

Salivary Markers for Oral Cancer Detection

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Abstract: Oral cancer refers to all malignancies that arise in the oral cavity, lips and pharynx, with 90% of all oral cancers being oral squamous cell carcinoma. Despite the recent treatment advances, oral cancer is reported as having one of the highest mortality ratios amongst other malignancies and this can much be attributed to the late diagnosis of the disease. Saliva has long been tested as a valuable tool for drug monitoring and the diagnosis systemic diseases among which oral cancer. The new emerging technologies in molecular biology have enabled the discovery of new molecular markers (DNA, RNA and protein markers) for oral cancer diagnosis and surveillance which are discussed in the current review.

Keywords: OSCC, DNA, mRNA, miRNA.

INTRODUCTION

Oral cancer refers to all malignancies arising from the lips, the oral cavity, and pharynx [1] and it affects more than 481,000 new patients worldwide. It is the sixth most common cancer in the USA [2]. The 90% of oral cancers are oral squamous cell carcinoma. This cancer, when found early, has an 80 to 90% survival rate. Despite this fact and the great treatment advances, the World Health Organization has reported oral cancer as having one of the highest mortality ratios amongst other malignancies with a death rate at five years from diagnosis at 45% [3]. This high morbidity rate can definitely be attributed to the late diagnosis of the disease [4]. At the moment, a lack in national screening programs together with a lack of definitive and satisfactory biological markers [5-7] for early oral cancer detection has resulted in late stage diagnosis of oral cancer [8]. An increasing number of systemic diseases and conditions, amongst them oral cancer, have been shown to be reflected diagnostically in saliva. Moreover, using saliva as a diagnostic fluid meets the demands for inexpensive, noninvasive, and accessible diagnostic methodology.

Attempts on Early Oral Cancer Detection

The most reliable method for the diagnosis of oral cancer is a tissue biopsy followed by a histopathological evaluation of the tissue specimen [9, 10]. This however takes as granted that a usually asymptomatic lesion will be detected by the patient who will be alerted and will then soon visit a dentist's or other practitioner's office [11]. Because oral cancers usually lack early signs, there have been in the past several attempts towards the direction of early oral cancer detection and attention has been drawn to cancer screening programs [12, 13]. Most oral cancer screening programs include the simple visual inspection [9, 14], whereas others attempt

the use of toluidine blue [15, 16], brush biopsy (exfoliative cytology) [17, 18], chemiluminescence [19, 20] and fluorescence imaging [21]. The last three screening methods in fact deal with the diagnosis of lesions that have already been detected by the patient, dentist or other clinician but a definitive diagnosis can only be made by a tissue biopsy.

However, according to Kujan *et al.* [22, 23], "there is not enough evidence to decide whether screening by visual inspection reduces the death rate for oral cancer and also no robust evidence exists to suggest that other methods of screening, toluidine, fluorescence imaging or brush biopsy are either beneficial or harmful".

Cancer Related Genetic Alterations Identified in Bodily Fluids

In the development of neoplastic disease, progressive genotypic and phenotypic alterations such as the activation of protooncogenes and oncooncogenes and inactivation of tumor suppressor genes -associated with tumorigenesis- are detected in the affected cells, establishing the model of multistep tumorigenesis [24]. It has been shown that identical mutations can be identified in bodily fluids draining a tumor [7], but, also lately in bodily fluids secreted away from the initial point where a solid tumor is developing [25, 26]. Nucleic acids and proteins related to cancer cells have been detected in plasma/serum [27-29], urine [30, 31], saliva [32, 33], bronchoalveolar lavage fluid [27], cerebrospinal fluid [34] and other bodily fluids. These nucleic acids and proteins have been used as molecular markers for the early diagnosis of the disease [33, 35, 36], recurrence markers [37] survival and metastasis predictors [38, 39] and decide the therapeutic approach [40, 41].

