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Study of Serum and Salivary Levels of Proinflammatory Cytokines, Potential Biomarkers in the Diagnosis of Oral Squamous Cell Carcinoma

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Abstract: Oral cancer is one of the prevalent cancers of the body and is one of the 10 most common causes of death. Oral squamous cell carcinoma (OSCC) accounts for over 90% of these tumors. The aim of this study was designed to detect biochemical markers in serum and saliva of oral squamous cell carcinoma patients and to evaluate their validity in monitoring and diagnosis. The level of certain proinflammatory cytokines in the serum and saliva of (30) patients with OSCC and (20) healthy individuals as control group was measured Levels of proinflammatory cytokines Interleukin 1α (IL-1α), Interleukin (IL-6), Interleukin (IL-8) and Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) was detected by enzyme linked immunosorbent assay (ELISA). Serum IL-6 and IL-8 level was detected at higher concentrations in patients with OSCC than the control group ($P<0.001$). No significant differences in serum IL-1 alpha and GM-CSF of patients with OSCC as compared with control group.The levels of IL-1 alpha, IL-6, IL-8 and GM-CSF in saliva showed significant increase in patients with OSCC when compared with control group. Salivary IL-1 alpha and GM-CSF was useful in the diagnosis of OSCC patients. Serum IL-6 was useful in the diagnosis of OSCC patients than salivary IL-6. Serum and salivary IL-8 were very useful in the diagnosis of OSCC patients and separating between OSCC patients and control group. From the results of the presents study, it can be concluded that cytokines are important in proinflammatory and proangiogenic responses and are detectable in serum and saliva of patients with OSCC. These cytokines increase the pathogenicity of OSCC and prove useful as biomarkers for diagnosis.

Key words: IL-1α · IL-6 · IL-8 GM-CSF

INTRODUCTION

Cancers of the oral cavity represent approximately 2-3% of all malignancies. Squamous cell carcinoma (SCC), which arises from the oral epithelium, accounts for over 90% of these tumors [1,2]. SCC of head and neck is the sixth most common human malignancy [3].

The survival of patients with oral cancer remains poor despite recent treatment advances. Five years survival is noticed in about 30-40% of patients with oral cancers. The short survival time is caused, largely, by late detection [4]. Public awareness of oral cancer as compared with other cancers is low and this contributes to delays in diagnosis [5]. However, the mouth can be examined by healthcare professionals with much greater ease and accuracy than other parts of the body. All healthcare workers need to be aware that a patient with an ulcer or white patch that persists beyond three weeks should be referred for further evaluation to an oral physician or to an oral and maxillofacial surgeon. The absence of definite early warning signs for most head and neck cancers suggests that sensitive and specific biomarkers are likely to be important in screening high-risk patients. A number of molecular markers have been used to detect these tumors with varying degrees of specificity and sensitivity [6].

Alterations in host immunity, inflammation, angiogenesis and metabolism have been noted to be prominent clinical features in patients with head and neck squamous cell carcinoma (HNSCC) [7,8]. These tumors induced T-Lymophocyte, granulocyte and neoangiogenesis responses in the local tumor microenvironment and have been associated with increased growth and metastasis and decreased survival

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rate[9]. Pathological changes in systemic responses have also been observed including induction of antibody and other acute-phase inflammatory protein responses[10]. The local and systemic nature of these responses suggests the hypothesis that cytokines with proinflammatory, proangiogenic and immunoregulatory activity are produced by SCC and could contribute in the pathogenesis of HNSCC [6-11].

In the present study, we assessed the expression of four important cytokines in the regulation of immune, inflammatory and angiogenesis responses in OSCC. Proinflammatory cytokines were most commonly detected, including IL-1α, IL-6, IL-8 and GM-CSF. These cytokines were detected in serum and saliva from patients with OSCC. The purpose of the present the study is: (1) to measure the level of certain proinflammatory cytokines in serum and saliva in patients with oral squamous cell carcinoma and a healthy control group, (2) to investigate the validity of proinflammatory cytokines as informative and useful biomarkers in the diagnosis of OSCC.

