

RESEARCH ARTICLE

Association of Serum and Salivary Tumor Necrosis Factor- α with Histological Grading in Oral Cancer and its Role in Differentiating Premalignant and Malignant Oral Disease

Rajkumar Krishnan^{1*}, Dinesh Kumar Thayalan¹, Rajashree Padmanaban¹, Ramya Ramadas¹, Ramesh Kumar Annasamy¹, Nirmala Anandan²

Abstract

Background: Oral squamous cell carcinoma (OSCC) is an important malignancy throughout the world; early detection is an important criterion for achieving high cure rate. Out of the many reported markers for OSCC, this study validated the efficacy of tumor necrosis factor- α (TNF- α) in differentially diagnosing premalignant oral lesions and OSCC. Also, the study aimed to correlate the levels of salivary and serum TNF- α with clinicopathologic factors. **Materials and Methods:** A prospective experimental laboratory study was designed. Serum and salivary samples from 100 subjects in each group of healthy control, premalignant disease (PMD) and OSCC were collected for the study following appropriate exclusion and inclusion criteria. Serum and salivary level of TNF- α was analysed by enzyme linked immunosorbent assay. The data obtained were subjected to appropriate statistical analysis. **Results:** Increased level of both serum and salivary TNF- α was observed in OSCC subjects compared to healthy control and PMD group. Receiver operator characteristic curve analysis and area under curve values showed high specificity and sensitivity for salivary TNF- α in differentiating OSCC from PMD and healthy controls. There was significant increase in TNF- α level in moderately and poorly differentiated lesion compared to well differentiated lesion and in stage IV of clinical stage. A positive correlation was observed only with histological grading of OSCC and TNF- α . **Conclusions:** Salivary TNF- α is proved to be superior for detecting OSCC. Increase in TNF- α with histological grading and clinical staging suggests a role in prognosis.

Keywords: Oral cancer - tumor necrosis factor- α - saliva - premalignant oral lesion

Asian Pac J Cancer Prev, 15 (17), 7141-7148

Introduction

Oral cancer is a major global health problem afflicting close to 300,000 people each year (Johnson et al., 2011). Malignant tumors of the oral cavity account for approximately 30% of all head and neck cancers, and 80% of these tumors are oral squamous cell carcinoma (OSCC). OSCC typically undergo rapid disease progression, though a subset exhibits slow progression and may live over a five year period from symptom onset and tumors acquire this phenotype by using (Warnakulasuriya, 2010) multiple mechanisms for escape, including defective antigen presentation, interference with tumor-T cell interaction, and production of immunosuppressive factors (Fang et al., 2013). Carcinogens, which are present in high concentration in tobacco and its products, are the leading cause of oral cancer and their incidence has increased in most countries over the last four decades (Vijaya et al., 2012). Despite the development of drugs that can bring about improvements in prognosis, survival and quality

of life, oral cancer remains a life threatening disease with a poor prognosis. The oral cavity is amenable to routine screening and clinical examination for malignant changes, therefore in theory, these changes should be more easily detected and diagnosed in the early stages leading to more effective management (Saleh et al., 2014). A community-based randomised controlled intervention trial to evaluate the efficacy of organised oral cancer screening and intervention programme in India demonstrated that multiple rounds of screening was effective in down-staging tumours and reducing oral cancer mortality particularly amongst individuals who are at high risk (Ramadas et al., 2003; Brocklehurst et al., 2010). Hence, recent guidelines encourage the use of screening examinations, such as a visual examination of oral cavity, in high-risk populations for the early detection of oral cancer (Patton et al., 2008). However, malignant oral cancer can be difficult to distinguish from benign oral lesion/conditions in patients screened with visual examination alone (Mehrotra et al., 2011). Clinical disease management is also hindered by

¹Department of Oral and Maxillofacial Pathology, ²Department of Biochemistry, SRM Dental College, SRM University, Chennai
*For correspondence: drrajumaromp@gmail.com

an often lengthy diagnostic process based predominately on clinical criteria and histopathological analysis of oral biopsy, the only available gold standard for detecting and confirming oral cancers. This is not only time consuming and requires experts but is also expensive to be used during screening programs. For this reason, diagnostic biomarkers for OSCC must be identified and validated to maximize treatment efficacy for future patients. This issue is of paramount importance given that therapies and outcomes greatly enhance when oral cancer is detected early in patients who are followed up for screening of oral cancer. Thus, there is an unmet need for clinical or laboratory tools distinguishing between the premalignant lesion/condition and OSCC.

To date no single clinical or biological indicator of OSCC has gained unanimous acceptance, although several attempts have been made to correlate salivary analyte with OSCC and patient prognosis. Though numerous pro-inflammatory mediators including IL-10 (Marzieh et al., 2013) have been extensively studied and have shown promise as candidate biomarkers, none have been fully validated or integrated into clinical practice (Shankar et al., 2012; Krishna et al., 2013; Zhang, 2013). One protein that has shown potential for OSCC diagnostic utility is tumor necrosis factor- alpha (TNF- α), a widely expressed pro-inflammatory cytokine, that is more abundant in saliva than in plasma (Kurokawa et al., 1998; Rhodus et al., 2005).

Tumor necrosis factor- α is a pleiotropic cytokine, which acts on a large variety of cells with wide ranging effects on individual cells. Amongst the haemopoietic actions of TNF, are the activation of macrophages/monocytes, lymphocytes, neutrophils and the promotion of coagulation. It has a dual role in NK cells depending on the target cell (Pfeffer, 2003; Morrison et al., 2005; Romero-Reyes et al., 2007). TNF- α is a cytokine that is produced early in the inflammatory cascade and has been shown to promote carcinogenesis (Watanabe et al., 1988; Komori et al., 1993; Jablonska et al., 1998).

