNA) on JOE/HEX/Yellow Channel, HPV-16 on FAM/Green Channel, HPV-18 on ROX/Orange channel.

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 e Bioscience, Bender-Med systems Vienna-Austria, observing all storage and handling precautions as per manufacturer's protocol. The quantification of cytokines IL_6 & IL_8 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 microwells of the kit. Human IL_6 & IL_8 present in the salivary samples or standards, binds to antibodies and adsorbed to microwells; a biotin-conjugated anti-human IL_6 & IL_8 antibodies binds to human IL_6 & IL_8 captured by the first antibody. Streptavidin-HRP binds to the biotin conjugated anti-human IL_6 & IL_8. After incubation, unbound biotin conjugated anti-human IL_6 & IL_8 and Streptavidin-HRP was removed by washing and substrate solution reactive with HRP was added to the 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 07 human IL_6 & IL_8 standard dilution and concentration of IL_6 & IL_8 was determined.

(b) Preparation of Reagents:

Wash buffer concentrate (25 ml) vial provided with the kit was poured in clean graduated glass cylinder and 475 ml 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 350 µI distilled water to lyophilized control and mixed gently to ensure homogenous complete solubilization.

(c) Test Protocol:

Total number of microwell strips required to test the desire number of samples plus microwell 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 in duplicate to all standard, blank and control wells on the plate as indicated on the label of the standard strips (A_1, A_2 to H_1, H_2). 100 µI of distilled water was added to designated sample wells. 50 µI of sampled saliva aliquots were added to designated wells. Microwell strips were covered with adhesive film provided and incubated at room temperature for three hours. Adhesive film removed and wells emptied. Microwell strips washed six times with 400 µI of wash buffer solution prepared earlier. After last wash microwell strips tapped on paper towel to remove excess wash buffer and place upside down on a wet absorbent pad. 100 µI of TMB substrate solution was added to all wells by micropipette including blank wells. Microwell strips incubated at room temperature for ten minutes. 100 µI 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 microwell 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 the IL_6 & IL_8 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 & IL_8 was determined to be 0.92 pg/ml and 1.3 pg/ml respectively. The concentration was designated as not detected (ND) below this level. Standard curve was plotted by mean absorbance of each standard concentration on the ordinate against human IL_6 & IL_8 concentration on the abscissa. (Figure-2)

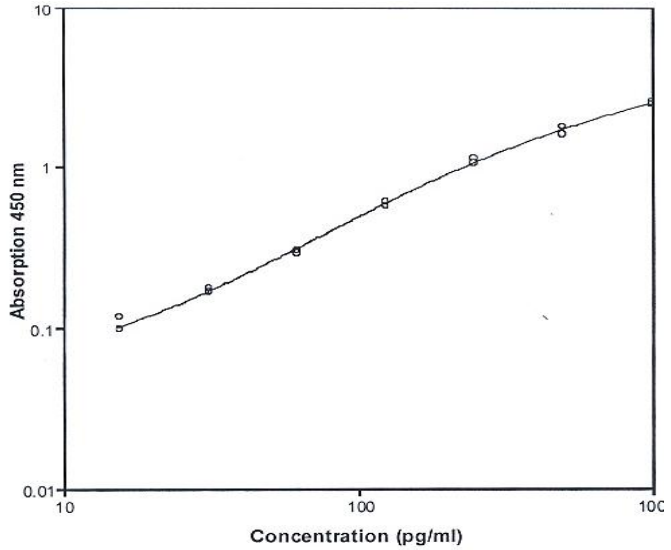

Figure-2: Representative standard curve for IL_6 and IL_8 Instant ELISA

To determine the concentration of IL_6 & 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 IL_6 & IL_8 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).

Data was entered and analyzed using computer SPSS version 16 (SPSS Inc. Chicago, IL, USA). Statistical analysis of the data was computed to see relationship between the groups by applying Pearson Chi-Square test. Quantitative data was analysed to look for relationship between the group by applying Kruskal Wallis One-way ANOVA test. For multiple comparission, between the groups, Post Hoc.2 sided Dunnet t test was applied. P-value of 0.05 was used as reference value.

