10.5005/jp-journals-10050-10096

REVIEW ARTICLE

Role of Salivary Biomarkers for Early Detection of Oral Squamous Cell Carcinoma

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ABSTRACT

Introduction: Oral cancer is a potentially fatal disease, which constitutes an important portion of tumors of the head and neck region. Among head and neck cancers, oral squamous cell carcinomas (OSCCs) constitute 90% of total cancers. Regardless of the fact that the oral cavity is easily accessible to the accumulation of carcinogens, most oral cancers are typically detected at an advanced stage leading to lower survival rate among subjects. Abnormal cellular products elucidated from malignant cells can be detected and measured in various body fluids including saliva, which constitute tumor markers. Saliva, an aqueous biological fluid, is in direct contact with the oral cancer lesion. Hence, the saliva in any stage of oral cancer constitutes abnormal deoxyribonucleic acid (DNA), acid (RNA), and protein molecules. Saliva, being a noninvasive diagnostic aid, can be an alternative to serum for early detection, status of chemotherapy regime, and also patient prognosis. This article aims at providing a brief overview of various salivary biomarkers and their implications in oral cancer.

Keywords: Carcinoma, Deoxyribonucleic acid, Epithelial, Ribonucleic acid, Saliva, Tumor markers.

How to cite this article: Gupta P. Role of Salivary Biomarkers for Early Detection of Oral Squamous Cell Carcinoma. Int J Adv Integ Med Sci 2017;2(3):155-160.

Source of support: Nil

Conflict of interest: None

INTRODUCTION

Oral cancer comprises cancers in the lip and oropharynx. They are the sixth most common type of cancers worldwide. Oral cancer has a 5-year mortality rate of approximately 50%,[1] which has not changed significantly over the last 50 years.[2] Oral cancers are thought to account for an estimated 650,000 new cancer cases and 350,000 cancer deaths worldwide per year.[3] There are certain high-risk regions that include south-central Asia for cancers of the oral cavity, and South America and western Asia for laryngeal cancers.[3]

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Among all the possible etiological factors, human papillomavirus (HPV) plays a significant role. It is well established that the major risk factors for oral cancer are tobacco and alcohol consumption constituting approximately 75% of cases. In patients consuming both products, the risks for cancer occur in a supraadditive fashion, and generally increase more than 35 times in patients who consumed more than 20 cigarettes and more than 4 drinks on any day or 14 per week.[3] The high morbidity rate in OSCC can be attributed to the delay in the diagnosis of the disease.[4]

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), (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, and (e) molecular biological aspects of the lesion.[5]

At present, the diagnosis of OSCC is through comprehensive clinical examination and histological analysis of suspicious areas, but it may remain undetected in hidden sites.[6] Since the molecular pathogenesis of oral cancer is complex, numerous studies have been done in this field to understand the possible role of molecular biological markers in cancers, which help in assessment of cancer risk, and potentiality of a lesion toward malignant transformation and also in predicting the prognosis.[7]

Currently, various researches are being carried out with the use of saliva as a diagnostic aid for early detection of OSCC. Saliva has the advantage of being readily available. In addition, the collection of saliva is noninvasive, safe, and patient compliant. Saliva, in cancer patients, contains significantly higher proportion of proteins, messenger RNA (mRNA), enzymes, and other chemicals in comparison with control samples. Since saliva is a fluid medium, therefore, oral cancer produces several abnormal cellular products, such as cellular DNA, proteins, mRNA, enzymes, and chemicals during carcinogenesis, which are released into saliva and can be extracted and analyzed by immunohistochemistry or other biochemical methods. These products are collectively termed as "tumor markers."[8] Various studies have been carried out in the field of oral cancer using tumor markers.[9,10]

These biomarkers may perhaps be important indicators of physiological or pathological conditions and provide information for the detection of oral cancer at any stage. Thus, they could serve as a widely available screening tool for diagnosis and prognosis of the disease process. There are two ways for studying the role of biomarkers in oral cancer, i.e., grade of epithelial dysplasia and presence/absence or the distributive pattern of the biomarkers.[11]