TNF- α is processed through the secretory pathway, and is reported to be involved in various pathogenic pathways of OSCC (Su et al., 2004; Lee et al., 2012). Various studies have demonstrated the diagnostic and prognostic utility of serum and salivary TNF- α for oral cancer detection (Kurokawa et al., 1988; Jablonska et al., 1998; Nakano et al., 1999; Rhodus et al., 2005; SahebJamee et al., 2008; Hsiao et al., 2009; Juretic et al., 2013). The role of TNF- α gene and its promoter polymorphisms has been an area of intensive research and has been studied as a potential determinant of disease susceptibility. A meta-analysis conducted suggested that there is increasing evidence that TNF- α polymorphisms may contribute to the risk of oral cancer and the development and spread of cancer (Cheng, 2013; Fang-Chun et al., 2013).

However, further studies are required to confirm and validate the diagnostic accuracy of TNF- α measurements in screening of OSCC in a population with higher prevalence of premalignant disease due to tobacco chewing/smoking habits. Also, there is lack of evidence of specificity and sensitivity of this marker with regard to detecting OSCC. Thus, with the above facts, the

objective of this study was to evaluate the utility of TNF- α as a biomarker for distinguishing between premalignant diseases (PMD included leukoplakia and submucous fibrosis) and malignant OSCC using a large study population. We evaluated TNF- α in both saliva and serum as a candidate diagnostic biomarker, and correlated levels of TNF- α with various clinicopathologic factors of OSCC subjects.

Materials and Methods

Study subjects recruitment

The recruitment of study subjects were done at outpatient department of SRM dental college, referrals from SRM General Hospital and private dental clinics, Chennai during January 2009 and December 2012. Study subjects were recruited by professionally qualified, well trained and experienced Oral Pathologists. The demographic details and information on previous history were collected.

Exclusion and inclusion criteria for study subjects

Healthy control group: The study subjects were recruited from student and staff volunteers from SRM Dental College and Hospital. Subjects were recruited with the following eligibility criteria: ages 18-65, male or female, no current use of prescribed or non-prescribed medication, no chronic/acute illnesses, no oral lesions no acute or sub-acute inflammation or infection, no pathological dry mouth syndrome, or inability to collect sufficient saliva samples on a reliable basis. Pregnant and lactating subjects were also excluded.

PML group: The study subjects were recruited from outpatient department of SRM dental college, referrals from SRM General Hospital and private dental clinics, Chennai. Subjects were recruited with the following eligibility criteria: ages 18-91, male or female, comprised of patients who had oral potentially malignant lesions/conditions which were clinically diagnosed and later histopathologically confirmed and who were not undergoing or having undergone any form of treatment for these lesions. Patients with underlying systemic illnesses and presence of oral inflammatory conditions such as gingivitis, periodontitis, and oral ulcers were excluded.

A total of 159 subjects were screened for potentially malignant lesion, before confirming the lesion with histopathological analysis, required amount of saliva and blood was collected and stored. Only samples of confirmed cases with potentially malignant lesion were included in the study. Due to rarity of erythroplakia occurrence in South India region, only leukoplakia and oral submucous fibrosis (OSMF) as potentially malignant lesions were included for the study. 50 in each group (leukoplakia and OSMF) were included for the study. Diagnosis of OSMF was made on the basis of clinical symptoms like difficulty in opening of mouth, palpable fibrotic bands and the lesions were graded with an established clinical grading system.

OSCC group: The study subjects were recruited from outpatient Department of SRM Dental College, referrals from SRM General Hospital and private dental clinics,

Chennai. Subjects were recruited with the following eligibility criteria: ages 18-91, male or female, comprised of patients who had oral squamous cell carcinoma which were clinically diagnosed and who were not currently undergoing or having undergone any form of definitive therapy for OSCC in the form of radiation, chemotherapy or any other adjunctive treatments. Before confirming with tissue histopathological analysis, the required amount of saliva and blood was collected and stored. Sample was collected from total of 139 subjects, out of which 39 were excluded for various reason, either non-confirmed OSCC by tissue biopsy or had other systemic illness apart from OSCC.

All oral lesions and conditions which report to the Outpatient department are subjected to routine exfoliative cytology studies with Papanicolaou stain and potassium hydroxide stain to rule out presence of candida hyphae.

Sample collection

Saliva collection: Saliva samples were collected between 9 and 11 A.M under non stimulatory condition. Participants were asked to refrain from eating, chewing and drinking at least one hour before collection. Subjects were asked to rinse out their mouth with water at least 5 min prior to saliva collection. Saliva samples were collected over a period of about 15 min in sterile plastic container. Samples were obtained by requesting subjects to swallow first, tilt their head forward, and expectorate all saliva into the centrifuge tubes for 10 min without swallowing. Following collection, the saliva was immediately centrifuged in a cooling centrifuge at 1800 rpm for 10 minutes at 4°C to remove debris. The resulting supernatant was separated into 1 ml aliquots and stored at -80°C freezer for further analysis. No more than one freeze-thaw cycle was allowed for each sample.

Blood collection and serum separation: 5 ml of peripheral blood were drawn from subjects using standardized phlebotomy procedures and allowed to clot. Handling and processing was similar for all three groups of patients. Blood samples were collected without anticoagulant into 10 ml centrifuge tubes and allowed to coagulate for 1 hour at room temperature. Sera were separated by centrifugation in a cooling centrifuge at 1500 rpm for 10 min at 4°C and all specimens were immediately aliquoted, frozen, and stored at -80°C freezer. No more than one freeze-thaw cycle was allowed for each sample.

Histopathological grading

The slides were observed under light microscope (4-40 X magnification) by experienced and skilled oral pathologist. Histopathological grading was done based on the following grading systems: *i*) OSMF- according to Pindborg and Sirsat (Pindborg et al., 1966), *ii*) Leukoplakia- according to WHO grading of epithelial dysplasia (Warnakulasuriya, 2001), *iii*) OSCC according to Modified Anneroth grading system (Anneroth et al., 1987).

