

## Review Article

### An insight into salivary markers in oral cancer

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#### ABSTRACT

Salivary diagnostics has fascinated many researchers and has been tested as a valuable tool in the diagnosis of many systemic conditions and for drug monitoring. Advances in the field of molecular biology, salivary genomics and proteomics have led to the discovery of new molecular markers for oral cancer diagnosis, therapeutics and prognosis. Oral cancer is a potentially fatal disease and the outcome of the treatment and prognosis largely depends on early diagnosis. Abnormal cellular products elucidated from malignant cells can be detected and measured in various body fluids including saliva and constitute tumor markers. This article discusses the various salivary tumor markers and their role in oral pre-cancer and cancer.

**Key Words:** DNA markers, oral cancer, protein markers, RNA markers, salivary markers, tumor markers

#### INTRODUCTION

Oral cancer is one of the major global public health problems and is the sixth most common human malignancy with a five year mortality rate of approximately 50%.<sup>[1]</sup> This has not changed significantly over the last 50 years.<sup>[2]</sup> A high prevalence of oral cancer in India and other Asian countries has been attributed to the influence of region specific epidemiological factors such as tobacco and betel quid chewing.<sup>[3]</sup> Another alarming scenario is the increasing incidence of oral squamous cell carcinoma (OSCC) in younger people owing to the heavy abuse of tobacco and tobacco related products.

The high morbidity rate in OSCC can be attributed to the delay in the diagnosis of the disease.<sup>[4]</sup> The diagnosis of oral cancer and/or the malignant

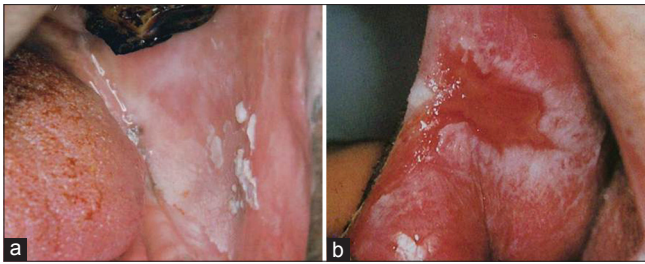
potential of an oral lesion is based on various aspects such as (a) etiology-associated with the use of tobacco, presence of factors such as detection of HPV (b) clinical appearance of the lesion (leukoplakic, erythroplakic, nodular, ulcerative, verrucous) [Figures 1 and 2] (c) location of the lesion - the high risk sites being floor of the mouth, ventrolateral aspect of the tongue etc., (d) histopathological aspects - presence of epithelial dysplasia [Figure 3] and (e) molecular biological aspects of the lesion.<sup>[5]</sup> A detailed discussion on the clinical and histopathological diagnosis and the biological aspects of cancer development are not within the scope of this review.

The diagnosis of epithelial dysplasia is based on a static picture though the histologic changes imply the possibility of a dynamic process. However, the problems in evaluating the significance of epithelial dysplasia arise from a lack of objectivity in the evaluation of established criteria, arbitrary division of the grading, and lack of sufficient knowledge of which criteria are important for the prediction of malignant potential.<sup>[6]</sup> A better understanding of the biological process of oral cancer have led to many studies in the field of molecular biological markers which help in assessment of cancer risk, potentiality

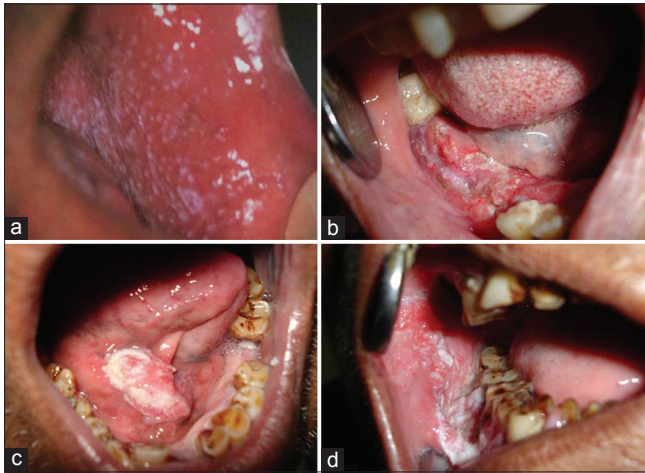
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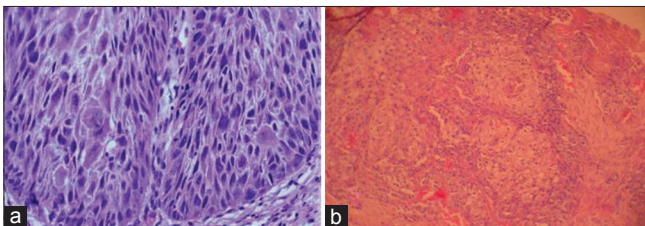
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**Figure 1:** Potentially malignant lesions (a) Leukoplakia and (b) Erosive Lichen planus



