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REVIEW

# Saliva as a Diagnostic Tool in Oral Squamous Cell Carcinoma – a Systematic Review with Meta Analysis

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## Abstract

Whole saliva is mainly composed of fluid produced by major and minor salivary glands. Major salivary glands including parotid, submandibular, and sublingual glands, are known to secrete fluid transported from serum as well as surrounding glandular tissues [1]. Beside the secretions from salivary glands, oral mucosa, periodontium, as well as oral microflora also contribute to the final content of whole saliva [1]. Whole saliva therefore represents a complex balance among local and systemic sources [2]. This allows for the application of saliva in the diagnosis not only for salivary gland disorders but also for oral diseases and systemic conditions [2]. The role of saliva as a diagnostic tool in detecting Oral Squamous Cell Carcinoma. Articles published in PUBMED, EMBASE, COCHRANE, GOOGLE, manual search and back references of the articles for last 5 years extracted 77 articles. Studies which considered saliva as a diagnostic tool were included. Statistical analysis with Receivers Operating Curve to establish sensitivity and specificity of the salivary biomarkers as a diagnostic tool to detect Oral Squamous Cell Carcinoma were included for meta analysis. The measure of effect with 95% confidence interval were meta analysed for 9 articles in which 308 healthy individuals compared with 340 patients with Oral Squamous Cell Carcinoma. Highly sensitive salivary biomarkers for detecting Oral Squamous Cell Carcinoma were MMP-9, Chemerin, Choline + Betaine + Pipecolinic Acid + I – Carnitine(confidence interval ranges from 0.83–1.0). The narrow confidence interval of 0.95 + (0.88–1.00) was seen for MMP-9 followed by 1.00 + (0.78–1.00) for chemerin. Highly specific biomarkers for Oral Squamous Cell Carcinoma were MMP-9 (specificity −100%,), Chemerin(specificity-100%), over expressed mi RNA 136 with specificity of 0.88(0.69–0.97), under expressed mi RNA 27B with specificity of 1.0(0.66–1.00). Saliva can be used as a diagnostic tool with highly sensitive and specific markers namely MMP-9, Chemerin for early detection of Oral Squamous Cell Carcinoma.

**Keywords** MMP-9 · Chemerin · Saliva · Markers

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## Introduction

Oral Squamous Cell Carcinoma is one of the ten most common cancers in the world due to increased tobacco usage. The survival rate of Oral Squamous Cell Carcinoma is poor due to its diagnosis at advanced stages. The diagnosis of Oral Squamous Cell Carcinoma at the early stage prevents extensive treatment and thus biomarkers can serve as a tool for diagnosis. One such diagnostic modality that has gained much relevance in the field of molecular biology has been the discovery of salivary biomarkers (DNA, RNA and protein markers).

Whole saliva is mainly composed of fluid produced by major and minor salivary glands. Major salivary glands including parotid, submandibular, and sublingual glands, are known to secrete fluid transported from serum as well as surrounding glandular tissues [1]. Beside the secretions from salivary glands, oral mucosa, periodontium, as well as oral microflora also contribute to the final content of whole saliva [1]. Whole saliva therefore represents a complex balance among local and systemic sources [2]. This allows for the application of saliva in the diagnosis not only for salivary gland disorders but also for oral diseases and systemic conditions [2].

The term, biomarker, refers to measurable and quantifiable biological parameters that can serve as indicators for health and physiology-related assessments, such as pathogenic processes, environmental exposure, disease diagnosis and prognosis or pharmacologic responses to a therapeutic intervention [3]. A cancer biomarker for a specific tumor type can provide vital information needed to successfully treat cancer. The ultimate goal in the discovery of biomarkers is to enhance the survivability of cancer through improved diagnostics and treatment [3]. Salivary diagnostics is a dynamic and emerging field utilizing nanotechnology and molecular diagnostics to aid in the diagnosis of oral and systemic diseases and using the salivary biomarkers for disease detection [4] .