All the specimens were blinded and two independent observers reviewed the histopathological specimens of

OSMF, leukoplakia and OSCC. Inter observer variability was evaluated by κ statistics and substantial agreement was arrived at. Very few cases which had disagreements were assessed with a consensus scoring system by a panel of pathologists

Clinical staging

Further clinical staging was done in OSCC confirmed subjects using computerised tomography scan and were classified based on TNM staging (Patel et al., 2005).

TNF- α estimation

Concentrations of salivary and serum TNF- α were quantified by commercially available ELISA kit (Syntron Bioresearch In., USA). The assay was carried out according to the manufacturer's instruction.

Briefly, the kit was based on sandwich ELISA method and procedure is as follows; to the precoated TNF- α antibody microplate, standards and sample were added, incubated (for 2 hours) and washed with buffer. To the washed plate detection antibody bound with horse radish peroxidase conjugate was added. The unbound antibody was washed and a chromogen substrate was added to the wells resulting in the progressive development of a blue coloured complex with the conjugate. The colour development was then stopped by the addition of stop solution turning the resultant final product yellow. The intensity of colour developed is proportional to the TNF- α present which was measured in a microplate reader (Robonik ELISA plate reader) at a wavelength of 450nm. The optical density obtained was then used for calculation of TNF- α present in each sample. The detection range of the kit was from a minimum of 25pg to a maximum of 1000pg/ml. The results were expressed as pg/ml of saliva or serum.

Statistical analysis

All statistical procedures were performed with SPSS version SPSS 18.0 (PASW statistics). Data were tested for normality using Shapiro-Wilk's test. Descriptive statistics (mean, median, interquartile range, standard deviation and standard error mean) was calculated. For data not normally distributed, nonparametric analyses were employed to assess differences between groups. The Kruskal-Wallis analysis of variance and the Mann-Whitney U test were used to evaluate differences between multiple group, and unpaired observations, respectively. Correlations were evaluated using the Spearman rank test. Graphs were plotted using GraphPad Prism for Windows ver. 5.0 (GraphPad Software, San Diego, CA, USA).

Receiver operator characteristic (ROC) curve analysis was performed, and the best cut off point was determined by the highest positive likelihood ratio (PLR) (sensitivity/[1-specificity]). The best cut-off value was defined as the test result with the highest sensitivity and specificity and that lied closest to the left upper corner of the curve. The area under the curve presented a direct measure of the diagnostic accuracy of the test. $p < 0.05$ was considered statistically significant.

Results

Characteristics of study subjects

Demographic details of the patients are outlined in Table 1. Briefly age and sex matched 100 control subjects, 100 premalignant lesions which included 50 each of leukoplakia and OSMF and 100 OSCC subjects were included. All the premalignant and OSCC subjects either had tobacco/pan chewing/smoking or alcohol intake habits. The potentially malignant lesion were from buccal mucosa (n=52), vestibule (n=4) together in buccal mucosa and vestibule (n=35), alveolar mucosa (n=3), palate (n=2), tongue (n=4). Similarly, the OSCC lesion were from buccal mucosa (n=64), alveolar mucosa (n=11), palate (n=4), tongue (n=19) and lip (n=2). Based on the histopathological grading of OSCC, 36 patients had well differentiated, 31 had moderately differentiated and 33 had poorly differentiated lesion. Also, the OSCC patients were categorized by clinical staging. There were 32 patients in stage I, in stage II there were 25 subjects, 25 subjects were categorized as in stage III and 11 patients were in stage IV. The study population in all the groups were predominantly males.

Table 1. Demographics and Sub Classification of Study Groups

Category	PML group			
	Control	Leukoplakia	OSMF	OSCC
Subjects (n)	100	50	50	100
Age range (years)	21-65	21-90	21-70	21-90
Sex				
Male	65	29	42	68
Female	35	21	8	32
Habits				
Tobacco chewing	-	28	42	34
Smoking	-	7	-	14
Smoking & chewing	-	14	8	38
Smoking & alcohol	-	1	-	9
Alcohol only	-	-	-	5
Site of Lesion				
Buccal mucosa	-	21	31	64
Vestibule	-	-	4	-
Buccal mucosa & Vestibule	-	20	15	-
Alveolar mucosa	-	3	-	11
Palate	-	2	-	4
Tongue	-	4	-	19
Lip	-	-	-	2
Histological differentiation of OSCC				
Well differentiated (WD)	-	-	-	36
Moderately differentiated (MD)	-	-	-	31
Poorly differentiated (PD)	-	-	-	33
TNM Staging of OSCC patients				
Stage I	-	-	-	32
Stage II	-	-	-	32
Stage III	-	-	-	25
Stage IV	-	-	-	11

Table 2. Comparison of TNF-α Levels in serum and saliva

Study Subjects	Serum TNF-α (pg/ml)	Salivary TNF-α (pg/ml)
Healthy Control (n=100)	3.9±2.	4.5±2.5
Premalignant Lesion subjects (n=50)	180.1±52.4*	136.8±59.6*
Premalignant Condition subjects (n=50)	166.5±49.4*	126.8±59.2*
Oral squamous cell carcinoma (n=100)	225.1±99.9*#	311.9±95.3*#

TNF-α levels in serum and saliva of OSCC and PML/C groups

The serum and salivary levels of TNF-α in OSCC and PML/C in comparison to control subjects are shown in Figure 1 A and B, respectively. The levels of serum TNF-α showed a significant increase (p<0.01) in the PML/C group (PML; median: 165.8, range: 130.1-325.3, PMC; median: 164.7, range: 108.2- 224.2 pg.ml) compared to control (median 3.9 pg/ml, range: 0.4-11.4 pg/ml). However, the level of serum TNF-α in the OSCC group (median 190.3 pg/ml, range: 101.7-591.7 pg/ml) were significantly greater than either PML/C (p<0.05) or control (p<0.05). Similar trend was observed with the salivary TNF-α levels (Figure 1B) where OSCC group showed higher levels (median: 289.3, range: 120.3-593.8 pg/ml) when compared with PML/C (PML; median 120.2, range: 13.5-401.2 pg/ml, PMC; median: 119.3, range: 36.3-302.5, p<0.05) and control (median 4.2, range: 0.4-12.8, p<0.001). Although salivary level in OSCC was significantly higher than serum level, there was no fold