3. Result:

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. (Table-1).

Gender	Group 'A'	Group 'B'	Group 'C'	Total
Males	25(71.4%)	24(68.6%)	21 (60%)	70 (66.7%)
Females	10(28.6%)	11(31.4%)	14 (40%)	35 (33.3%)
Subtotal	**35 (100%)**	**35 (100%)**	**35 (100%)**	**105 (100%)**

Table-1: Gender distribution

Overall minimum age was 20 years and maximum was 80 years with a mean of 43.2 years. (Table-2)

Group	Numbers of cases	Minimum	Maximum	Mean	Std. Deviation
A	35	20.00	75.00	39.3	15.9
B	35	26.00	80.00	46.7	14.4
C	35	23.00	70.00	43.5	13.7

Table-2: Descriptive analysis of age

In group 'A', oral submucosal fibrosis (OSF) was found in majority of the cases. 27(77.1%) OSF were observed alone or in combination with other pre-neoplastic lesion. Leukoplakia was detected in 10(28.6%) cases alone or in combination, Erythroplakia was found in 06(17.1%) cases again alone or in combination with other pre-neoplastic lesions. 02(5.7%) cases were diagnosed as lichen planus alone on examination. As regards to combination of PML, 02(5.7%) cases were of leukoerythroplakia, 06(17.1%) cases were leukoplakia with OSF & 03 (8.6%) cases were of erythroplakia with OSF. In all the cases of OSF (n-27), cheek, palate and anterior faucial pillars were involved causing trismus from grade I to III in most of the cases. Out of 10 leukoplakia cases 09(90%) were found on cheek mucosa and 01(10%) found on lateral border of tongue. Out of 06 cases of erythroplakia 04(66.7%) were found on cheek mucosa and 02(33.3%) were found on lateral border of tongue. Both the cases of lichen planus were found on cheek involving retro molar trigone (RTM) (Table-3).

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: Types & sites of pre-malignant lesions Group 'A' (n-35)

The subjects recruited in group 'B', histologically proven OSCC were staged according to the 2002-3 American Joint Committee on Cancer (AJCC),Tumour-Node-Metastasis (TNM) classification. As regards to TNM staging, 02(5.7%) cases were T_1 stage, 12(34.3%) were T_2, 18(51.4%) were T_3 and 03(8.6%) were T_4 stage at presentation. 20(57.1%) cases were having no clinically palpable nodes (N_0), 9(25.7%) were having N_1, 05(14.3%) were having N_2 and 01(2.9%) was having N_3 nodal status. Distant metastasis 'M' was not found clinically in any of the recruited subjects (Table-4).

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

In group 'A', HPV-16 was detected in salivary samples of 3 (8.6%) cases while HPV-18 was not detected in salivary samples of any of group 'A' individual. In group 'B', HPV-16 genotype was detected in the salivary samples of 07 (20%) cases while HPV-18 was detected in 06 (17.1%) cases. Mixed HPV-16 and HPV-18 genotypes were found in 02 (5.7%) cases. In group 'C', HPV-16 was detected in 03(8.6%) cases while HPV-18 genotype was not detected in any of the salivary samples of the subjects recruited in this group.

Statistical analysis was performed to compare the qualitative relationship between the groups by using Pearson Chi-Square test. The relationship of salivary HPV-16 detection by Real-time PCR was not found significant between the groups (P-0.245) while the relationship of salivary HPV-18 detection was found significant (P-0.002). (Table-5)

S. No	Genotypes	Group 'A'	Group 'B'	Group 'C'	Total
1.	HPV-16	03(8.6%)	07(20%)	03 (8.6%)	13 (12.4%)
2.	HPV-18	Nil	06(17.1%)	Nil	06 (5.7%)
3.	Mixed* (HPV 16 & 18)	Nil	02 (5.7%)	Nil	02 (01.9%)
Total		**03 (8.6%)**	**15 (42.9%)**	**03 (8.6%)**	**21 (20.0%)**