Salivary biomarkers are not only used for detecting oral carcinomas but evidence from several studies has proved its extensive support in diagnosing breast, lung, pancreatic and ovarian cancers [5]. The salivary proteome has been characterised in several diseases: Oral Squamous Cell Carcinoma and oral leukoplakia, chronic graft-versus-host disease Sjögren's syndrome and other autoimmune disorders such as SAPHO, schizophrenia and bipolar disorder, and genetic diseases like Down's Syndrome and Wilson disease. [4]

These salivary biomarkers play a non-invasive role in the diagnosis and surveillance of oral cancer lesions makes it a most sensitive and specific, screening method in diagnosis, staging, and follow-up. The validation of sensitive and specific biomarkers identified by ROC has not been systematically reviewed. The literature review of these biomarkers will substantiate the utilization of saliva as a diagnostic tool to diagnose Oral Squamous Cell Carcinoma.

The salivary markers if found to be sensitive and specific, it can be used for screening large population and it can serve as a effective tool in early identification of Oral Squamous Cell Carcinoma.

In the case of OSCC, if the malignancy is detected in the T1 stage, the survival rate at 5 years is 80%, while if it is detected in T3 and T4 stage it is 20–40% [6]. Therefore the aim of this study is to systematically review the studies on salivary biomarkers as a diagnostic tool for the detection of Oral Squamous Cell Carcinoma.

## Materials and Methods

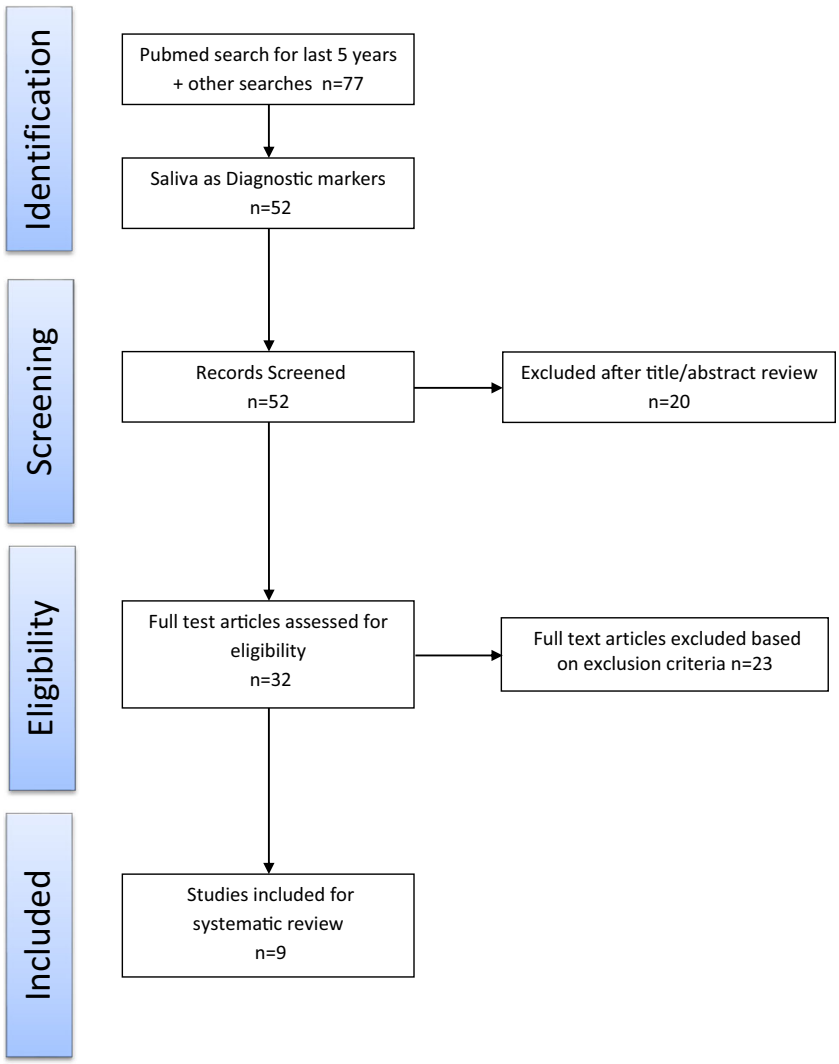
Articles published in PUBMED, EMBASE, COCHRANE, GOOGLE, manual search and back references of the articles for last 5 years extracted 77 articles. Studies which considered saliva as a diagnostic tool were included. Statistical analysis with Receivers Operating Curve to establish sensitivity and specificity of the salivary biomarkers as a diagnostic tool to detect Oral Squamous Cell Carcinoma were included for meta analysis. The measure of effect with 95% confidence interval were meta analysed for 9 articles in which 308 healthy individuals compared with 340 patients with Oral Squamous Cell Carcinoma.