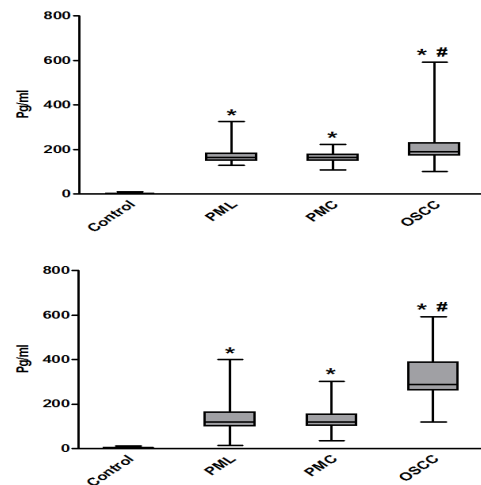


Figure 1. The Levels of TNF-α were Measured by ELISA in the Serum (A) and Saliva (B) of Healthy Control, PML/C and OSCC Subjects. Box and Whisker plots show range, inter-quartile range and median. The statistical significance is shown as * when compared to healthy control and # compared PML/PMC subjects. p<0.05 was considered to be statistically significant

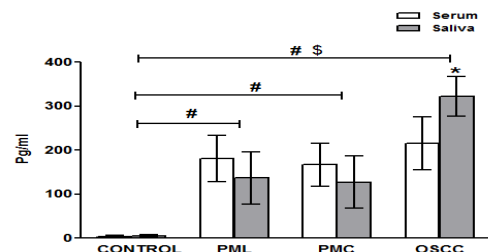


Figure 2. The levels of TNF-α Compared between Serum and Saliva of Healthy Control, PML/C and OSCC Subjects. The results are expressed as mean±SD. The statistical significance is shown as *when compared within group, #when compared with healthy control group and \$ when compared with PML/PMC group. p<0.05 was considered to be statistically significant

increase with regard to salivary TNF- α level than serum TNF- α level in control and PML/C (Figure 2).

Serum and salivary TNF- α levels based on histological grading

For 100 patients with OSCC, TNF- α concentrations were compared according to histological differentiation (Figure 3 A and B). Median and interquartile range of serum TNF- α concentrations were 178.3 (105.8-284.3), 197.8 (101.7-582.8) and 219.5 (116.5-591.7) pg/ml for well differentiated, moderately differentiated (MD) and poorly differentiated (PD) lesions, respectively. The median and interquartile range of salivary TNF- α levels were 281 (120.3-390.4), 293.9 (178.5-593.8) and 310.2 (195.7-544.4) pg/ml for WD, MD and PD lesions. Significant difference in TNF- α was noted with MD ($p < 0.05$) and PD ($p < 0.01$) when compared with WD in both serum and saliva. No significant difference with serum and salivary TNF- α levels were found depending on type and histological grading in PMD (data not shown).

Serum and salivary TNF- α levels based on clinical staging in OSCC

The level of TNF- α in serum and saliva were also compared based on the clinical staging of OSCC patients (Figure 4 A and B). In serum, the median and interquartile range for stage I is 178.3 (101.7-394.9), stage II is 193.2 (125.3-325.3), stage III is 184.7 (148.8-270.1) and stage IV is 489.3 (226.2-591.7). The median and interquartile range of TNF- α in for stage I is 270.1 (120.3-218.7), stage II is 289.3 (1780.2-267.5), stage III is 290.1 (190.2-390.4) and stage IV is 501.4 (390.1-593.8) pg/ml, respectively. In both, serum and salivary TNF- α levels were significantly increased in IV ($p < 0.01$) compared to all other stages.

Diagnostic utility of salivary TNF- α versus serum TNF- α

ROC curve analyses revealed that salivary TNF- α could serve as valuable biomarker for differentiating PMD from OSCC with an AUC of 0.981 (95%CI: 0.968 - 0.995). On the other hand serum TNF- α showed an AUC of 0.865 (95%CI: 0.822-0.908) (Figure 5). Significant difference between salivary and serum AUC was found ($p < 0.001$). At a cut-off value of 175pg/ml, the salivary TNF- α showed a sensitivity and specificity of 97% and 83%, respectively. Contrarily, with the same cut off value of in serum, the specificity and sensitivity was reduced to 75% and 72% respectively). Table 3 represents the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of serum and salivary TNF- α , above 85% of both PPV and NPV was observed only in salivary TNF- α .

Clinicopathologic correlation with serum and salivary TNF- α

The level of TNF- α in serum and saliva was correlated with clinicopathologic factors using the univariate analysis employing Spearman rank test and is illustrated in Table 4. Clinicopathologic factors included for correlation were, age, sex, habits, site of lesion, histopathologic grading and clinical staging. No significant correlation between age, sex, habit and site of lesion and serum/salivary biomarker levels. A positive significant correlation was found only