**Figure 2:** Various clinical presentations of oral cancer (a) Erythroleukoplakia (b) Ulcerative (c) Nodular/verrucoid (d) Ulcero-proliferative



**Figure 3:** Photomicrograph showing (a) Severe dysplasia (b) Moderately differentiated OSCC

of a lesion towards malignant transformation and also in predicting the prognosis.<sup>[7]</sup>

Neoplastic process gives/produces several abnormal cellular products which can be detected in various body fluids and on the surface of cancer cells either by biochemical methods or by immunohistochemistry; such products that are detected and measured are known as “tumor markers”.<sup>[8]</sup> These tumor markers aid the clinician greatly if the clinico-pathological picture is not accurately suggestive of malignancy and/or is indicative if the picture would soon change. Circulating tumor markers for OSCC have been investigated in various studies<sup>[9-14]</sup> and these have

shown relatively moderate sensitivity and specificity values. Investigators over the years have used two approaches for the study of markers of malignant development: Epithelial dysplasia on one hand and oral cancer on the other are characterized by the presence/absence or the pattern of distribution of the marker in question. In general, the marker is characterized as a promising tool if the reaction pattern in epithelial dysplasias is similar to that in carcinomas and/or if the aberrant reaction pattern is positively related to the grade of epithelial dysplasia. These biological markers for determining future cancer development in oral pre-malignant lesions can be broadly divided into (a) genomic markers, including DNA content (ploidy) chromosome aberration, and changes in expression of oncogenes and tumor suppressor genes; (b) proliferation markers; and (c) differentiation markers, including keratins and carbohydrate antigens which will be discussed.

Saliva, a watery and frothy substance produced in the mouth of human and most other animals contains informative components that have been used as diagnostic markers for human disease. An increasing number of systemic diseases and conditions, amongst them oral cancer, have been shown to be reflected diagnostically in saliva. Saliva as a diagnostic fluid meets the demand for an inexpensive, non invasive and accessible in the diagnosis, prognosis prediction, as well as for monitoring the patients post therapy status in oral cancer.<sup>[14]</sup>

## MATERIALS AND METHODS

Literature search in Medline, PubMed and Scholar Google was performed using the key words salivary markers, tumor markers in saliva, oral cancer, salivary diagnostics, DNA markers, Protein markers and RNA markers. Manual search from various printed articles was also performed.

## DISCUSSION

### Tumor marker

A tumor marker is a substance present in or produced by a tumor or tumors' host in response to the tumor's presence that can be used to differentiate a tumor from normal tissue or determine the presence of a tumor based on measurement in blood or secretions.<sup>[15]</sup> Lehto, Ponten.<sup>[16]</sup> have defined tumor markers as “specific, novel or structurally altered cellular macromolecules or temporarily spatially

or quantitatively altered normal molecules that are associated with malignant (and in some cases benign) neoplastic cells.” Tumor markers may be unique genes or their products that are formed only in tumor cells or they may be genes or gene products that are found in normal cells but are aberrantly expressed in unique locations in the tumor cells.<sup>[17]</sup> The distinguishing biological characteristics of tumor cells such as the capacity for invasion, metastasis, unlimited proliferation, evasion of apoptosis and angiogenesis are all mediated by complex molecular pathways; any one of these components are potential tumor markers.<sup>[18]</sup>

### An ideal tumor marker

A biological marker should have certain characteristics that are applicable in all situations. Kaplan and Pesce<sup>[19]</sup> have suggested the following criteria for an ideal tumor marker:

1. Be easy and inexpensive to measure in readily available body fluids.
2. Be specific to the tumor being studied and commonly associated with it.
3. Have a stoichiometric relationship between plasma levels of the marker and the associated tumor mass.
4. Have an abnormal plasma level, urine level or both in the presence of micro-metastases, that is, at a stage when no clinical or presently available diagnostic methods reveal their presence.
5. Have plasma levels, urine levels or both, that are stable and not subject to wild fluctuations. If present in the plasma of healthy individuals, exist in a much lower concentration than that found in association with all stages of cancer.