* Both inclusive

Pearson Chi Square analysis between the groups

P- value for HPV-16, 0.245 - Not significant

P- value for HPV-18, 0.002 - Significant

Table-5: High-risk human papilloma virus-16 &18 detected by Real-time PCR

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 majority of the cases IL_8 was consistently found raised. 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. Majority of the subjects in this group were also having consistently raised salivary IL_8 levels. It is observed that IL_8 is consistently found raised in group 'A' & 'B'. In group 'C', IL_6 was not detected in any of the subject while IL_8 was found to be 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.

Significant association was found between the groups by Pearsons Chi-Square analysis (P-<0.0001) for salivary detection of IL_6 by ELISA. Salivary detection of IL_8 was also found to have significant relation between the groups by Pearson Chi-Square analysis (P-<0.0001). (Table-6)

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-06: Qualitative analysis; salivary detection of cytokines IL_6 & IL_8 by ELISA

Quantitative analysis of IL_6 & IL_8 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 7.9pg /ml and maximum was 330.3pg/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 by ELISA. As the numeric data was skewed and unevenly distributed, non-parametric analysis was performed to see the relationship between the groups by applying Kruskal Wallis one-way ANOVA test, which shows significant relationship of concentration of IL_8 between the groups (P-<0.0001).

Post Hoc analysis for multiple comparison by two sided, Dunnet.t-test, shows significant association of salivary concentration of IL_8 between oral PNL group and controls(P-0.001) and OSCC group and controls (P-<0.0001)(Table-07).

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

Table-07: Quantitative analysis; salivary detection of cytokines IL_6 & IL_8 by ELISA

4. Discussion:

The role of high-risk oncogenic HPV in pre-malignant and malignant oral lesions has been an issue of extreme controversy with conflicting data reported by numerous studies [9] [10] [11] [12]. Most controversial issues relating to role of HPV in head and neck cancer, particularly OSCC are frequency of HPV, viral load acquisition through oro-genital route or otherwise and its diagnostic methodology [13].

There are substantial evidences that high risk HPV-16 and 18 are associated with oral, laryngeal and other head and neck cancers but conflicting data has been reported in the literature regarding its prevalence and meta-analysis shows heterogeneity in different reports as regards to its association in different sub-sites of head and neck regions [11] [12] [13]. Its prevalence reported in OSCC vary from <5% to 100% [13] [14] [15] [16] [17].

Modes of transmission of HPV in head and neck mucosal districts have not been fully resolved. It is proposed that multiple pathways are involved including auto-infection by hand. HPV association was found to be more pronounced with the history of perverted sexual practices but its validity is not well documented [18] [19].

Orogenital contact, peri-natal transmission and sexual transmission specially with oral sex, multiple sex partners and possibly mouth to mouth transmission as in kissing had been implicated [20] [21]. It is believed that oral sex, including fellatio and cunnilingus, is the main mode of transmission of HPV infection to oral cavity [13]. In the west, HPV is one of the most common viruses transmitted by sexual behaviors in both males and females. In many Muslim dominated countries including Pakistan, religious and socio-cultural values strictly discourage sexual perversions and promiscuity. Therefore protection from sexually transmitted HPV infection is taken for granted.

In Pakistan, HPV screening for both OSCC and cervical cancer is not commonly practiced due to socio-cultural reasons that prohibit asking enquiries related to risk factors for acquiring HPV load like oral sex and multiple partners. Therefore the data relating to acquisition of HPV infection is grossly deficient [22] [23]. The gravity of the situation is further aggravated due to stigmas attached to privacy of sexual practices. Many of the patients are either shy of expressing true situation or retaliate due to socio-cultural and religious taboos that prohibit investigation of all matters pertaining to sex and sexually transmitted diseases. This is the major barrier for epidemiological surreys related to HPV incidence and prevalence in Pakistan. As the sexual non-promiscuity in our society could well be a myth, so the knot is still tied relating to association of HPV in OSCC. In the present study, we faced the same barrier. Consequently, enquires related to risk factors for acquisition of viral load were not included in data collection performa. Although HPV is not always spread through sexual activities but it does appeared to be a predominant mode of transmission. Wealth of information's suggesting growing evidences in favour of causative role of HPV in OSCC at least as co-factor [12] [13] [14] [15] [16] [17] [18].