Classification of Cancer Biomarkers[12]

- *Based on Biomolecules*
  - DNA biomarkers
  - RNA biomarkers
  - Protein biomarkers
  - Glyco biomarkers
- *Based on Disease State*
  - Prediction biomarkers
  - Detection biomarkers
  - Diagnosis biomarkers
  - Prognosis biomarkers
- *Based on Other Criteria*
  - Pathological biomarkers
  - Imaging biomarkers
  - *In silico* biomarkers

An Ideal Tumor Marker

A biological marker is one that possesses certain characteristics as described below. Kaplan and Pesce[13] have suggested the following criteria for an ideal tumor marker:[14]

- Should be easily available in body fluids
- Should be inexpensive when measured in laboratory settings
- Be specific to the tumor being studied and commonly associated with it
- Have a stoichiometric relationship between plasma levels of the marker and the associated tumor mass
- Have an abnormal plasma level, urine level, or both in the presence of micro-metastases, i.e., at a stage when no clinical or presently available diagnostic methods reveal their presence.
- 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 have stated that the ideal tumor marker should relate to the clinical setting and comply with the following:[14]

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

Biomarkers for Oral Cancer Applications[15]

- Biomarkers predict the earliest change in the malignant transformation of epithelial cell.
- The early identification of markers will, therefore, help the clinicians to institute various preventive measures and necessary therapeutic measures at the earliest.
- Biomarkers will reveal various genetic and molecular changes in the early, intermediate, and late stages of oral cancer.
- Monitor progression or recurrence and treatment compliance.
- Useful in early stages of cancer drug development.
- Determine efficacy and safety of chemopreventive agents

Conjectures about Probable Mechanisms that Lead to the Presence of Genotypic and Phenotypic Markers in the Saliva[11]

Salivary cell-free nucleic acids and proteins may be derived from serum or can be locally produced. In the salivary glands, acinar cells will produce nucleic acids and proteins, which later gain entry into the oral cavity through intracellular routes (active transport or passive diffusion) or extracellular routes (ultrafiltration through tight junctions) or through gingival crevicular fluid. The cancerous cell in all stages will release cellular DNA and proteins in saliva as a result of cell necrosis, apoptosis, and trauma. Another mechanism leading to the release of mRNA, microRNA (miRNA), and proteins in the saliva is through exosomes originating from the endoplasmic reticulum. They are thought to play a role in the cell-free intercellular communication (Table 1).

Molecular Markers for Oral Cancers

There are numerous biomarkers that have a significant role in OSCC patients. They are classified as DNA biomarkers, RNA biomarkers, or protein markers (Table 2).

Table 1: Possible mechanism of entry of biomarkers into the saliva

Serum-derived biomarkers	*Locally produced biomarkers*
Through normal salivary secretion	Cell necrosis, lysis
Active transport from cell membrane	Apoptosis
Passive diffusion	Trauma
Ultrafiltration through tight junctions	Through exosomes
Outflow of crevicular fluid	

Table 2: Molecular markers for oral cancers

Changes in the cellular DNA	*Altered RNA transcripts*	*Altered protein markers*
• Allelic loss on chromosome	• Presence of IL-8, IL-1B	• Increase in IL-6, IL-8
• Mitochondrial DNA mutations	• S100 calcium binding protein P (S100 P)	• Elevated CD-44
• Cyclin D1 gene amplification	• Dual-specificity phosphatase 1 (DUSP 1)	• Elevated level of defensin-1
• Increase in Ki67 marker	• H3 histone family 3A (H3F3A)	• Inhibitors of apoptosis (IAP)
• Hypermethylation of genes	• Ornithine decarboxylase antizyme	• SCC-associated antigen (SCC-Ag)
• Presence of HPV and EBV genomes		• Carcinoembryonic antigen
		• Serum tumor marker (CA125)
		• Lactate dehydrogenase
		• RNS
		• Intermediate filament protein (cyfra-21)
		• MMPs
		• Insulin growth factor
		• Immunoglobulin G

IL: Interleukin

Changes in the Cellular DNA

Point mutations, deletions, translocations, amplifications and methylations, cyclin D1, epidermal growth factor receptor, microsatellite instability, and HPV presence are the typical changes present in the host DNA of dysplastic or cancer cells. Femiano and Scully,[16] based on their research, have found that the malignant potential is more in premalignant lesions with higher DNA content than lesions with normal DNA content irrespective of any histopathological grading of tumor. Therefore, cancers with higher DNA content predict the aggressiveness of the tumor[17] and such cancers have greater probability for spread through perineural invasion and lymph node metastasis.