MATERIALS AND METHODS

Patients: Fifty individuals were employed in this study. Thirty patients suffered from lesions that were diagnosed clinically and histologically as oral squamous cell carcinoma (OSCC). Their age ranged between 22-84 years, (16 males and 14 females). Patients admitted to the Maxillofacial Centers in Surgical Specialty Hospital in Baghdad and Oral Surgery Department of the College of Dentistry, University of Baghdad during the period from February till September 2009. Twenty three patients were newly diagnosed untreated primary lesions, whereas 7 cases represented recurrent lesions after previous therapy. Twenty healthy persons were used as control (10 males and 10 females); they didn't have medical history of any chronic or acute diseases; their age ranged between 13-63 years. Patients were evaluated by full medical history to exclude any existing systemic diseases such as diabetes or hypertension and periodentitis that may affect the parameters to be examined. Patients with such medical were excluded from the study.

Fluids Collection and Preparation:

Blood Sample: Ten ml of venous blood were aspirated from antecubital vein from each individual using plastic syringe and 21 gauge stainless needles. The whole blood was collected into plain polyethylene tube until blood clot formation. The clots were separated from the wall of the tube using a wooden applicator stick. The serum was separated by centrifugation at 3000 rpm for 10 minutes and then transferred immediately into another tube and divided into 5 equal parts and frozen at (-20°C) for subsequent analysis.

Saliva: Five to six ml of unstimulated (resting) whole saliva was collected two minutes after the patients had rinsed his mouth several times with tap water. The accumulated saliva in the floor of the mouth was drawn by a plastic disposable pipette and collected into a plastic polyethylene tube of 10 ml capacity. Saliva sample collected from OSCC patients as well as from the normal individuals. The collection period was 20 minutes and sampling time was always between 10 AM -1PM.The collected saliva was centrifuged at 3000 rpm for 10 minutes; this was done within one hour after collection to eliminate debris and cellular matter. The centrifuged supernatants were divided into 5 equal parts. All samples were stored frozen at (-20°C) in polyethylene tubes until assayed.

Immunological Assay

Determination of Serum and Salivary Interleukin Levels: Enzyme linked immunosorbent assay (ELISA) kits for specific cytokines were used (Immuntech, a beckman compant, Marseille, France) according to the manufacturer's protocol.

Statistical Analysis: Data are calculated and interred into a computerized data base structure. Statistical analyses were done by using SPSS (Statistical Package for Social Sciences). Frequency distribution for selected variable was done first. The non-normally distributed variables are described by median and interquartile range instead of mean ±SD. The difference in median between two groups was assessed by Mann-Whitney tests. Receiver Operating Characteristic (ROC) curve analysis was used to discriminate diseased cases from normal cases and can also be used to compare the diagnostic performance of two or more laboratory or diagnostic tests. ROC is a graph that plots the true positive rate in function of the false positive rate at different cut-off points.

RESULTS

Assessment of Interleukins Level: As shown in Table (1) there were a highly significant difference in serum IL-6, IL-8 among OSCC patients (median 10pg/ml, median 80pg/ml) respectively in comparison to that of control group (5pg/ml,17.5pg/ml) respectively ($p<0.001$). There were no

Table 1: Level of interleukins in serum among OSCC patients and control group using Mann-Whitney test.

Interleukins	Controls (n=20)	(Oral Cancer) (n=30)	P
Serum IL-1 alpha concentration			[NS]
Range	(0 - 5)	(0 - 137.5)	
Median	1.3	3.8	
Interquartile range	(0 - 5)	(0 - 12.5)	
Serum IL-6 concentration			<0.001
Range	(0 - 10)	(2.5 - 87.5)	
Median	5	10	
Interquartile range	(2.5 - 5)	(5 - 17.1)	
Serum IL-8 concentration			<0.001
Range	(0 - 40)	(0 - 645)	
Median	17.5	80	
Interquartile range	(5 - 25)	(31.2 - 120)	
Serum GM-CSF concentration			[NS]
Range	(0 - 0)	(0 - 112.5)	
Median	0	0	
Interquartile range	(0 - 0)	(0 - 0)	

Table 2: Level of interleukins in saliva among OSCC patients and control group Mann-Whitney test.