Saliva as a Perfect Diagnostic Medium

Whole saliva is the product of the secretions of the 3 major salivary glands (parotid, submandibular, sublingual) and the numerous minor salivary glands mixed with crevicular fluid, bronchial and nasal secretions, blood constituents from wounds or bleeding gum, bacteria, viruses, fungi, exfoliated

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epithelial cells and food debris [42, 43]. Saliva has been long proposed and used as a diagnostic medium [44-46] because it is easily accessible and its collection is non-invasive, not time-consuming, inexpensive, requires minimal training and can be used for the mass screening of large population samples [46, 47].

Whole saliva can be collected with or without stimulation. Stimulation can be performed with masticatory movements or by gustatory stimulation (citric acid) [48]. Stimulated saliva however, it can be collected in larger quantities, is a little bit altered in content [49]. Unstimulated saliva can be collected by merely spitting in a test tube or by leaving saliva drool from the lower lip [50] and it is more often used for the diagnosis or follow up of systemic diseases.

Saliva has long been used for the monitoring of drug abuse (drugs and addictive substances) such as cocaine, heroin, amphetamine, barbiturates [51] etc. Moreover salivary testing has largely performed for the diagnosis of HIV-infection [52, 53]. Analysis of salivary parameters such as salivary flow rate, pH, buffer capacity, lactobacillus, and yeast content, presence of IgG, IgM and anti-La autoantibodies and raised protein levels such as that of lactoferrin and cystatin C as has been proposed for the diagnosis of Sjogren's syndrome [54, 55]. Concerning cancer diagnostics and follow up altered levels of certain mRNA molecules [33, 56] have been detected in saliva in oral cancer patients and of certain proteins in several cancers [25, 26, 57].

Speculations about Possible Mechanisms that Lead to the Presence of Genotypic and Phenotypic Markers in the Saliva

Cell-free nucleic acids and proteins in saliva may derive from serum or can be locally produced [58].

Serum derived nucleic acids and proteins in the saliva may be part of the normal salivary secretion (by the acinar cells) [59] or come there either *via* intracellular routes such as active transport or passive diffusion [60] from the serum to saliva across cell membranes or extracellular routes such as ultrafiltration through tight junctions [61] or as constituents of the outflowing crevicular fluid.

Cell free nucleic acids and proteins in saliva however can be locally produced by cell necrosis, lysis or apoptosis and

trauma and may even be actively released by normal epithelial or cancerous cells. Cell necrosis is a possible mechanism leading to the release of cell free nucleic acids and proteins in the saliva and this idea is also supported by the large amount of DNA in the plasma of patients with cancers in an advanced stage. Moreover, mounting evidence exists concerning the presence of cell-free nucleic acids and proteins in apoptotic bodies [62] which also protect these molecules from degradation [63]. The active release of these molecules in exosomes or microvesicles is another strong possibility [64]. Exosomes or microvesicles are released by living cells. They are membrane vesicles, 40–100-nm in diameter [65], originating from the endoplasmic reticulum and are released when fused with the cell membrane. They contain mRNA [66], miRNA [67] and proteins [68, 69] and are thought to play a role in the cell-free intercellular communication [69-71], Table 1.

Salivary Markers for Oral Cancer Detection

Molecular markers for the diagnosis of OSCC can be requested in 3 levels; (I) changes in the cellular DNA, which result in (II) altered mRNA transcripts, leading to (III) altered protein levels (intracellularly, on the cell surface or extracellularly). All these markers are summarized in Table 2.

Changes in the Cellular DNA

Typical changes in the host DNA of dysplastic or cancer cells include point mutations, deletions, translocations, amplifications and methylations, cyclin D₁, epidermal growth factor receptor (EGFR), microsatellite instability and HPV presence.

Allelic loss on chromosomes 9p has been observed in OSCC [72]. Mitochondrial DNA mutations have also been useful targets to detect exfoliated OSCC cells in saliva. They have been identified in 46% of head and neck cancers. The same mitochondrial DNA mutations were detected in 67% of saliva samples from OSCC patients by direct sequencing alone [73]. p53 gene mutations are also present in approximately one-half of head and neck cancers [74, 75]. Using plaque hybridization, Boyle *et al.* [75] identified tumor specific p53 mutations in 71% saliva samples from patients with head and neck cancer.