Loss of heterozygosity (LOH) is defined as loss of genomic material in one of the chromosomal pair. Based on various studies, it has been shown that presence of tumor suppressor gene (TSG) in the area of LOH predicts early malignant transformations in potentially malignant disorders.[18] This helps the clinician to identify high- and low-risk lesions in the context of management. Studies have demonstrated frequent LOH in chromosome 3p, 9q, 13q, and 17p as an early event in oral carcinogenesis.[19,20]

Mitochondrial DNA mutations have also been useful to detect exfoliated OSCC cells in saliva.[20] Such mutations have been identified in 46% of head and neck cancer and in 67% of saliva samples from OSCC patients by direct sequencing.[21]

The p53 TSG gene located on chromosome 17p13.1 shows mutation in 50 to 70% of epithelial tumors, and LOH of p53 allele has been reported in 20% of oral cancer.[22] Boyle et al[22] showed mutations in the p53 TSG in 71% salivary samples in head and neck cancer patients using plaque hybridization. Other genes related to p53 such as p16, p27, p63, p73 are found to be altered in oral cancer.

In cancer cells, aberrant methylation of TSGs is a common finding and it has been reported that promoter hypermethylation of p53 TSG gene is a suitable biomarker in OSCC patients. Cyclin D1 gene amplification has been related to the poor prognosis in patients with OSCC.[23]

In saliva of OSCC patients, the levels of Ki67 marker were found to be increased while 8-oxoguanine DNA glycosylase, phosphorylated-Src, and mammary serine protease inhibitor (Maspin) were found to be decreased as evident in various studies.[24]

The presence of HPV and Epstein–Barr virus (EBV) genomic sequence has been identified as possible DNA markers in detecting OSCC and tumor progression.[25]

RNA as a Biomarker

The RNA has been found to be informative marker, and salivary RNA signatures have been identified for oral cancer. The RNAases are the enzymes that are associated with the degradation of RNA present in saliva.[26] However, cell-free RNA is present in saliva both in intact and fragmented forms. It has been speculated that salivary mRNA is contained in apoptotic bodies or actively released in exosomes or micro vesicles.[11] Researchers compared the clinical accuracy of saliva with that of blood RNA biomarker for oral cancer detection and found that RNA biomarkers in saliva have a sensitivity and specificity of 91 and 71% respectively, when compared with blood RNA biomarkers.[27]

Li et al, based on their research findings, concluded that a variety of mRNA molecules were upregulated in the saliva of OSCC patients.[28]

Seven mRNA molecules and their function have been described (Table 3).[20]

miRNA as a Biomarker

The miRNAs are considered as potential biomarkers found in saliva of patients with OSCC. They are short

Table 3: mRNA molecules in saliva and their functions

mRNA transcripts	*Functions*
1 IL-8	Angiogenesis, replication, cell adhesion, chemotaxis, cell cycle arrest, and immune response
2 IL-1ß	Signal transduction, proliferation; inflammation and apoptosis
3 Dual specificity phosphatase 1	Protein modification, signal transduction, and oxidative stress
4 Ornithine decarboxylase antizyme 1	Polyamine biosynthesis
5 H3 histone, family 3A	DNA binding activity
6 S100 calcium binding protein P	Protein and calcium ion binding
7 Spermidine N1-acetyltransferase	Enzyme and transferase activity