Interleukins	Controls (n=20)	(Oral Cancer) (n=30)	P
Salivary IL-1 alpha concentration			<0.001
Range	(187.5 - 675)	(175 - 1000)	
Median	225	968.8	
Interquartile range	(187.5-497.9)	(389.4 - 1000)	
Salivary IL-6 concentration			0.05
Range	(10-25)	(2.5 - 722.5)	
Median	15	39.4	
Interquartile range	(10.6 - 20)	(12.1 - 312.5)	
Salivary IL-8 concentration			<0.001
Range	(300 - 785)	(515 - 2000)	
Median	550	1495	
Interquartile range	(300 - 676.3)	(701.7 - 2000)	
Salivary GM-CSF concentration			0.05
Range	(0 - 0)	(0 - 195)	
Median	0	0	
Interquartile range	(0 - 0)	(0 - 43.1)	

significant difference in serum IL-1α and GM-CSF between OSCC patients and control group as shown in Table (1).

Table (2) demonstrates that there were a highly significant difference ($p<0.001$) in salivary IL-1 alpha and IL-8 levels of OSCC patients (median 968.8pg/ml, 1495pg/ml) respectively in comparison to that of control group (225pg/ml, 550pg/ml) respectively. There were a significant difference in salivary IL-6 and GMCSF levels of OSCC patients in comparison to that of control group ($p<0.05$) (Table2).

Correlation- Coefficient of Study Parameters: Serum IL-1alpha showed a highly significant correlation with serum IL-6 ($r = 0.480$) ($P<0.01$) and a significant weak correlation with salivary IL-6 ($r = 0.400$) ($P<0.05$). There was a significant correlation between serum IL-6 and salivary IL-6($r=0.648$) ($P<0.01$).There was a significant correlation between serum and salivary GM-CSF ($r = 0.544$) ($P<0.01$).

Cut-Off Values for Salivary and Serum Interleukins

Interleukin-1 Alpha: As shown in Fig.1 the area under ROC curve for serum IL-1α was not significantly different (0.64) from 0.5 value of an equivocal test, while the area under ROC curve for salivary IL-1α was significantly higher(0.82) from 0.5 value of an equivocal test (<0.001).

For salivary IL-1α the cut-off value of highest sensitivity used for screening purpose (positive test ≥ 228.8 pg/ml) yields a sensitivity of (93.3%) and a reasonable specificity of (55%), at the other extreme the cut-off value of highest specificity used for diagnostic purpose (100% diagnostic) was (= 740.0 pg/ml). The optimum cut-off value of highest accuracy (740.0pg/ml) yields a highest specificity of 100% and a reasonable sensitivity of 63.3%.

Intreleukin -6: Fig. 2 revealed that the areas under ROC curve for serum and salivary IL-6 were significantly higher (0.8) and (0.73) respectively from 0.5 value of an equivocal test.

For serum IL-6 that can be used for screening purpose of highest sensitivity (positive test = 3.8 pg/ml) yields a sensitivity of 86.7% and a specificity of 40%, at the other extreme the cut-off value with highest specificity (100% diagnostic) was (= 11.1 pg/ml) yields a sensitivity of 33.3%. The optimum cut-off value of highest accuracy (7.5 pg/ml) yields a highest specificity of 90% and a reasonable sensitivity of 63.3%, while for salivary IL-6 the cut-off value of highest sensitivity (positive test = 11 pg/ml) yields a sensitivity of (80%) and a reasonable specificity of (25%), at the other extreme the cut-off value with highest specificity (100% diagnostic) was (= 26.3 pg/ml) yields a sensitivity of (60%). The optimum cut-off value of highest accuracy (76%) was equal to specific one with reasonable sensitivity of (60%).

Interleukin-8: Fig. 3 revealed that the areas under ROC curve for serum and salivary IL-8 were significantly higher (0.9) from 0.5 value of an equivocal test.