Table 1. Mechanisms that Lead to the Presence of Genotypic and Phenotypic Markers in the Saliva

Cell-free nucleic acids & proteins in saliva	Serum derived	Normal salivary secretion
		Passive diffusion
		Active transport
		Ultrafiltration through tight junctions
		Outflow of crevicular fluid
	Locally produced	Cell necrosis, lysis
		Apoptosis
		Trauma
		Active release

Table 2. Molecular Markers for the Diagnosis of Oral Squamous Cell Carcinoma

Changes in the cellular DNA	Altered mRNA transcripts	Altered protein markers
Allelic loss on chromosomes 9p	Presence of IL8	Elevated levels of defensin-1
Mitochondrial DNA mutations	Presence of IL1B	Elevated CD44
p53 gene mutations	DUSP1 (dual specificity phosphatase 1)	Elevated IL-6 and IL-8
Promoter hypermethylation of genes (p16, MGMT, or DAP-K)	H3F3A (H3 histone, family 3A)	Inhibitors of apoptosis (IAP)
Cyclin D1 gene amplification	OAZ1 (ornithine decarboxylase antizyme 1)	Squamous cell carcinoma associated antigen (SCC-Ag)
Increase of Ki67 markers	S100P (S100 calcium binding protein P)	Carcino- embryonic antigen (CEA)
Microsatellite alterations of DNA	SAT (spermidine/spermine N1-acetyltransferase)	Carcino-antigen (CA19-9)
Presence of HPV		CA128
		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 (IgG)
		s-IgA
		Insulin growth factor (IGF)
		Metalloproteinases MMP-2 and MMP-11

Promoter hypermethylation of several genes has been reported in head and neck cancer. Rosas *et al.* identified aberrant methylation of at least one of three genes (p16, MGMT, or DAP-K) in OSCC. Abnormal promoter hypermethylation was also detected in the matched saliva sample in 65% of OSCC patients [76].

Cyclin D1 gene amplification has been found to be associated with poor prognosis in OSCC [77]. In another study Ki67 markers 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 [78].

Microsatellite alterations of DNA were also observed in the saliva of patients with small cell lung cancer [79]. In the same study it was further demonstrated that 93% of the patients with microsatellite instability in tumor DNA also had similar microsatellite alterations in the corresponding plasma DNA.

The presence of HPV (human papilloma virus) and Epstein Barr virus genomic sequences have been identified as possible DNA molecular markers in detecting OSCC and tumor progression [80, 81].

Altered mRNA Transcripts

RNA for years was thought to quickly degrade in saliva due to the various RNases that saliva contains [82]. Despite the opposite reports [83], cell-free RNA molecules however, seem to exist in saliva both intact but also fragmented [84]. An intriguing question that remains to be answered is the mechanism by which mRNA in saliva is protected by degradation. A speculation is that salivary mRNA is contained in apoptotic bodies [63, 64] or actively released in exosomes or microvesicles [66, 68,70]. Lately microRNAs, small RNA molecules, 18-24 molecules in length, that seem to regulate transcription were also discovered existing in saliva [85-87].

mRNA detection in saliva has been extensively reported enabling body fluid identification in Forensic Medicine [88, 89]. Moreover mRNA markers in the saliva have been proposed for the diagnosis of primary Sjögren's syndrome [90] and for the identification of sleep drive both in flies but also in humans [91].

Various mRNA molecules were found up-regulated in the saliva of patients suffering from OSCC by the team of Li *et al.* [33]. Seven mRNA molecules: transcripts of: 1. IL8 (interleukin 8) playing a role in angiogenesis; replication; calcium-mediated signaling pathway; cell adhesion; chemo-

taxis; cell cycle arrest; immune response, 2. IL1B (interleukin 1B) which takes part in signal transduction; proliferation; inflammation and apoptosis 3. DUSP1 (dual specificity phosphatase 1) with a role in protein modification; signal transduction and oxidative stress, 4. H3F3A (H3 histone, family 3A) having a DNA binding activity, 5. OAZ1 (ornithine decarboxylase antizyme 1) taking part in polyamine biosynthesis 6. S100P (S100 calcium binding protein P) with a role in protein binding and calcium ion binding, and 7. SAT (spermidine/spermine N1-acetyltransferase) which takes part in enzyme and transferase activity- were found significantly elevated in OSCC patients rather than in healthy controls [56].