Etiological role of HPV in head and neck carcinogenesis was first proposed by Syrjanen et al in 1983 [24]. subsequently, several studies have supported this proposal on the basis of well proved epitheliotropic nature of HPV, morphological similarities between oral and pharyngeal mucosa with genital epithelium and strongly established role of high risk HPV in cervical cancer [8] [9] [10] [25] [26] [27].

HPV detection was investigated in normal subjects separately or along with PML and / or OSCC groups as control comparison groups. In a study from Quetta, the capital city of Balochistan province of Pakistan reported detection rate of HPV DNA in 24.5% (47/200) in a group of Pakistani subject visiting the dental department having normal oral cavity. They used oral tissue scraping for DNA detection by Real-time PCR method. Out of 47 HPV-positive cases, 4(2%) contained HPV-16 and 11(6%) contain HPV-18 genotypes [28]. High prevalence of HPV (81%) was reported in the normal oral mucosa of Japanese adults.[29] From Magnolia, HPV detection was reported to be 25% in normal oral cavities [30].

These studies do not co-relate with our findings, as we found HPV-16 in 03/35 controls and HPV-18 DNA was not detected in any of the salivary samples of normal controls with Real-time PCR. The difference of viral prevalence is very pronounced in normal oral mucosa. Very wide range from 0% to 100% is reported in the literature [31].

From Karachi, the biggest Metropolitan city of Pakistan a study was reported on the molecular analysis of HPV detection in tissue blocks by Dot Blot and in-situ hybridization and PCR method. They found 17.7% cases of OSCC positive for HPV-16 & 18 DNA on PCR and 14.6% cases showed viral DNA by NISH. While in only 4.6% cases of oral PML, the viral DNA was detected in biopsy tissues. They concluded that high risk HPV-16 & 18 has contributing co-factor role rather than mandatory causative role in oral carcinogensis [22].

In a study from Allahabad, India reported HPV detection rate of 27.4% in SMF while it was detected in 31.53% cases of OSCC on brush biopsy specimen by Hybrid Capture II test. In the same study, PCR was done on the same specimen for the detection of E6 DNA of HPV-16. They found detection rate of 25.96 in SMF cases and 32.43% in cases of OSCC. They conclude that there is slight difference in detection rates between Hybrid Capture II test and PCR [32]. Same group from India reported high risk HPV detection rate of 31.42%(33/105) in oral PML (OSF) by Hybrid capture II test [33]. Our findings regarding detection of HPV DNA in OSCC cases is quite close to the findings of Allahabad study but the findings in oral PML (OSF) does not co-relate. Another study from Karnataka, India found salivary detection rate of 54.2% for HPV-18 in OSCC cases. They reported multiple mixed HPV infection in 4.18% cases of OSCC in India [34]. Multiple mixed infection of high risk HPV was reported as 5.2% from Japan [29]. These finding are in accord to our study where we found mixed HPV-16 & 18 infection in 02(5.7%) salivary samples of OSCC cases.

A study from Southern Iran reported salivary detection of HPV DNA in 6.38% (3/47) cases of OSCC and none in normal controls [35]. Another study from Iran showed salivary detection of HPV-16 in 27.3% of OSCC and 20% of controls by PCR method. One patient of OSCC and non in controls group was found to have HPV-18 in the salivary samples. As the sample size was small (n-22), they conclude that they were unable to support the detection of HPV in saliva rinses as a diagnostic method of OSCC [14]. Our findings co-relate with this study in the sense that we also found more HPV-16 DNA in salivary samples of OSCC cases.