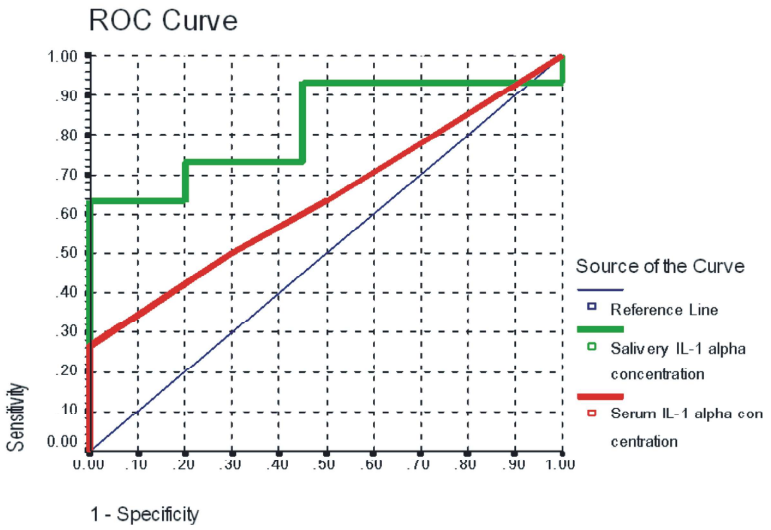

Fig. 1: ROC curve showing the tradeoff between sensitivity (true positive) and false positive (1-specificity) of all available cut off values for salivary and serum IL-1 alpha concentration in the diagnosis of OSCC.

	Area	P
Serum IL-1 alpha concentration	0.64	[NS]
Salivary IL-1 alpha concentration	0.82	<0.001

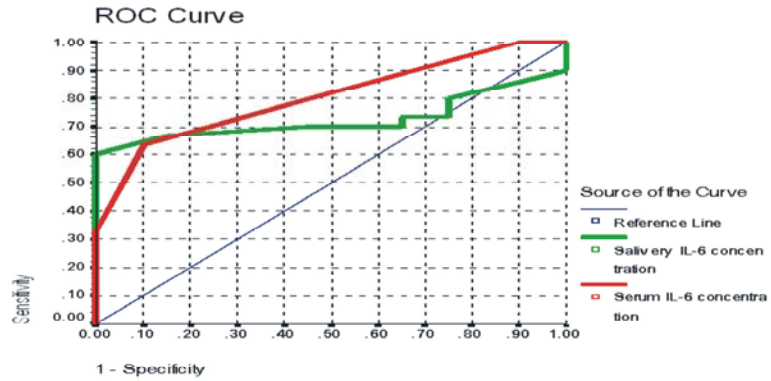

Fig. 2: ROC curve showing the tradeoff between sensitivity (true positive) and false positive (1-specificity) of all available cut off values for salivary and serum IL6 concentration in the diagnosis of OSCC.

	Area	P
Serum IL-6 concentration	0.80	<0.001
Salivary IL-6 concentration	0.73	<0.01

The cut-off value for serum IL-8 of highest sensitivity that used for screening purpose (= 22.5 pg/ml) yields a sensitivity of (93.3%) and a reasonable specificity of (60%), at the other extreme the cut-off value with highest specificity (100% diagnostic) was (= 45 pg/ml). The optimum cut-off value of highest accuracy (45 pg/ml) yields a highest specificity of (100%) and a reasonable sensitivity of (66.7%), while for salivary IL-8 the cut-off value of highest sensitivity (=407.5pg/ml) yields a sensitivity of (100%) and a specificity of (40%), at the other extreme the cut-off value with highest specificity (100%) was (= 867.5 pg/ml). The optimum cut-off value of highest accuracy (80%) was equal to specific one with reasonable sensitivity of (70%).

Granulocyte Monocyte-Colony Stimulating Factor: As shown in Fig. 4 the area under ROC curve for serum GM-CSF was not significantly different (0.58) from 0.5

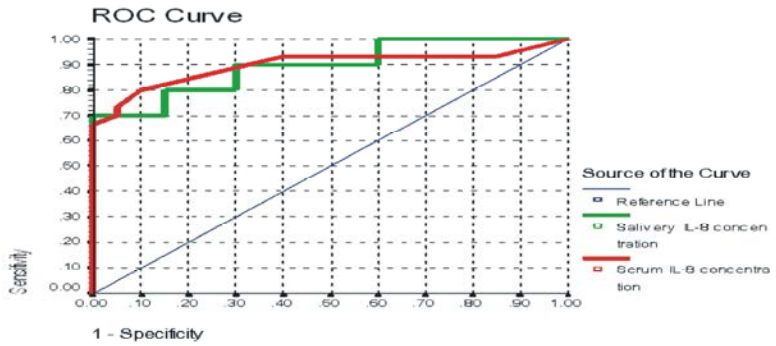

Fig. 3: ROC curve showing the tradeoff between sensitivity (true positive) and false positive (1-specificity) of all available cut off values for salivary and serum IL8 concentration in the diagnosis of OSCC.