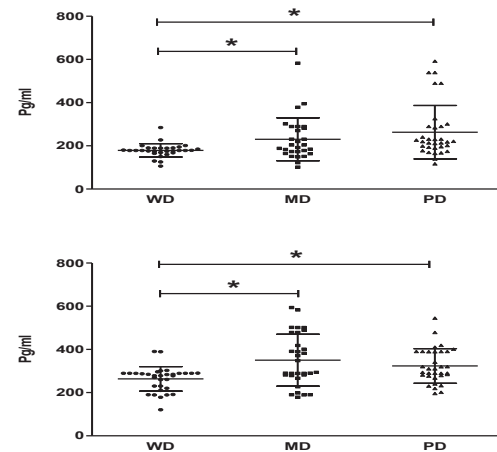


Figure 3. The Serum (A) and Salivary (B) TNF- α levels Compared in OSCC Subjects Based on Histological Grading [Well Differentiated (WD), Moderately Differentiated and Poorly Differentiated (PD)]. Each dot represents TNF- α level of each individual subject and the horizontal line represent mean value. The statistical significance is shown as * when compared to WD and # compared to MD. $p < 0.05$ was considered to be statistically significant

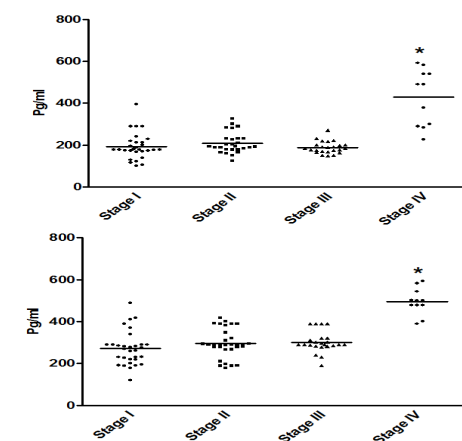


Figure 4. The Serum (A) and Salivary (B) TNF- α levels Compared in OSCC subjects Based on Clinical Staging. Each dot represents TNF- α level of each individual subject and the horizontal line represent mean value. The statistical significance is shown as * when compared to stage I, # when compared to stage II, \$ when compared to stage III. $p < 0.05$ was considered to be statistically significant

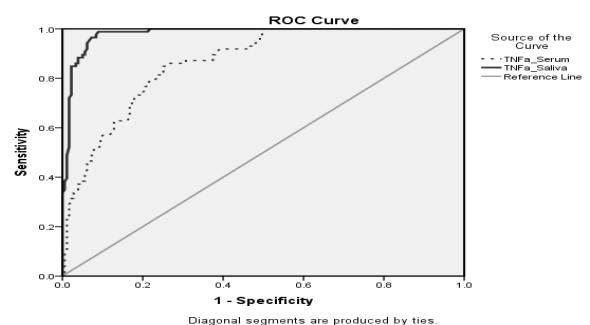


Figure 5. ROC Curve Analysis of TNF- α Marker in Saliva and Serum for Predicting OSCC when Compared to PML

Table 3. Diagnostic Utility of Salivary and Serum TNF- α

Diagnostic parameters	Saliva	Serum
Cut off value	175 pg/ml	175 pg/ml
Sensitivity	97%	75%
Specificity	83%	72%
Area under Curve	0.96	0.86
PPV	85.00%	72.80%
NPV	96.50%	74.20%

Table 4. Correlation between Serum and Salivary TNF- α levels with Clinicopathologic Factors in OSCC patients

Factors	Sample size (Total n=100)	Serum TNF- α (cut off 175pg/ml)	Salivary TNF- α (cut off: 175pg/ml)
Age			
£40 years	42	32 (10>cut off)	39 (5> cut off)
>40 years	58	35 (15>cut off)	48 (10>cut off)
		p<0.13	p<0.192
Sex			
Male	68	50 (18>cut off)	58 (10>cut off)
Female	32	25 (7>cut off)	27 (5>cut off)
		p<0.065	p<0.065
Habit			
Tobacco chewing	34	26 (8>cut off)	30 (4>cut off)
Smoking only	14	11 (3>cut off)	12 (2> cut off)
Smoking & chewing	38	28 (10>cut off)	33 (5> cut off)
Alcohol only	9	07 (2>cut off)	06 (3>cut off)
Smoking & chewing	5	03 (2>cut off)	04 (1>cut off)
		p<0.092	p<0.088
Site of lesion			
Buccal mucosa	64	50 (16>cut off)	57 (7>cut off)
Alveolar mucosa	11	07 (4>cut off)	08 (3>cut off)
Palate	4	02 (2>cut off)	03 (1>cut off)
Tongue/Lip	21	18 (3> cut off)	17 (4>cut off)
		p<0.089	p<0.103
Histopathological grading			
Well differentiated	36	24 (12>cut off)	29 (7>cut off)
Moderately differentiated	31	24 (7>cut off)	25 (6>cut off)
Poorly differentiated	33	27 (6> cut off)	31 (2>cut off)
		p<0.05*	p<0.05*
Clinical staging			
Staging I	32	22 (10>cut off)	28 (4>cut off)
Staging II	32	24 (8>cut off)	26 (6>cut off)
Staging III	25	21 (4>cut off)	21 (4>cut off)
Staging IV	11	08 (3>cut off)	10 (1>cut off)
		p<0.068	p<0.098

between histopathologic grading TNF- α (p<0.05) levels in both serum and saliva.

Discussion

Oral cancer is generally diagnosed at advanced stages of the disease. Most OSCC is not diagnosed until an advanced stage, which has been one of the major reasons for minimally improved survival rate over the years (Yi-Shing, 2011). Moreover, the fact that the survival of the patients with stages I-II of the disease ranges from 60% to 80% suggests the potential for a high treatment rate with earlier detection of the disease (Patton et al., 2008; Mehrotra et al., 2011).

Clinically, it is important to note that the therapeutic modality currently offered to patients with OSCC is based on traditional stage predicted indices [based on tumor node metastasis (TNM) criteria] and on histological grading. Unfortunately, these predictors are subjective and often

lack sensitivity and hence are relatively unreliable, as often two tumors with identical staging and grading behave in different fashions, and although one responds to therapy, the other is lethal. Furthermore, these methods do not reflect the aggressiveness of tumors, prognosis, and response to therapy (Rafael et al., 2006).