Inclusion criteria for the literature review were Last 5 years articles, articles which used Receiver Operating Curve for statistical analysis as it gives sensitivity and specificity of the diagnostic tool and human studies. Another inclusion criteria were articles that estimated saliva as a diagnostic tool rather estimating its prognostic role in Oral Squamous Cell Carcinoma. Invitro studies, animal studies were excluded. The entire search methodology is depicted in flow chart 1.

## Statistical Analysis

Revman version 5.3 was used for statistical analysis. The sensitivity and specificity of each study were meta-analysed. The outcome measure is plotted in Forest plot. The sensitivity and specificity with 95% confidence interval were calculated and pooled in meta analysis. There was no heterogenity in methods used for analysing the salivary biomarkers. The studies used appropriate methods for analysing particular biomarker. The biomarkers are heterogenous so by cumulating their sensitivity and specificity identified by ROC would yield the outcome whether saliva can be used as a diagnostic tool for Oral Squamous Cell Carcinoma. It would also yield

Chart 1




high sensitive and specific markers for oral squamous cell carcinoma.

## Result

Saliva can be used as a diagnostic tool with highly sensitive and specific markers (MMP-9, Chemerin) in early detection of Oral Squamous Cell Carcinoma. Systematic review with meta analysis revealed highly sensitive salivary biomarkers for detecting Oral Squamous Cell Carcinoma as MMP-9, Chemerin, Choline + Betaine + Pipecolinic Acid + I-Carnitine(confidence interval ranges from 0.83–1.0). The narrow confidence interval of 0.95 + (0.88–1.00) was seen for MMP-9 followed by 1.00 (0.78–1.00) for chemerin. Highly specific biomarkers for Oral Squamous Cell Carcinoma were MMP-9 (specificity −100%,), Chemerin(specificity-100%), over expressed mi RNA 136 with specificity of 0.88(0.69–0.97), under expressed mi RNA 27B with specificity of 1.0(0.66–1.00). The result had been tabulated in Tables 1 & 2. The meta-analysis forest plot is depicted in Fig. 1.

## Discussion

Nine articles in which 308 healthy individuals were compared with 340 Oral squamous cell carcinoma patients [6–11, 13, 14, 16]. 50% of the articles included potentially malignant disorders and various stages of oral squamous cell carcinoma also as study population. Since it did'nt meet the criteria of diagnostic role of salivary biomarkers in identifying the early stages of oral squamous cell carcinoma, it had been excluded.

Thirty percent of the study used ELISA to analyse salivary biomarkers [8, 11, 14]. 30% of the study utilised Real Time-PCR as a analytical method [9, 10, 13] .Luminex Bead Based Multiplex Assay was used by Lee et al. for detecting IL-1β, IL-6, IL-8, TNFα [7]. Malhotra et al. analysed Cyfra 21–1 by Electro Chemiluminescent Immunoassay and RT-PCR

**Table 1** Description of the studies comparing salivary biomarkers in patients with oral squamous cell carcinoma and control, included in the meta analysis