Area Under the Curve	Area	P
Serum IL-8 concentration	0.90	<0.001
Salivary IL-8 concentration	0.90	<0.001

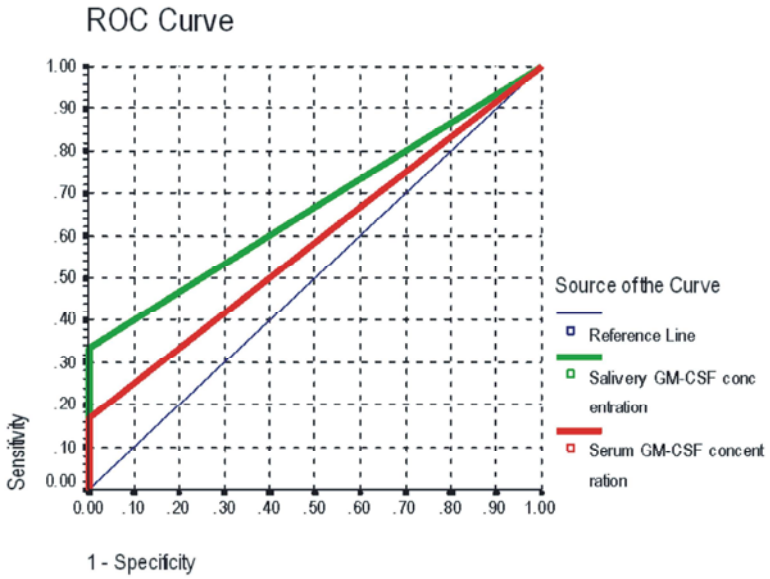

Fig. 4: ROC curve showing the tradeoff between sensitivity (true positive) and false positive (1-specificity) of all available cut off values for salivary and serum GM-CSF concentration in the diagnosis of OSCC.

Area Under the Curve	Area	P
Serum GM-CSF concentration	0.58	[NS]
Salivary GM-CSF concentration	0.67	<0.05

value of an equivocal test, while the area under ROC curve for salivary GM-CSF was significantly different (0.67) from 0.5 value of an equivocal test (<0.05).

The optimum cut-off value for salivary GM-CSF of (= 16.3 pg/ml) yields a highest accuracy (60%) with highest specificity of (100%) and reasonable sensitivity of (33.3%).

DISCUSSION

Several proinflammatory cytokines including IL-1α, IL-6, IL-8 and GM-CSF, were detected in serum and saliva of patients with OSCC [6,11,12]. Concentrations of serum IL-1α and GM-CSF were detected at the limits of sensitivity of the assays and no significant differences were detected in serum of patients with OSCC and control

group. These findings were similar to that reported by Chen, *et al.* 1999[11] who demonstrated that there were no significant differences in serum IL-1alpha and GM-CSF of patients with HNSCC and control group. Although IL-1α and GM-CSF were not detected at significant levels in serum (Table1), these two cytokines were detected at higher concentrations in saliva of patients with OSCC in comparison to that of control group (Table2).

The present study as well as others concerning oral cancer confirms the existence of significant correlation between serum IL-1α and serum IL-6 ($r=0.480$) ($p<0.01$) and between serum IL-1α and salivary IL-6 ($r=0.400$) ($p<0.05$). These findings were in agreement with Woods, *et al.* 1998[13] who concluded that IL-1 strongly up- regulated IL-6. Several studies (11, 14, 15) revealed that IL-1α play an important role in inducing the expression of cytokines during inflammation and IL-1α may have a central role in the regulating the local immune response through the enhancement or induction of cytokine production by tumor and/or resident tissue cells.

Detection of IL-1alpha and GM-CSF in saliva holds a potential role for OSCC diagnosis as the area under ROC curve for salivary IL-1α (0.82) was higher than the determined of an equivocal test of 0.5 with optimum cut-off value of highest accuracy (positive test =740.0 pg/ml) yields a specificity of 100% and a sensitivity of 63.3% and the area under ROC curve for salivary GM-CSF (0.67) was higher than the determined of an equivocal test of 0.5 with optimum cut-off value of highest accuracy (positive test =16.3 pg/ml) yields a specificity of 100% and a sensitivity of 33.3%, thus, these cytokines have been reported to play an important role in the initiation of local inflammation and activation of lymphocyte responses as well as serve as useful biomarkers in the diagnosis of OSCC than serum IL-1 alpha and serum GM-CSF. Moreover significant increase in the concentrations of serum and salivary IL-6 and IL-8 among OSCC patients when compared with that of control group were detected (Table1 and Table 2).