Consequently, there is a need to devise critical tools for the early detection of OSCC and this is associated with finding specific markers of oral tumors. Unfortunately, neither aetiology nor pathogenesis of OSCC is still completely known even though there is intensive research of the disease. A better knowledge of the disease has led to the study of newer biological or molecular diagnostic factors that may complement or even replace some of the less objective conventional parameters. One of the proteins that have shown potential for OSCC diagnostic utility is TNF- α , a widely expressed pro-inflammatory cytokine during transformation and progression of oral cancers (Jablonska et al., 1998; Kurokawa et al., 1998; Nakano et al., 1999; Jablonska et al., 2001).

TNF- α is processed through the secretory pathway, and, is reported to be involved in various pathogenic pathways of OSCC (Romero-Reyes et al., 2007; Hohberger et al., 2008; Lee et al., 2012). It is known that the TNF-TNF receptor system plays an important role in inflammation, angiogenesis, programmed cell death, and proliferation, which are all crucial components in malignant transformation (Romero-Reyes et al., 2007; Hohberger et al., 2008). Its anticancer property is mainly through inducing cancer death (Curnis et al., 2002; Partheniou et al., 2001). However, TNF- α also stimulates proliferation, survival, migration and angiogenesis in most tumors that are resistant to TNF- α induced cytotoxicity, resulting in tumor promotion (Nakano et al., 1999; Arnott et al., 2002; Il'yasova et al., 2005). TNF- α is a double-edged sword that could be either pro- or anti-tumorigenic. Various research reports strongly suggests that TNF- α and its soluble receptors could be useful in many types of cancer detection and staging or predicting prognosis including oral cancers (Dobrzycka et al., 2009; Fuksiewicz et al., 2010; Kotowicz et al., 2010).

TNF- α produced in oral cancers may be able to promote tumorigenesis by increasing local vasculature and by inducing tissue remodelling (Arnott et al., 2002). Production of TNF- α by OSCC cells could result in resistance to the cytotoxic action of host derived TNF- α or exogenous recombinant TNF- α administered in a therapeutic setting. TNF- α has been used in several clinical trials in cancer patients and has resulted in no significant improvements in survival (Sherman et al., 1988; Daniel et al., 2008). Local production by tumor cells and their consequent resistance may in part explain these disappointing results. The present study also proves that local production of TNF- α is high as reflected by significantly high salivary TNF- α level compared to systemic levels (serum TNF- α). This is in concordance with various other earlier reported studies (Jablonska et al., 1997; Kurokawa et al., 1998; Nakano et al., 1999; SahebJamee et al., 2008).

There are few studies that have addressed the diagnostic potential of routine TNF- α measurement in

screening of OSCC. Rhodus et al., 2005 showed that TNF- α , IL-1 α , IL-6, and IL-8 were elevated in the whole unstimulated saliva of subjects with OSCC compared with PML and controls (Rhodus et al., 2005). In another study they analyzed and compared the level of TNF- α , IL-1, 6 and 8 in whole unstimulated saliva among oral lichen planus patients with dysplasia and individuals of control and OSCC. In moderate and severe dysplasia, the level of each cytokine was significantly higher than control. Mahnaz Saheb Jamee et al., 2008 studied the concentrations of TNF- α , IL-1, 6 and 8 and concluded that the concentration of salivary cytokine in OSCC was higher than control group (SahebJamee et al., 2008). But, there are no studies in a population like India with higher prevalence due to tobacco chewing/smoking habits and little is known about the sensitivity and specificity of this marker. Hence, the present study has addressed this issue.

In accordance with the previous studies (Rhodus et al., 2005; Brailo et al., 2006; SahebJamee et al., 2008), the results of the present study demonstrate higher level of TNF- α production in OSCC compared to premalignant disorder and healthy control subjects, specifically salivary TNF- α is significantly higher than serum level in OSCC groups. Also, ROC curve analysis along with diagnostic parameter calculation reveals salivary TNF- α to be a better medium for detecting OSCC. Furthermore, correlation studies showed a positive correlation with histological grading in OSCC. No other clinicopathologic factors showed any significant correlation with TNF- α level in serum or saliva. However, a significant increase of serum and salivary TNF- α level in Stage IV of clinical staging denotes that it is involved in accelerating the disease progression. Similar observation was reported by Jablonska et al. (1997) wherein they observed a relation between clinical staging and increase in serum TNF- α level in oral cavity cancer (Jablonska et al., 1997). TNF- α has also been shown to be a potential monitoring molecule for transformation of premalignant to malignant condition in oral cancer (Brailo et al., 2012; Juretic et al., 2013). Thus, the results of the present study also substantiate the observation of earlier reports wherein we observed a significant increase in TNF- α in premalignant disorder group but the increase was significantly lower than OSCC group. The difference between serum and salivary TNF- α level was also not significant in PMD group, suggesting tumor cells to be source of TNF- α in OSCC, which may probably rise the level in saliva the local environment for the oral carcinoma tissue. But this needs to be proved by further laboratory investigation.

Finally, this study proves salivary TNF- α to be a potential biomarker for optimal diagnosis of OSCC and identification of such early changes may be useful in selecting patients for early interventional therapies. On the other hand, the level of this marker has to be assessed in other oral inflammatory disorder as well; this is one of the limitations of the study. Though earlier reports have shown significant difference in TNF- α with inflammatory disease and OSCC, this has to be confirmed in large population for considering TNF- α as a screening/diagnostic marker at clinics for routine use.