In addition to the above, they<sup>[19]</sup> have stated that the ideal tumor marker should relate to the clinical setting and comply with the following:

1. They should prognosticate a higher or lower risk for eventual development of recurrence.
2. They should change as the current status of the tumor changes over time.
3. They should precede and predict recurrences before they are clinically detectable.

### Saliva and its diagnostic potential

The ability to use saliva to monitor a patient’s health and disease state is a highly desirable goal for health promotion and health care research. Saliva has long been proposed and used as a diagnostic medium<sup>[20]</sup> as it is easily accessible, collection is noninvasive,

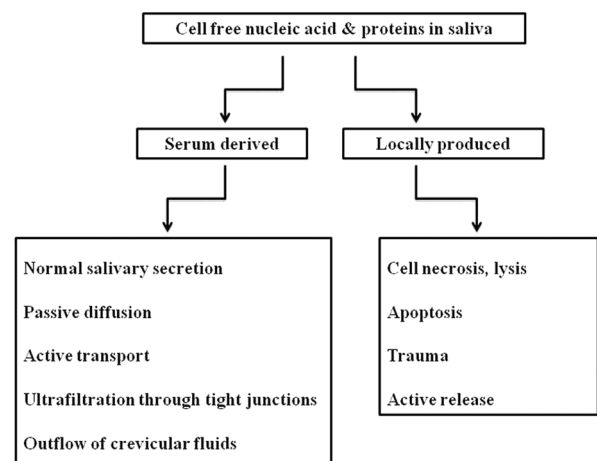
not time consuming, inexpensive and can be used for mass screening purposes.<sup>[21]</sup> A major drawback to using saliva as a diagnostic fluid has been the notion that informative analytes generally are present in lower amounts than in serum.<sup>[22]</sup> However with new techniques of detecting small quantities of salivary components including proteins and messenger Ribonucleic acid (mRNA), almost anything that can be measured in blood can be measured in saliva. Hence, saliva is considered as the blood stream of oral cavity. Alterations in the levels of certain mRNA molecules<sup>[23]</sup> and of certain proteins have been detected in several cancers,<sup>[24]</sup> including oral cancers indicating their possible use in cancer detection and follow-up.

### Mechanism for the presence of biomarker in saliva

Though the exact mechanism for the presence of these tumor markers in saliva is not known, they may be either derived from serum or can be produced locally.<sup>[25]</sup> Figure 4<sup>[25-37]</sup> summarizes the possible mechanism that leads to the presence of biomarkers in saliva.

### Salivary biomarkers for oral cancer detection

Several salivary tumor markers are found to be significantly increased in the saliva of oral cancer patients.<sup>[14,38]</sup> Molecular markers for the diagnosis of oral cancer can be quested in 3 levels;<sup>[39]</sup> changes in the cellular Deoxyribonucleic acid (DNA) which results in altered mRNA transcripts leading to altered protein levels intracellularly, on the cell surface or extracellularly. Markopoulos *et al.*,<sup>[39]</sup> have summarized the molecular markers for the diagnosis of OSCC [Table 1].



**Figure 4:** Mechanism that lead to the presence of molecular markers in saliva

**Table 1: Molecular markers for oral cancer<sup>[39]</sup>**

Changes in cellular DNA	Altered mRNA transcripts	Altered protein markers
Allelic loss on chromosome	Presence of IL-8	Elevated levels of Defensin-1
Mitochondrial DNA mutations	Presence of IL-1 $\beta$	Elevated CD44
P <sup>53</sup> gene mutations	Dual specificity phosphatase 1 (DUSP1)	Elevated IL-6, IL-8
Promoter hypermethylation of genes	H3F3A (H3 histone, family 3A)	Inhibitors of apoptosis (IAP)
Cyclin D1 gene amplification	Ornithine decarboxylase antizyme 1 (OAZ1)	Squamous cell carcinoma associated antigen (SCC-Ag)
Increase in Ki67 markers	S100 calcium binding protein P (S100P)	Carcino-embryonic antigen (CEA)
Micro satellite alterations of DNA	Spermidine/spermine N1-acetyl transferase (SAT)	Carcino-antigen (CA19-9), CA128
Presence of HPV and EBV virus genome		Serum tumor marker (CA125)
		Intermediate filament protein (Cyfra 21-1)
		Tissue polypeptide specific antigen (TPS)
		Reactive nitrogen species (RNS)
		8-OHdG DNA damage marker
		Lactate dehydrogenase (LDH)
		Immunoglobulin G (IgG)
		s-IgA
		Insulin growth factor (IGF)
		Metalloproteinases