Kremier etal identified HPV-16 and 18 genotypes in 16.0% and 3.9% respectively in head and neck cancers. In systematic review of literature they calculated overall prevalence of HPV in 25% head and neck cancers versus 35.6% in oro-pharyngeal and 23.5% in OSCC [36].

In a meta-analysis (1988-2007), the incidence of HPV in head & neck SCC versus OSCC, it was observed that pooled prevalence of HPV in the tissue samples of head and neck SCC was 34.5% while in cases of OSCC, it was 38.1% [37]. These findings are in close proximity to our study.

Significant association is reported in OSCC with HPV-16 and to a lesser extent with HPV-18.In the salivary samples HPV-16 was the most detectable virus reported in the literature [10] [11] [12] [13] [20] [21] [22] [34] [35] [36] [37] [38]. Low copy numbers of HPV was found in the oral PML in the previous studies [39 40].These findings are in conjunction with our study.

Comparing these data from various studies, the results are highly variable and controversial. No clear cut association or relation of HPV infection with oral PNL and OSCC has so far emerged. Our findings are consistent with many of the reported studies in literature. We are unable to support definitive causative or mandatory initiator role of HPV in oral carcinogenesis in our etiologically distinct population where use of areca nut and chewable tobacco is very prevalent in the society. It is difficult to provide suggestion for routine salivary screening for the presence of HPV infection in absence of peculiar habits due to socio-cultural and economic constrains. We also could not suggest HPV vaccination in routine use for HPV related OSCC as advocated for cervical cancer in which HPV has a strongly established etiological role.

Multifunctional pro-inflammatory, pro-angiogenic cytokines such as tumour necrosis factor alpha (TNF ∞) interleukin (IL_6& IL_8) are raised in OSCC and has important role in carcinogenesis. There are evidences that these cytokines are produced in a dysregulated fashion in OSCC and that they have roles in cell growth, invasion, interruption of tumour suppression, immune status and even survival [15] [16] [41] [42].

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 quality of life and can serve as a guide for planning strategies for future novel treatment plan of targeted anti-leukotrein therapy [43] [44].

One could argue that higher levels of salivary cytokines might be the result of an innocent or benign epithelial discontinuity lesion and surronding inflammation, not directly related to PNL or OSCC. It is said that periodontal and gingival disease and smoking can also affect concentration of salivary cytokines but it's not proved. There is remote possibility of altered cytokines levels to be impaired by these condition.

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 PNL & OSCC rather than influenced by local inflammatory, gingival or periodontal disease and smoking. Our findings are in conjunction with the studies reported by St; John MA etal [45] and Brailo V etal [46].

Studies demonstrated that IL_6 levels play a pivotal role in suppression of host immune response to prevent neoplastic change.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. Vucicevic Boras V et al reported significantly higher salivary concentration of IL_6 in OSCC cases compared to normal control.[47] Katakura A et al reported higher salivary levels of IL_6 in OSCC patients than healthy controls [48]. Duffy SA and colleagues measured high concentration of IL_6 in OSCC cases compared to normal individuals [49]. Sato J et al found significantly increased salivary concentration of IL_6 in oral cancer cases compared to controls [50]. Brailo V and associates found increases salivary levels of IL_6 and TNF ∞ in patients with oral leukoplakia as compared to healthy individuals. They postulate that alteration in these cytokines may play a significant role in development of oral leukoplakia [51]. Gasche JA and colleagues co-related high levels of salivary and serum IL_6 with the grade of tumour and found that it is reduced after effective treatment with surgery, radiotherapy, chemotherapy or combination. They proposed its utility for follow-up monitoring the treatment response [41]. Culig Z and co-workers found increased salivary expression of IL_6 levels in patients with OSCC. They believed that this high level of salivary IL_6 concentration is considered to be bad prognostic factor in terms of treatment out comes as it is a pro-tumourogenic cytokine [44].

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 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. 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, (20/35{57.1%}) even at advanced staged disease.

IL_8 levels promote tumour suppression and have anti-tumour activity through various extracellular and intracellular pathways.