Acknowledgements

The authors thank the Patients and healthy volunteers in the study for their willingness to contribute towards this study. The authors disclose no conflict of interest

References

- Anneroth G, Batsakis J, Luna M (1987). Review of the literature and a recommended system of malignancy grading in oral squamous cell carcinomas. *Scandinavian J Dental Res*, **95**, 229-9.
- Arnott CH, Scott KA, Moore RJ, et al (2002). Tumour necrosis factor-alpha mediates tumour promotion via a PKC alpha- and AP-1-dependent pathway. *Oncogene*, **21**, 4728-8.
- Brailo V, Vucicevic-Boras V, Cekic-Arambasin A, et al (2006). The significance of salivary interleukin 6 and tumor necrosis factor alpha in patients with oral leukoplakia. *Oral Oncol*, **42**, 370-3.
- Brailo V, Vucicevic-Boras V, Lukac J, Biocina-Lukenda D, et al (2012). Salivary and serum interleukin 1 beta, interleukin 6 and tumor necrosis factor alpha in patients with leukoplakia and oral cancer. *Medicina Oral, Patologia Oral Y Cirugia Bucal*, **17**, 10-5.
- Brocklehurst P, Kujan O, Glenny AM, et al (2010). Screening programmes for the early detection and prevention of oral cancer. *Cochrane Database Syst Rev*, **11**, 1-27.
- Chen F-C, Zhang F, Zhang Z-J, et al (2013). Tumor necrosis factor- α gene polymorphisms and risk of oral cancer: evidence from a meta-analysis. *Asian Pac J Cancer Prev*, **14**, 7243-9.
- Cheng K, Zhao YJ, Liu L, Wan JJ (2013). Tumor necrosis factor- α 238 G/A polymorphism and risk of hepatocellular carcinoma: evidence from a meta-analysis. *Asian Pac J Cancer Prev*, **14**, 3275-9.
- Curnis F, Sacchi A, Corti A (2002). Improving chemotherapeutic drug penetration in tumors by vascular targeting and barrier alteration. *J Clin Investigation*, **110**, 475-82.
- Daniel D, Wilson NS (2008). Tumor necrosis factor: renaissance as a cancer therapeutic? *Current Cancer Drug Targets*, **8**, 124-31.
- Dobrzycka B, Terlikowski SJ, Kowalczyk O, Kinalski M (2009). Circulating levels of TNF-alpha and its soluble receptors in the plasma of patients with epithelial ovarian cancer. *European cytokine network*, **20**, 131-4.
- Fang-Chun C, Zhang F, Zhi-Jiao Z et al (2013). Tumor necrosis factor- α gene polymorphisms and risk of oral cancer: evidence from a meta-analysis. *Asian Pac J Cancer Prev*, **14**, 7243-9.
- Fang L, Lin S, Fang-Fang H, Qiao-Er C (2013). Effects of fasl expression in oral squamous cell cancer. *Asian Pac J Cancer Prev*, **14**, 281-5.
- Fuksiewicz M, Kowalska M, Kotowicz B, et al (2010). Serum soluble tumour necrosis factor receptor type I concentrations independently predict prognosis in patients with breast cancer. *Clinical chemistry and laboratory medicine: CCLM/ FESCC*, **48**, 1481-6.
- Hohberger L, Wuertz BR, Xie H, Griffin T, Ondrey F (2008). TNF-alpha drives matrix metalloproteinase-9 in squamous oral carcinogenesis. *The Laryngoscope*, **118**, 1395-9.
- Hsiao SH, Lee MS, Lin HY, et al (2009). Clinical significance of measuring levels of tumor necrosis factor-alpha and soluble interleukin-2 receptor in nasopharyngeal carcinoma. *Acta oto-laryngologica*, **129**, 1519-3.
- Il'yasova D, Colbert LH, Harris TB, et al (2005). Circulating levels of inflammatory markers and cancer risk in the health