Elevated serum IL-6 level was in accordance with other studies[6,11,16,17] who demonstrated a significant increase of IL-6 level in serum of patients with OSCC. This finding is also in agreement with the finding of other investigators, who stated a significant increase in the level of serum IL-6 in patients with other forms of cancers like ovarian cancer [18], renal cell carcinoma [19], colorectal cancer [20], esophageal squamous cell carcinoma [21] and cervical cancer [22].

Elevation of IL-6 produced by OSCC indicates that it promote immune unresponsiveness and induction of wasting, Cachexia and hypercalcemia, all of which are observed in patients with OSCC who have a poor prognosis [6,20-24]

In this study, the area under ROC curve for serum IL-6 (0.8) of patients with OSCC was higher than the determined of an equivocal test of 0.5 with optimum cut-off value that can be used for diagnosis purpose of highest accuracy (positive test≥7.5pg/ml) yields a specificity of 90% and a sensitivity of 63.3%, indicated that serum IL-6 serve as useful biomarker in the diagnosis of OSCC than salivary IL-6 since the area under ROC for salivary IL-6 (0.73) was higher than the determined of an equivocal test of 0.5 with optimum cut-off value of highest accuracy (positive test = 26.3 pg/ml) yields a specificity of 100% and a sensitivity of 60%.

Elevated serum IL-8 was in accordance with results of Chen and coworkers 1999 [11], who demonstrated a significant increase of IL-8 level in serum of patients with OSCC.

This finding is in agreement with finding of other investigators, who demonstrated a significant increase in serum IL-8 in patients with colorectal cancer [21], hepatocellular carcinoma [25], metastatic melanoma [26] and endometrial cancer [27].

Several studies reported that IL-8 was detected in tumor specimens and primary cell cultures from patients with HNSCC [12,28-32].

IL-8 level was detected at higher concentrations in saliva of patients with OSCC than control group. This finding was supported by John, *et al.* 2004 [6], who demonstrated that IL-8 was detected at higher concentrations in saliva of patients with OSCC than control group.

Elevated IL-8 level suggested that it was play an important role in the stimulation of angiogenesis, proliferation and chemotaxis of granulocytes and macrophages which are prominent constituents in the stroma of OSCC [6,9,20,26]

In this study, detection of IL-8 in saliva and serum holds great potential for OSCC diagnosis as the area under ROC curve for both serum and salivary IL-8 was (0.9) which is higher than the determined of an equivocal test of 0.5 with optimum cut-off value of highest accuracy(= 45 pg/ml) yields a specificity of 100% and a sensitivity of 66.7% for serum IL-8 and optimum cut-off value of highest accuracy (=867.5pg/ml) yields a specificity of 100% and a sensitivity of 70% for salivary IL-8. These findings were in agreement with other studies [6-11].

In the present study, there were no significant relationship between serum and salivary IL-1alpha, IL-6, IL-8 and GM-CSF levels and degree of tumor differentiation, lesion status, clinical staging and duration of the disease this may be due to small sample used in this study.

The detection of elevated expression of proinflammatory cytokines in serum and saliva from cancer patients suggests that such cytokine expression may play a role in the increased pathogenicity of OSCC by providing a growth advantage.

These variations in serum cytokine concentrations observed among individual patients with OSCC, indicates that serum levels of cytokines may also depend in part upon individual host inflammatory responses within the tumor and biologically active cytokines contribute to altered immune status in OSCC patients [33].

The explanation of increased salivary cytokines concentration among individual patients with OSCC is that these cytokines play a role in the initiation of local inflammation and activation of lymphocyte responses so it is not specific markers since the oral cavity may be a site for several inflammatory conditions especially in elderly like periodentitis.

The use of the fluid phase of saliva has unique advantages over the use of exfoliated cells. Depending on the location of the tumor, one may not be able to easily access and swab the tumor bed. Although salivary biomarkers could not identify the site from which the tumor originated, they could identify patients at risk. The ability to analyze saliva would therefore be beneficial in the diagnosis of OSCC. The use of saliva has been criticized as a diagnostic medium since informative analyses are generally present in lower amounts than in serum [6, 33] saliva based test could be a cost-effective adjunctive tool in the diagnosis of patients with OSCC.

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