DNA: Deoxyribonucleic acid; mRNA: messenger Ribonucleic acid; HPV: Human Papilloma Virus; EBV: Epstein Barr Virus

### Alterations in host cellular DNA

DNA markers are universal i.e., there is not a single tumor cell with in the lesion which does not contain its genetic material. They originate from dead cells, are detected in the early stage of tumorigenesis and are absolutely oncospecific; showing a direct cause-and-effect relationship with tumorigenesis. However, tissue specificity of DNA markers is very low.<sup>[40]</sup> There are several changes in the host DNA of dysplastic or cancer cells which include point mutation, deletion, translocation, amplification, methylation, cyclin D1, epidermal growth factor (EGFR), microsatellite instability and presence of HPV. Sudbo, *et al.*,<sup>[41]</sup> and Femiano, *et al.*,<sup>[42]</sup> have found that premalignant lesions with aneuploidy transform into malignancy more frequently than lesions with normal DNA content irrespective of the histopathological grade of dysplasia. DNA aneuploidy appears to be associated with advanced stage carcinomas, perineural invasion and lymph node metastasis.<sup>[43]</sup> Hence, DNA content of a tumor may help in predicting the aggressiveness of the tumor.

Loss of heterozygosity (LOH) is defined as loss of genomic material in one of the chromosomal pair. Studies have shown that LOH in regions that contain a known human suppressor gene is an early predictor of malignant transformation of precancerous lesion.<sup>[44]</sup> This helps the clinician to identify high and low risk lesions in the context of management. Studies<sup>[45-47]</sup> have demonstrated frequent LOH in

chromosome 3p, 9q, 13q and 17p as an early event in oral carcinogenesis. Rosin, *et al.*,<sup>[48]</sup> in their study found that allelic loss at 3p and 9q increase the risk of malignant transformation by 3.8 fold and the risk of further increase to 33 fold when LOH occurs at chromosomes 4q, 8p, 11q, 13q and 17p in addition to the former.

Mitochondrial DNA mutations have also been useful to detect exfoliated OSCC cells in saliva.<sup>[39]</sup> Such mutations have been identified in 46% of head and neck cancer and in 67% of saliva samples from OSCC patients by direct sequencing.<sup>[49]</sup>

p53 gene located on chromosome 17p13.1 exhibits mutation in 50-70% of epithelial tumors<sup>[50-51]</sup> and LOH of p53 allele has been reported in 22% of pre-cancer and 20% of oral cancer. However, the prognostic significance of p53 in oral cancer is yet to be established although there are multiple studies comparing the expression of p53 in premalignant lesions and malignancies.<sup>[52-53]</sup> Boyle *et al.*,<sup>[51]</sup> using plaque hybridization identified tumor specific p53 mutations in 71% saliva samples from patients with head and neck cancer. Other genes such as p16, p27, p63, p73 related to p53 and cell cycle have been found to be altered in varying degrees in oral cancer.<sup>[50]</sup>

Promoter methylation, an alternate form of gene silencing, which depends on the epigenetic factor has been described to be involved in OSCC.<sup>[50]</sup> The main genes to be methylated are CDKN2A, CDH1,

MGMT, DAPK1,<sup>[50,54]</sup> CDKN2A which are involved in the retinoblastoma pathway of the cell cycle appear to be methylated in 23-67% of primary OSCC's. CDH1 a gene responsible for cell adhesion, promotes metastasis when mutated and shows promoter methylation in up to 85% of tumors.<sup>[54]</sup> Rosas, *et al.*,<sup>[55]</sup> identified aberrant methylation of at least one of these genes (p16, MGMT, DAP-K) in OSSC and detected promoter hyper methylation in 65% of matched saliva samples in OSCC patients.