In the previous studies by Wong DJ reported significantly raised IL_8 concentration at protein levels in OSCC at a cut off value of 600 pg/ml. IL_8 concentration at m RNA 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 [22].

Arellano-Garcia ME and associates found high expression of salivary IL_8 and IL_1 beta levels in OSCC cases compared to healthy control [53].Brinkmann O and colleagues validated three protein and four transcriptomes biomarkers including IL_8 (Protein & mRNA levels). They found raised salivary levels of all these markers in OSCC. They proposed that profiling the combination of these markers would discriminatory and reproducible in different ethnic cohorts [54]. Shah FD and co-workers 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 [5].

St John MA et al found increased salivary concentration of IL_8 and higher serum levels of IL_6 in patients with OSCC. They did not considered gingival or periodontal conditions as a possible source of these cytokines on the notion that their results for salivary IL_8, not for IL_6 levels were so significant that they suggested OSCC as a major contributor of IL_8 levels in saliva. According to them their findings overweigh any potential background contribution from host's potential inflammatory conditions [45]. We observed the same logic in our study.

Rhodus NL and colleagues showed that TNF∞, IL-1∞, IL_6 and IL_8 were elevated in whole unstimulated saliva of the subjects with OSCC (n-13), compared with oral PML (n-13) and controls (n-13). In another study by the same group, reported comparison and analysis of the levels of TNF∞, IL_1∞, IL_6 and IL_8 levels in unstimulated whole saliva between oral lichen planus cases with dysplasia, OSCC and controls. They found that in oral lichen planus with moderate to severe dysplasia, the levels of each cytokines were significantly higher than controls but the levels of IL_6 and IL_8 were significantly lower than OSCC cases [15] [55]. Lee JM et al 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 [56].

There are number of possible reasons for relative discrepancies observed in these studies reported in literature. Variability observed in these biochemical parameters may be attributed to different life styles, distinct geographic regions, genetic differences, ethnicity and indulgence in peculiar habits. In our study salivary levels of IL_8 were detected in majority of the cases and were found consistently raised in oral PNL and OSCC carcinoma cases (PML-74.3% & OSCC-94.3%). There is a significant difference in salivary concentration of IL_8 between PNL & OSCC compared to controls. These findings are in close concordance with the reported studies.

Inspite 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. 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 [53] [57] [58].

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.

Oral Fluid Nano Sensor Test (OFNASET) is a stat of art hand held automated saliva based device which has revolutionarize 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 [59] [60].

The present study is believed to be the first of its kind in Pakistan that investigated biomarkers of oral PNL & 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 our etiologically distinct population.

5. Conclusion:

Host response towards HPV infection for the causation of oral cancer cannot be fully established possibly due to small sample size. More over different genetic makeup, environmental and geographic differences, indulgence in peculiar risk factor habits and different sexual practices compared to west due to socio-cultural and religious restrictions may be the factors responsible. Our study is unable to support definite causative or mandatory initiator role of HPV infections in oral carcinogenesis. It may have a co-factor role rather than just a passenger virus.

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 raised in oral PNL & 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 biomarker for oral carcinogenesis.

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LEGENDS:

Table-1: Gender distribution

Table-2: Descriptive analysis of age

Table-3: Types & sites of pre-malignant lesions Group 'A' (n-35)

Table-4: TNM staging of OSCC Group 'B' (n-35)

Table-5: High-risk human papilloma virus-16 &18 detected by Real-time PCR

Table-06: Qualitative analysis; salivary detection of cytokines IL_6 & IL_8 by ELISA

Table-07: Quantitative analysis; salivary detection of cytokines IL_6 & IL_8 by ELISA

Figure-01: Crossing of fluorescence curves detected in one go; internal control (Human DNA) on JOE/HEX/Yellow Channel, HPV-16 on FAM/Green Channel, HPV-18 on ROX/Orange channel.

Figure-2: Representative standard curve for IL_6 and IL_8 Instant ELISA