Altered Protein Markers

Several salivary protein markers for OSCC have been investigated in various studies and have shown relatively moderate sensitivity and specificity values relative to prognosis prediction.

For example, defensins are peptides which possess antimicrobial and cytotoxic properties. They are found in the azurophil granules of polymorphonuclear leukocytes [92, 93]. Elevated levels of salivary defensin-1 were found to be indicative for the presence of OSCC, since higher concentrations of salivary defensin-1 were detected in patients with OSCC compared with healthy controls [94].

In another study soluble CD44 [95] was found to be elevated in the majority of patients with OSCC and distinguished cancer from benign disease with high specificity. Whereas the solCD44 test lacks sensitivity by itself, methylation status of the CD44 gene seems to complement the solCD44 test and provides very high sensitivity and specificity for the detection of OSCC.

St John *et al.* [32] investigated whether IL-6 and/or IL-8 could serve as informative biomarkers for OSCC in saliva. Interleukin 8 was detected at higher concentrations in saliva, while IL-6 was detected at higher concentrations in serum of patients with OSCC. Thus, they concluded that IL-8 in saliva and IL-6 in serum hold promise as biomarkers for OSCC.

A group of leading researchers [33, 56, 96-99] using new and sophisticated approaches, such as, Luminex Multianalyte Profiling (xMAP) technology, shotgun proteomics, capillary reversed-phase liquid chromatography with quadruple time-of-flight 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. For example Arellano-Garcia *et al.* [100] using Luminex xMAP technology showed that both IL-8 and IL-1 β were expressed at significantly higher levels in OSCC subjects.

Other salivary biomarkers which have been shown to be significantly altered in OSCC patients as compared with healthy controls are inhibitors of apoptosis (IAP) [101], squamous cell carcinoma associated antigen (SCC-Ag) [102-104], carcino-embryonic antigen (CEA) [102, 103], carcino-antigen (CA19-9) [102, 104], CA128 [102, 104], serum tu-

mor marker (CA125) [105], intermediate filament protein (Cyfra 21-1) [106-108], tissue polypeptide specific antigen (TPS) [108, 109], reactive nitrogen species (RNS) and 8-OHdG DNA damage marker [106], lactate dehydrogenase (LDH) and immunoglobulin (IgG) [107], s-IgA [110], insulin growth factor (IGF) [110], metalloproteinases MMP-2 and MMP-11 [110].

CONCLUSIONS

For years saliva is tested as a diagnostic fluid and compared to blood (serum or plasma) in parameters such as sensitivity, specificity and applicability of the method, cost and duration of the procedure, patient compliance [111] etc. Due to the recent advances and emerging technologies in molecular biology new molecular markers (DNA, RNA and protein markers) have been discovered existing in the saliva in measurable quantities [112]. OSCC can be diagnosed with high sensitivity and specificity by merely testing saliva samples from the subjects. This does not of course undermine the value of screening tests by visual examination neither the importance of the tissue biopsy.

Despite the scepticism in the scientific community and the conservatism of the patients, saliva seems to emerge as a valuable tool in cancer diagnostics and mass population screening. In our opinion much attention must be given to the saliva collecting method. An attempt to integrate the simultaneous testing of different salivary molecular markers in order to raise the possibility of an accurate diagnosis by simply using micro- and nano-electrical-mechanical systems biosensors is on the way raising much hope in its future applications [113].

Finally, since the present methods are not ready for immediate clinical use as diagnostic tools, much work is necessary and it can be envisaged that simple, fast, portable and cost-effective clinical diagnostic systems could be available in the near future.

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