Amplification and over expression of c-MYCIN-MYC has been observed in 20-40% of oral cancers. Das, *et al.*,<sup>[56]</sup> have reported amplification of 11q13, containing 1NT2, HST1 and Cyclin D oncogenes in 30-50% of patients with oral cancer. Cyclin D1 gene amplification has been found to be associated with poor prognosis in OSCC.<sup>[57]</sup> STAT 3 expression and activation was found in 82% of oral cancers related to chewing tobacco, while there was no expression in normal mucosa and premalignant lesions.<sup>[58]</sup>

Levels of Ki67 marker were increased while 8-oxoguanine DNA glycosylase, phosphorylated-Src and mammary serine protease inhibitor (Maspin) were found decreased in the saliva of patients with OSCC.<sup>[59]</sup>

The presence of HPV and EBV virus genomic sequence have been identified as possible DNA markers in detecting OSCC and tumor progression.<sup>[60]</sup>

### RNA as a biomarker

RNA has been found to be a robust and informative marker and salivary RNA signatures have been identified for oral cancer. RNA for years was thought to degrade in saliva due to the various RNAases that is present in saliva.<sup>[61]</sup> However, cell free RNA is present in saliva both in intact and fragmented forms.<sup>[62]</sup> It has been speculated that salivary mRNA is contained in apoptotic bodies<sup>[29,30]</sup> or actively released in exosomes or micro vesicles.<sup>[32,34,35]</sup> Researchers<sup>[63]</sup> compared the clinical accuracy of saliva with that of blood RNA biomarker for oral cancer detection and found four RNA biomarkers that have a sensitivity and specificity of 91% and 71% and a collective receiver operator characteristic (roc) value of 0.95.<sup>[18]</sup>

Studies have found seven mRNA molecules; (1) IL-8,<sup>[63-64]</sup> (2) IL1 $\beta$  (interleukin 1)<sup>[63-64]</sup> which take part in signal transduction, proliferation, inflammation and apoptosis, (3) DUSP1<sup>[64]</sup> (dual specificity phosphatase 1) with a role in protein modification, signal transduction and oxidative

stress, (4) H3F3A<sup>[64]</sup> (H3 histone, family 3A) having a DNA binding activity, (5) OAZ1<sup>[63,64]</sup> (ornithine decarboxylase antizyme 1) taking part in polyamine biosynthesis, (6) S100P<sup>[64]</sup> (S100 calcium binding protein P) with a role in protein binding and calcium ion binding and (7) SAT<sup>[63,64]</sup> (spermidine/spermine N1-acetyltransferase) which takes part in enzyme and transferase activity, to be significantly elevated in OSCC patients rather than in healthy controls.

### Protein markers

Protein markers are differentiation antigens of corresponding normal tissue and characterize a certain stage of its maturation. They originate from live cells and show high tissue specificity. However, they may be detected in other pathologies as well.<sup>[40]</sup> Salivary protein markers have shown moderate sensitivity and specificity as prognostic markers.<sup>[39]</sup> A group of leading researchers<sup>[64-66]</sup> using new and sophisticated approaches, such as Luminex Multianalyte Profiling (xMAP) technology, shotgun proteomics, capillary reversed-phase liquid chromatography with quadruple time off light mass spectrometry and matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS) has contributed significantly in recent years to the research in saliva for cancer diagnosis. Their studies have shown that saliva contains proteins that may serve as biomarkers for OSCC, since 46 peptides/proteins were found at significantly different levels between the OSCC and control groups.

Carbonylation (indicative of oxidative damage to proteins) has attracted a great deal of attention in cancer research because of its irreversible and irreparable nature, which becomes cytotoxic and is associated with cancer.<sup>[67]</sup> It is currently reported that a substantial increase in salivary carbonyls (246%) is seen in OSCC patients and points to the fact that there is a significant free radical attack to which the epithelial cells are exposed.<sup>[59]</sup>

Metalloproteinases such as MMP-9,<sup>[59]</sup> MMP-11,<sup>[68]</sup> MMP-2<sup>[68]</sup> are significantly altered in OSCC. MMP 9 are shown to degrade type IV collagen, a major type in the basement membrane, other types of collagen (V, VII, X), elastin and fibronectin.<sup>[59]</sup> Studies<sup>[68,69]</sup> have shown high MMP-9 expression in stromal cells surrounding the invading front of metastasizing tumors.<sup>[69-70]</sup> MMP-9 polymorphism was shown to have a strong association with increased risk for developing OSCC.<sup>[71]</sup> Shpitzer, *et al.*,<sup>[59]</sup> found a 39%

increase in MMP-9 with a sensitivity of 100% and specificity of 79% in OSCC patients.