| S.No. | First author | Sample size | Molecules | AUC | Cut off value | Sensitivity | Specificity |
|---|---|---|---|---|---|---|---|
| 1 | Lee LT et.al. [7] | OSCC-41 Healthy-24 | IL-1β | Greater than 0.7 | | 60.98% | 79.17% |
| | | | IL-6 | | | 82.93% | 70.83% |
| | | | IL-8 | | | 65.85% | 79.17% |
| | | | TNFα | | | 39.02% | 100% |
| 2 | Ghallab Na [8] | OSCC-15 Healthy −15 | MMP-9 | 0.99 | 260.32 | 100% | 100% |
| | | | Chemerin | 0.88 | 6.29 | 100% | 100% |
| 3 | Malhotra R et.al. [9] | OSCC-50 Healthy-50 | Cyfra 21–1 | Higher | 8.5 ng/ml | 93.8% | 84.3% |
| 4 | Zahran F et.al. [10] | OSCC-40 Healthy −20 | $m_i$RNA-21 | 0.73 | | 65% | 65% |
| | | | $m_i$RNA-145 | 0.68 | | 60% | 70% |
| | | | $m_i$RNA-184 | 0.86 | | 80% | 75% |
| 5 | Rajkumar et al. [11] | OSCC-100 Healthy-100 | IL-8 | | 950 pg/ml | 85% | 93% |
| 6 | Wang Q et al. [12] | OSCC-30 Healthy-60 | N-Leucine +N-Phenylalanine | | | 92.3% | 91.7% |
| 7 | Momen. Heravi F et al. [13] | OSCC-9 Healthy −9 | $m_i$RNA-27B (over expressed) | | 14.3 | 85.71% | 100% |
| | | | $m_i$RNA-136 (under expressed) | | 10.8 | 88.89% | 100% |
| 8 | Rajkumar et.al. [14] | OSCC-100 Healthy-100 | Cyfra 21–1 | | >10.4 | 75% | 75% |
| 9 | Wang Q et.al [15] | OSCC-30 Healthy-30 | Choline+betaine+ pipecolinic acid+ I-carnithine | 0.926<br>0.759<br>0.994<br>0.708 | | 100% | 96.7% |

*OSCC* patients with Oral Squamous Cell Carcinoma

whereas Rajkumar et al. analysed Cyfra 21–1 by ELISA [9, 14]. 20% of the article used Ultra Performance Liquid Chromatography) [12, 15]. These assays were highly sensitive to identify markers (both genetic and protein marker).

Salivary biomarkers is defined as a pharmacological or physiological measurement that is used to predict a toxic event; a specific molecule in the body, which has a particular feature that makes it instrumental for measuring disease progression or the

**Table 2** Meta analysed result with confidence interval 95%

| S. No. | Molecules | True positive | False positive | False negative | True negative | Sensitivity (95% CI) | Specificity (95% CI) |
|---|---|---|---|---|---|---|---|
| 1 | IL-1β(Lee et al) [7] | 25 | 5 | 16 | 19 | 0.61(0.45,0.75) | 0.79(0.58,0.93) |
| 2 | IL-6(Lee et al) [7] | 34 | 7 | 7 | 17 | 0.83(0.68,0.93) | 0.71(0.49,0.87) |
| 3 | IL-8(Lee et al) [7] | 27 | 5 | 14 | 19 | 0.66(0.49,0.80) | 0.79(0.58,0.93) |
| 4 | IL-8(RajKumar et al) [11] | 170 | 7 | 30 | 93 | 0.85(0.79,0.90) | 0.93(0.86,0.97) |
| 5 | TNF α (Lee et al) [7] | 27 | 5 | 14 | 19 | 0.66(0.49,0.80) | 0.79(0.58,0.93) |
| 6 | MMP-9 (Ghallab Na) [8] | 30 | 0 | 0 | 15 | 1.00(0.88,1.00) | 1.00(0.78,1.00) |
| 7 | Chemerin (Ghallab Na) [8] | 30 | 0 | 0 | 15 | 1.00(0.88,1.00) | 1.00(0.78,1.00) |
| 8 | Cyfra 21–1 (Malhotra R et.al) [9] | 47 | 8 | 3 | 42 | 0.94(0.83,0.99) | 0.84(0.71,0.93) |
| 9 | Cyfra 21–1 (Rajkumar et.al.) [14] | 150 | 25 | 50 | 75 | 0.75(0.68,0.81) | 0.75(0.65,0.83) |
| 10 | $m_i$RNA-21 (Zahran F et.al.) [10] | 65 | 7 | 35 | 13 | 0.65(0.55,0.74) | 0.65(0.41,0.85) |
| 11 | N-Leucine + N-Phenylalanine (Wang Q et al) [12] | 28 | 5 | 2 | 55 | 0.93(0.78,0.99) | 0.92(0.82,0.97) |
| 12 | $m_i$RNA-27B over expressed(Momen. Heravi F et al) [13] | 21 | 0 | 4 | 9 | 0.84(0.64,0.95) | 1.00(0.66,1.00) |
| 13 | $m_i$RNA-136 under expressed (Momen. Heravi F et al) [13] | 22 | 0 | 3 | 9 | 0.88(0.69,0.97) | 1.00(0.66,1.00) |
| 14 | Choline+betaine+ pipecolinic acid+ I-carnithine (Wang Q et.al) [15] | 30 | 1 | 0 | 29 | 1.00(0.88,1.00) | 0.97(0.83,1.00) |