- aging and body composition cohort. *Cancer Epidemiol Biomarkers Prev*, **14**, 2413-8.
- Jablonska E, Kiluk M, Markiewicz W, et al (2001). TNF-alpha, IL-6 and their soluble receptor serum levels and secretion by neutrophils in cancer patients. *Arch Immunol Ther Exp*, **49**, 63-9.
- Jablonska E, Kiluk M, Piotrowski L, et al (1998). Tumor necrosis factor-alpha and soluble tumor necrosis factor receptors in the culture supernatants of polymorphonuclear cells and peripheral blood mononuclear cells from cancer patients. *Eur Cytokine Network*, **9**, 155-9.
- Jablonska E, Piotrowski L, Grabowska Z (1997). Serum Levels of IL-1b, IL-6, TNF-a, sTNF-RI and CRP in Patients with Oral Cavity Cancer. *Pathology Oncol Res, POR*, **3**, 126-9.
- Johnson NW, Warnakulasuriya S, Gupta PC, et al (2011). Global oral health inequalities in incidence and outcomes for oral cancer: causes and solutions. *Adv Dental Res*, **23**, 237-6.
- Juretic M, Cerovic R, Belusic-Gobic M, Brekalo Prso I, Kqiku L, Spalj S, et al (2013). Salivary levels of TNF-alpha and IL-6 in patients with oral premalignant and malignant lesions. *Folia Biologica*, **59**, 99-102.
- Komori A, Yatsunami J, Suganuma M, et al (1993). Tumor necrosis factor acts as a tumor promoter in BALB/3T3 cell transformation. *Cancer Res*, **53**, 1982-5.
- Kotowicz B, Kaminska J, Fuksiewicz M, et al (2010). Clinical significance of serum CA-125 and soluble tumor necrosis factor receptor type I in cervical adenocarcinoma patients. *Int J Gynecol Cancer*, **20**, 588-92.
- Krishna Prasad RB, Sharma A, Babu HM (2013). An insight into salivary markers in oral cancer. *Dental Res J*, **10**, 287-95.
- Kurokawa H, Yamashita M, Yamashita Y, Murata T, Miura K, Kajiyama M (1998). Estimation of tumor necrosis factor-alpha in the diagnosis, the prognosis and the treatment follow-up of oral squamous cell carcinoma. *Fukuoka Igaku Zasshi*, **89**, 312-20.
- Lee SH, Hong HS, Liu ZX, et al (2012). TNFalpha enhances cancer stem cell-like phenotype via Notch-Hes1 activation in oral squamous cell carcinoma cells. *Biochem Biophys Res Commun*, **424**, 58-64.
- Marzieh H, Azadeh AT, Gita R, et al (2013). Tissue expression, serum and salivary levels of IL-10 in patients with head and neck squamous cell carcinoma. *Asian Pac J Cancer Prev*, **14**, 1681-5.
- Mehrotra R, Gupta DK (2011). Exciting new advances in oral cancer diagnosis: avenues to early detection. *Head and Neck Oncol*, **3**, 33.
- Morrison MD, Reiley W, Zhang M, Sun SC (2005). An atypical tumor necrosis factor (TNF) receptor-associated factor-binding motif of B cell-activating factor belonging to the TNF family (BAFF) receptor mediates induction of the noncanonical NF-kappaB signaling pathway. *J Biol Chem*, **280**, 10018-24.
- Nakano Y, Kobayashi W, Sugai S, Kimura H, Yagihashi S (1999). Expression of tumor necrosis factor-alpha and interleukin-6 in oral squamous cell carcinoma. *Jpn J Cancer Res*, **90**, 858-66.
- Navazesh M (1993). Methods for collecting saliva. *Annals New York Acad Sci*, **694**, 72-7.
- Partheniou F, Kelsey SM, Srinivasula SM, et al (2001). c-IAP1 blocks TNFalpha-mediated cytotoxicity upstream of caspase-dependent and -independent mitochondrial events in human leukemic cells. *Biochem Biophys Res Commun*, **287**, 181-9.
- Patel SG, Shah JP (2005). TNM staging of cancers of the head and neck: striving for uniformity among diversity. *CA Cancer J Clin*, **55**, 242-58.
- Patton LL, Epstein JB, Kerr AR (2008). Adjunctive techniques for oral cancer examination and lesion diagnosis: a systematic review of the literature. *J Am Dent Assoc*, **139**, 896-905.
- Pfeffer K (2003). Biological functions of tumor necrosis factor cytokines and their receptors. *Cytokine Growth Factor Rev*, **14**, 185-91.
- Pindborg JJ, Sirsat SM (1966). Oral submucous fibrosis. *Oral Surg Oral Med Oral Pathol*, **22**, 764-79.
- Rafael N, Gideon B, Thomas S, Raphael F (2006). Concomitant analysis of salivary tumor markers. A new diagnostic tool for oral cancer. *Clin Cancer Res*, **12**, 3979-84.
- Ramadas K, Sankaranarayanan R, Jacob BJ, et al (2003). Interim results from a cluster randomized controlled oral cancer screening trial in Kerala, India. *Oral Oncol*, **39**, 580-8.
- Rhodus NL, Cheng B, Myers S, Miller L, Ho V, Ondrey F (2005). The feasibility of monitoring NF-kappaB associated cytokines: TNF-alpha, IL-1alpha, IL-6, and IL-8 in whole saliva for the malignant transformation of oral lichen planus. *Molecular carcinogenesis*, **44**, 77-82.
- Rhodus NL, Ho V, Miller CS, Myers S, Ondrey F (2005). NF-kappaB dependent cytokine levels in saliva of patients with oral preneoplastic lesions and oral squamous cell carcinoma. *Cancer Detect Prev*, **29**, 42-5.
- Romero-Reyes M, Head C, Cacalano NA, Jewett A (2007). Potent induction of TNF-alpha during interaction of immune effectors with oral tumors as a potential mechanism for the loss of NK cell viability and function. *Apoptosis*, **12**, 2063-75.
- Saheb Jamee M, Eslami M, Atarbashi-Moghadam F, Sarafnejad A (2008). Salivary concentration of TNFalpha, IL1 alpha, IL6, and IL8 in oral squamous cell carcinoma. *Med Oral Patol Oral Cir Bucal*, **13**, 292-5.
- Saleh A, Kong YH, Vengu N, et al (2014). Dentists' perception of the role they play in early detection of oral cancer. *Asian Pac J Cancer Prev*, **15**, 229-7.
- Shankar AA, Routray S (2012). Trends in salivary diagnostics - A 5-year review of oral oncology (2007-2011). *Oral Oncol*, **48**, 22-3.
- Sherman ML, Spriggs DR, Arthur KA, et al (1988). Recombinant human tumor necrosis factor administered as a five-day continuous infusion in cancer patients: phase I toxicity and effects on lipid metabolism. *J Clin Oncol*, **6**, 344-50.
- Su TR, Chang KL, Lee CH, et al (2004). Expression of tumor necrosis factor-alpha and its soluble receptors in betel-quid-chewing patients at different stages of treatment for oral squamous cell carcinoma. *Oral Oncol*, **40**, 804-10.
- Vijaya M, Malathi M, Shivashankara AR (2012). Lipid Peroxidation and antioxidant vitamin status in oral cavity and oropharyngeal cancer patients *Asian Pac J Cancer Prev*, **13**, 5763-65.
- Warnakulasuriya S (2001). Histological grading of oral epithelial dysplasia: revisited. *J Pathol*, **194**, 294-7.
- Warnakulasuriya S (2010). Living with oral cancer: epidemiology with particular reference to prevalence and life-style changes that influence survival. *Oral Oncol*, **46**, 407-10.
- Watanabe N, Niitsu Y, Umeno H, Kuriyama H, Neda H, Yamauchi N, et al (1988). Toxic effect of tumor necrosis factor on tumor vasculature in mice. *Cancer Res*, **48**, 2179-83.
- Yi-Shing LC, John W (2011). Advances in diagnostic adjuncts for oral squamous cell carcinoma. *The Open Pathology J*, **5**, 3-7.
- Zhang A, Sun H, Wang P, Wang X (2013). Salivary proteomics in biomedical research. *Clin Chim Acta*, **415**, 261-5.