IL-6, IL-8 are post inflammatory cytokines that play a prominent role in immune host defense responses to infection, they are found to stimulate angiogenesis, influence tissue remodeling and take part in regulation of cell proliferation and differentiation. They are identified as important mediators of cancer development and powerful activators of not only apoptosis but also anti apoptotic signaling cascade<sup>[72]</sup> and hence play a role in early detection of oral pre-malignancies and OSCC. Rajkumar, *et al.*,<sup>[38]</sup> in their study found concentration of IL-6 and IL-8 in saliva to be significantly increased. St John, *et al.*,<sup>[73]</sup> detected higher levels of IL-8 in saliva of OSCC patients. Studies have found significant increase in the levels of tumor marker in patients with OSCC compared to dysplastic oral lesions and controls, thus suggesting its diagnostic value as a marker of malignant transformation in oral premalignant lesions.<sup>[74]</sup> Arllano-Garcia, *et al.*,<sup>[75]</sup> using Luminex MAP technology showed that both IL-8 and IL-1 $\beta$  were expressed at significantly higher levels in OSCC patients.

Cytokeratins are cytoskeletal intermediate filaments present in almost all normal and malignant epithelial cells.<sup>[76]</sup> In malignant epithelial cells, the activated protease increases degradation of cytokeratin free filaments into the blood. Increased levels of Cyfra 21-1 in saliva has been found in OSCC.<sup>[10,11,14]</sup>

Defensins are peptides which possess antimicrobials and cytotoxic properties. They are found in azurophilic granules of polymorphonuclear leukocytes.<sup>[77-78]</sup> Mizukawa, *et al.*,<sup>[79]</sup> found elevated levels of salivary Defensin 1 in OSCC patients compared with healthy controls. Some of the other salivary biomarkers which are significantly altered in OSCC patients as compared with healthy controls are inhibitors of apoptosis (IAP),<sup>[80]</sup> squamous cell carcinoma associated antigen (SCC-Ag),<sup>[13,14]</sup> carcino-embryonic antigen (CEA),<sup>[12,14]</sup> carcino-antigen (CA19-9),<sup>[13,14]</sup> CA128,<sup>[13]</sup> serum tumor marker (CA125),<sup>[14,81]</sup> tissue polypeptide specific antigen (TPS),<sup>[14,82]</sup> reactive nitrogen species (RNS),<sup>[9]</sup> lactate dehydrogenase (LDH)<sup>[11]</sup> and immunoglobulin G (IgG),<sup>[11]</sup> s-Ig A,<sup>[68]</sup> insulin growth factor (IGF).<sup>[68]</sup>

#### Uses of tumor markers

Recent technological advances in molecular biology has led to discovery of new tumor markers and thus

increased its scope of use in oral biology. Tumor markers have a role in the diagnosis, prognosis, formulating the treatment and in the detection of recurrence of cancer. Chan and Sell.<sup>[15]</sup> have summarized the potential uses of tumor markers as follows:

- (a) screening in general population
- (b) differential diagnosis in symptomatic patients
- (c) clinical staging of cancer
- (d) estimating tumor volume
- (e) prognostic indicators for disease progression
- (f) evaluating the success of treatment
- (g) detecting recurrences
- (h) Monitoring responses to therapy
- (i) radioimmunolocalization of tumor masses and
- (j) Determining direction for immunotherapy.

## CONCLUSION

Present day screening tests by visual examination, or other well established and time tested diagnostic tools such as vital staining with toluidine blue, brush biopsy, chemiluminescence, tissue autofluorescence and the diagnostic gold standard being tissue biopsy followed by histopathological evaluation for OSCC are still critical for the diagnosis of potentially malignant lesions and malignancies. The field of salivary diagnostics is a broad, complex and crosscutting area of scientific research with enormous potential to impact the diagnosis of various diseases including oral cancer. The significant increase in tumor markers in saliva is quite encouraging as saliva offers many advantages such as saliva harvesting is noninvasive thus making it an effective alternative to serum testing. Though at present no single tumor marker can validate the presence or prognosis of disease, a panel of biomarkers would be more helpful. Recent advances and emerging technologies such as nanotechnology, proteomic and genomics make salivary diagnostics a highly sensitive tool. The present methods are not ready for immediate direct use and much needs to be done to come up with a fast, simple, cost effective clinical diagnostic system. Saliva will possibly outperform other media in the diagnosis of not only cancer but other diseases as well.

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