| Test | TP | FP | FN | TN | Sensitivity (95% CI) | Specificity (95% CI) |
|---|---|---|---|---|---|---|
| 1 IL1B | 25 | 5 | 16 | 19 | 0.61 [0.45, 0.76] | 0.79 [0.58, 0.93] |
| 2 IL-6 | 34 | 7 | 7 | 17 | 0.83 [0.68, 0.93] | 0.71 [0.49, 0.87] |
| 3 IL-8 | 27 | 5 | 14 | 19 | 0.66 [0.49, 0.80] | 0.79 [0.58, 0.93] |
| 4 IL-8 By Rajkumar K et al | 170 | 7 | 30 | 93 | 0.85 [0.79, 0.90] | 0.93 [0.86, 0.97] |
| 5 TNF Alpha | 27 | 5 | 14 | 19 | 0.66 [0.49, 0.80] | 0.79 [0.58, 0.93] |
| 6 MMP-9 Ghallab NA et al | 30 | 0 | 0 | 15 | 1.00 [0.88, 1.00] | 1.00 [0.78, 1.00] |
| 7 Salivary Chemerin Ghallab NA et al | 30 | 0 | 0 | 15 | 1.00 [0.88, 1.00] | 1.00 [0.78, 1.00] |
| 8 CYFRA 21-1 Malhotra R et al | 47 | 8 | 3 | 42 | 0.94 [0.83, 0.99] | 0.84 [0.71, 0.93] |
| 9 CYFRA 21-1 Rajkumar K et al | 150 | 25 | 50 | 75 | 0.75 [0.68, 0.81] | 0.75 [0.65, 0.83] |
| 10 Mi RNA-21 Zahran F et al | 65 | 7 | 35 | 13 | 0.65 [0.55, 0.74] | 0.65 [0.41, 0.85] |
| 11 N - Leucine + N - Phenylalanine Wang Q et al | 28 | 5 | 2 | 55 | 0.93 [0.78, 0.99] | 0.92 [0.82, 0.97] |
| 12 Mi RNA - 27B Momen Heravi F et al | 21 | 0 | 4 | 9 | 0.84 [0.64, 0.95] | 1.00 [0.66, 1.00] |
| 13 Mi RNA - 136 Momen Heravi F et al | 22 | 0 | 3 | 9 | 0.88 [0.69, 0.97] | 1.00 [0.66, 1.00] |
| 14 Choline + Betaine + Pipecolonic Acid + l- Carnithine | 30 | 1 | 0 | 29 | 1.00 [0.88, 1.00] | 0.97 [0.83, 1.00] |

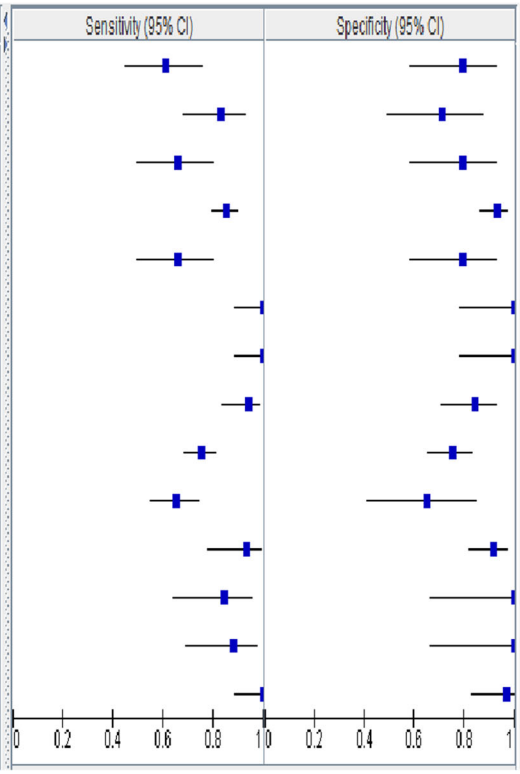



**Fig. 1** Forest plot from the meta-analysis showing the sensitivity and specificity with 95% cofidence interval depicting the association of salivary biomarkers for patients with Oral Squamous Cell Carcinoma