(19–25 nucleotides) transcripts of RNA associated with posttranscriptional regulation by the RNA-induced silencing complex. The miRNAs are thought to play a vital role in cellular growth and differentiation, apoptosis, and immune function. The miRNA content has been found to be increased in various cancer types, ranging from 10 to 100 times the normal amount. The miRNA has been proved to be a useful marker when compared with mRNA in discriminating solid and poorly differentiated tumors. In contrast to studies indicating high content of miRNAs, one study (Park et al)[29] reported significantly reduced levels of miRNAs, such as miR-125a and miR-200a (tumor suppressors) in the saliva of patients with OSCC when compared with control group. Recently, it is also shown that salivary miR-31 (implicated in tumorigenesis) was appreciably superior in all stages of oral cancer, and that salivary miR-31 was more copious than blood miR-31, representing the oral tumor origin of this biomarker.[29]

Proteins as Biomarkers

Proteins are highly tissue-specific molecules originating from the living cells. Protein markers are considered as differentiation antigens of corresponding normal tissue and typify a certain stage of its maturation. Increased content of various protein biomarkers has been detected in many pathologies in head and neck region including OSCC.[30] Salivary protein markers are moderately sensitive and specific as prognostic markers.[21]

Carbonylation (indicative of oxidative damage to proteins), because of its irreversible and irreparable nature and its association with cancer, has attracted a great deal of attention in cancer research. 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.[24]

The cytoskeletal intermediate filaments are present in almost all normal and malignant epithelial cells as a result of increased activity of proteases enzymes, which causes degradation of filaments. These intermediate filaments are termed as "cytokeratins." Salivary samples in OSCC patients contain increased levels of cytokeratins (Cyfra 21-1).[10,31]

In addition to cytokeratins, many cytokines, such as IL-6, IL-8 are thought to play a role in host defense mechanism and inflammatory process. They play a prominent role in tissue remodeling, angiogenesis, cellular proliferation, and differentiation. They are identified as important mediators of cancer development and powerful activators of not only apoptosis, but also antiapoptotic signaling cascade and, hence, play a role in early detection of oral premalignancies and OSCC.[32] Several studies reported increased levels of cytokines IL-6 and IL-8 in salivary samples of OSCC patients.[33]

Altered profiles of matrix metalloproteinases (MMP-9, 11) have been reported in many variants of OSCC. Among them, MMP-9 polymorphism was shown to have a strong association with increased risk for developing OSCC.[34] The MMP-9 causes degradation of basement membrane type-IV collagen, collagen (V, VII, X), elastin, and fibronectin.[23]

Defensins are peptides found in azurophilic granules of polymorphonuclear leukocytes. They are known to possess antimicrobials and cytotoxic properties. Studies conducted by Mizukawa et al[35] reported increased salivary levels of defensin 1 in OSCC patients compared with control group.

Other salivary biomarkers that are significantly altered in OSCC patients as compared with healthy controls are inhibitors of apoptosis, SCC-Ag, RNS,[9] IGF.[23] The lactate dehydrogenase[28] and immunoglobulin G,[28] tissue polypeptide specific (PPS) antigen,[36] carcinoembryonic antigen,[36] and carcinoantigen (CA19-9, 128).[37]

Uses of Salivary Biomarkers

Recent technological advances in molecular biology have led to discovery of new tumor markers and, thus, increased its scope of use in oral biology. Salivary biomarkers perform a vital role in early detection of, formulating proper diagnosis, prognosis, treatment plan, and detection in case of recurrence. Chan and Sell[38] have summarized the potential uses of salivary tumor biomarkers as follows:

- Screening in general population
- Differential diagnosis in symptomatic patients
- Clinical staging of cancer
- Estimating tumor volume
- Prognostic indicators for disease progression

- Evaluating the success of treatment
- Detecting recurrences
- Monitoring responses to therapy
- Radioimmunolocalization of tumor masses
- 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. Advancements in the field of molecular biology have led to many new tumor markers identified in the saliva. Since saliva is an abundant fluid present in the oral cavity, it offers many advantages, such as easily accessibility and inexpensive laboratory testing, making it an effective alternative to serum testing. Further, precise diagnostic aids, such as proteomic, genomics, and nanotechnology make saliva an excellent medium not only for diagnosis of OSCC, but for many other diseases as well.

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