effects of treatment [17]. The salivary biomarkers obtained from literature were IL-6, IL-8, TNFα, MMP-9, Chemerin, Cyfra 21–1, miRNA-21,miRNA-145,miRNA-184, IL-8, N-Leucine +N-Phenylalanine, miRNA-27B (over expressed), miRNA-136 (under expressed), Cyfra 21–1,Choline+betaine+ pipecolinic acid+ I-carnithine. Among these highly sensitive salivary biomarkers were MMP-9, Chemerin and Choline+betaine+ pipecolinic acid+ I-carnithine. Highly specific biomarkers for Oral Squamous Cell Carcinoma were MMP, Chemerine, Over expressed mi RNA 136, Under expressed mi RNA 27B. N-Leucine and N-Phenylalanine also has high sensitivity and specificity of 0.93(0.78,0.99) and 0.92(0.82,0.97) respectively. The pooled sensitivity and specificity for Cyfra 21–1 ranges from 0.68 to 0.99 and 0.65 to 0.93 respectively. The cytokines were moderately sensitive and specific. IL-1β, IL-6, IL-8, TNFα were the cytokines that has been analyzed. Among these TNFα has high specificity.

The highly sensitive salivary markers are MMP-9 and Chemerin which has 100% sensitivity. Based on the study population they both have confidence interval of 0.88 to 1.00. MMP-9 and Chemerin also have highest specificity of 100% with confidence interval of 0.78 to 1.00. Randomised Control Trial could be done to use MMP-9 and chemerin as a salivary biomarker for screening and diagnosing Oral Squamous Cell Carcinoma. Chemerin, is a novel adipokine secreted by adipose tissues and a variety of other tissues, has been shown to exert important regulatory functions in various aspects of human physiology/ pathophysiology via G protein-coupled receptors, such as CMKLR1, GPR1, and CCRL2 [18]. Chemerin can increase morphogenesis and proliferation of endothelial cells (ECs), induce migration of ECs, activate key angiogenic pathways, and induce angiogenesis in tumor cells [18].

One hallmark of cancer is the degradation of the extracellular matrix (ECM), which is caused by proteinases [19]. In oral cancers, matrix metalloproteinases (MMP), especially MMP-9, are associated with this degradation. MMP-9 s also release cytokines, chemokines, and growth factors from their proforms or their cryptic sites [20].

Combination of Choline, betaine, pipecolinic acid and I-carnithine has highest sensitivity of 100% with confidence interval of 1.00(0.88,1.00). They also have specificity of 0.97(0.83,1.00).

Choline is important as a precursor of acetylcholine, as a methyl donor in various metabolic processes, and in lipid metabolism. In tumor cells, choline metabolism is the main method of phosphorylation metabolism [15]. The excessive proliferation of cancer cells requires the high level conversion of choline into phosphatidylcholine for membrane synthesis [15]. Betaine functions very closely with choline as a methyl donor and also plays a role in the synthesis of L-carnitine [15]. Pipecolinic acid is a cyclic imino acid, which is produced during the degradation of lysine [15]. Its level is increased in OSCC probably because the OSCC cells have upregulated

lysine metabolism [15]. L-carnitine is an essential factor in fatty acid metabolism and its most important known metabolic function is the transport of fat into the mitochondria of muscle cells [15]. The L-carnitine content is slightly lower in OSCC patients probably because fatty acid metabolism is downregulated in OSCC [15].

miRNA-21, miRNA-27B (over expressed) and miRNA-136 (under expressed) are the miRNA expressed in oral squamous cell carcinoma. miRNA-27B (over expressed) and miRNA-136 (under expressed) have 100% specificity. MicroRNAs (miRNAs) are short (19-to-25 nt) single stranded non-coding RNAs, that bind to complementary sequences present usually in the 3′ untranslated region (UTR) of target messenger RNAs [21]. MicroRNAs are encoded throughout the genome with a vast majority located in intergenic regions (anywhere between 57 and 69%), followed by intronic regions (~12 to 17%), exonic (~5%), long-noncoding (5%) and repeat regions (~8%) [22]. Nevertheless, around 50% of these genomic regions are frequently prone to alterations in various cancers and are collectively termed as cancer-associated genomic regions (CAGRs) [23]. Thus its expression is usually associated with oral squamous cell carcinoma.

Combination of N-Leucine and N-Phenylalanine has high sensitivity and specificity of 0.93(0.78,0.99) and 0.92(0.82,0.97) respectively. This could be analysed using UPLC.

L-Phenylalanine is an essential amino acid and the precursor for tyrosine. Like tyrosine, it is the precursor of catecholamines in the body (tyramine, dopamine, epinephrine and norepinephrine) [24]. The decreased L-phenylalanine level in OSCC saliva appears to be the result of enhanced energy metabolism or upregulation of the appropriate biosynthetic pathways, and required cell proliferation in oral cancer tissues [25]. L-Leucine as a branched chain amino acids is critical to human life and is solely to fats metabolism [9]. It stimulates protein synthesis, increase reutilization of amino acids in many organs and reduce protein breakdown [9]. Substantial amounts of L-leucine are generated by protein breakdown [9]. The intermediates of L-leucine become involved in the tricarboxylic acid (TCA) cycle when there is a shortage in energy supply [26]. In OSCC, L-leucine content in saliva decreased as compared to the healthy people, probably because the increased metabolic utilization by the TCA cycle in oral cancer cells [27] . Additional, it may also be associated with cancer cachexia and enhanced protein synthesis in excessive proliferation of cancer cells [9].

The cytokines were chronic inflammatory mediators which has been associated with cancer. They alter the matrix proteins and promote angiogenesis in tumor cell. Interleukin-8 (IL-8) is a chemo attractant cytokine produced by a variety of tissue and blood cells [28]. IL-8 functions in immune surveillance, inflammation, and angiogenesis [29]. IL-8 acts as a potent angiogenic factor in tumors by modulating endothelial cell proliferation and migration [29]. Lee et al. and Rajkumar et al. identified it as a salivary biomarker with moderate sensitivity and specificity [7, 14]. Comparison of two study revealed ELISA would be the better test to detect IL-8 in saliva [7, 14]. On pooling, the CI ranges from 0.49 to 0.90 for sensitivity and 0.58 to 0.97 for specificity [7, 14].

Interleukin-1β (IL-1β) is a potent pro-inflammatory cytokine that is crucial for host-defence responses to infection and injury [30]. It induces prostaglandin synthesis, neutrophil influx and activation, T cell activation and cytokine production, B cell activation and antibody production, and fibroblast proliferation and collagen production [31]. The sensitivity of IL-1β as salivary biomarker for oral squamous cell carcinoma was 0.61(0.45,0.75) and its specificity was 0.79(0.58,0.93).

Interleukin 6 (IL-6), promptly and transiently produced in response to infections and tissue injuries, contributes to host defense through the stimulation of acute phase responses, hematopoiesis, and immune reactions [25]. Interleukin-6 (IL-6), is secreted by different cell types including macrophages, T- and B-lymphocytes, fibroblast, endothelial cells, keratinocytes and cancer cells [27]. It has better sensitivity than other cytokines.

Cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) exert biological effects on tumor development [32]. They are produced by a variety of cells including macrophages, fibroblasts and endothelial cells, as well as neoplastic cells [32]. TNF-α has similar sensitivity and specificity as that of IL8.

CYFRA 21-1 is the serum soluble fragment of cytokeratin 19 and was first described in the mid 1990s [33]. In oral squamous cell carcinoma (OSCC), to early detect tumor, it usually links to the squamous cell component of cancer. Cytokeratins are structural proteins forming the subunits of epithelial intermediary filaments [34]. The pooled sensitivity and specificity for Cyfra 21–1 ranges from 0.68 to 0.99 and 0.65 to 0.93 respectively. Since all these cytokines have average sensitivity and specificity they can be used as a prognostic markers rather than a diagnostic markers.

Limitations for this study is that others molecules which shows moderate sensitivity and specificity has to be further investigated.

## Conclusion

Saliva can be used as a diagnostic tool with highly sensitive and specific markers (MMP-9, Chemerin) for early detection of Oral Squamous Cell Carcinoma.



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