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An Insight on salivary biomarkers for oral Cancer

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Abstract

Oral cancer is one of the major public health concerns with 1-2% of all types of cancers worldwide. The 90% of oral cancer issues have been increased due to smoking and alcohol consumption all over the world. The major hindrance is that the most of the oral cancer patients undergo diagnosis at the advanced clinical stages, i.e., III and IV stages. Due to the delayed diagnosis, the morbidity rate is potentially high with minimum of 5 year survival rate in 20-25% of the patients. Cancerous tumors exhibit various biological characteristics at the most similar stages of development because of which detection of cancer hits a lag. Hence, there is an utmost need for the development of effective biological markers that can diagnose the condition at earlier stages. This paper is a review giving details on the biomarkers available for oral cancer diagnosis through noninvasive approach using saliva.

Keywords: Saliva biomarker; Oral cancer

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Introduction

Oral cancer is one of the major public health concerns with 1-2% of all types of cancers worldwide. It is the 6th most common human malignancy with 50% mortality rate/5 years [1,2]. Cancer can evolve in various locations in the oral cavity such as lining of cheeks, tongue, floor of mouth and lips etc. The 90% of oral cancer issues has been increased due to smoking and alcohol consumption all over the world [3,4]. But the highest prevalence is observed in Asian countries due to their regional influence on tobacco and betel chewing [3]. The incidence rate is also elevating among younger generation who are prone towards consumption of commercially available tobacco related products.

The major hindrance is that the most of the oral cancer patients undergo diagnosis at the advanced clinical stages, i.e., III and IV stages. Due to the delayed diagnosis, the morbidity rate is potentially high with minimum of 5 years survival rate in 20-25% of the patients [5-7]. The prognosis is conducted on the basis of clinical staging system of tumor-lymph node-metastasis of the disease (TNM system) and the initial diagnosis of any kind of malignancy in oral tissues is based on the following criteria:

1. Etiology- data on current and previous addiction towards tobacco, presence of HPV or any other viral infectious factors.
2. Clinical features of the lesion (Leukoplakic, erythroplakic, nodular, ulcerative, verrucous).
3. Identification of the location of the lesion - floor of the mouth being at the highest risk followed by ventrolateral areas of the tongue etc.
4. Histopathological observations - presence of epithelial dysplasia.
5. Molecular biological aspects of the lesion [7].

The histopathological diagnosis for epithelial dysplasia is solely dependent on the static picture which can hint on possibility of changes in dynamic system. However, this evaluation cannot be reliable due to lack significance in the objectives, analytical grading, and also insufficient knowledge to predict the potency of malignancy [8] and the above mentioned system is not optimal since tumors may exhibit variant biological characteristics at the most similar stages of development. Hence, the development of additional aspects or biological markers that can determine the diseases effectively is of paramount importance [9].

The cancerous cells produce several abnormal/unique cellular secretions during their natural processes into the surrounding

environment. These secretions could be detected in the bodily fluids by biochemical methods or by immunohistochemistry. The ones that are detected and measured are known as "tumor markers" [10]. These tumor markers serve the clinicians to a very great extent in determining the malignancy picture accurately. The circulating tumor markers for oral cancer patients have been studied by various researchers [11-16] and have suggested having moderate sensitivity and specificity. They have used two distinct approaches to study the markers for malignant development: 1. epithelial dysplasia and [2] Oral cancer. They characterized the issue by the presence/absence or the pattern of distribution of the marker in question. That is the marker is graded as an effective tool if the reaction pattern in epithelial dysplasias is similar to that in carcinomas or if the deviated reaction is proportionately related to the grade of epithelial dysplasia. Based on different biological aspects, the markers can be broadly classified into:

(a) Genomic markers- involves DNA content (ploidy), studies aberrations, and changes in expression of oncogenes and tumor suppressor genes.

(b) Proliferation markers- involves proteomics.

(c) Differentiation markers, including keratins and carbohydrate antigens etc.

One promising criteria for the discovery of new biomarkers is the identification of protein profile of body fluids that could be used to characterize a specific disease. Saliva is one such fluid in the body that has gained least insight. Though saliva research is working from decades, at the present condition it has been recognized with great interest as a diagnostic fluid. The main reason behind this approach is that harvesting of saliva is simple and non-invasive [8]. Furthermore, certain studies have shown that saliva contains various signaling molecules that can indicate cancer potency [17-19]. In addition, cancer cells can also give rise to extracellular vesicles (EVs) which can deliver molecules (such as proteins, mRNA, microRNA, rRNA, tRNA, DNA and lipids) that have been suggested to involve saliva for their intracellular signaling mechanisms towards distant target cells [20]. Although many potential biomarkers for oral cancer have been identified in human saliva, the role of such molecules in oral cancer is not completely understood [17-19]. Saliva 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 [16]. Hence studies on Saliva must be developed to confirm the as potential characteristics as a biomarker.

Literature Review

Literature search was done on PubMed, Google Scholar and Pro Quest using the key words Salivary Biomarkers, oral cancer, Saliva as a diagnostic fluid, Biological markers, DNA, Protein markers in saliva. Manual search was also performed. The findings were then summarized.

Tumor markers

A tumor marker is a substance present in or produced by a tumor or the host cell of a tumor in response to its presence that can be used to differentiate a tumor from normal tissue. In addition, determine the presence of a tumor based on measurement in blood or secretions [21]. Tumor markers has been defined 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." [21]. 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 differently expressed in unique sites in the tumor cells [22]. The distinct biological features of tumor cells are the capacity for invasion, metastasis, uncontrolled proliferation, evasion of apoptosis and angiogenesis which are mediated by critical molecular pathways. Thus, any one of these molecular components can be raised as a potential tumor marker [23,24].

Saliva as a perfect diagnostic medium

Human saliva is a clear, slightly acidic (pH=6.0-7.0) biological fluid containing a mixture of secretions from multiple salivary glands, including the parotid, submandibular, sublingual gland and other minor glands beneath the oral mucosa mixed with crevicular fluid, bronchial/nasal secretions, blood constituents, bacteria, viruses, fungi, exfoliated epithelial cells and food debris [25,26]. Saliva has been termed as a potential diagnostic medium [27-29]. Since it's easily accessible and collection is non-invasive, inexpensive, requires minimal training and can be used for the mass screening of large population [29,30]. Whole saliva can be collected with or without stimulation. Stimulation can be performed with masticatory movements or by gustatory stimulation (citric acid) [31]. Stimulated saliva however, it can be collected in larger quantities [32]. Unstimulated saliva can be collected by merely spitting in a test tube or by leaving saliva drool from the lower lip [33] and it is commonly used for the diagnosis of systemic disease. A major drawback to use saliva as a diagnostic fluid was that the informative analytes were present in minute quantities in comparison to serum [34]. However with new emerging techniques, detection of small quantities of salivary components including proteins and messenger Ribonucleic acid (mRNA), everything that can be measured in blood/serum can be measured in saliva. With this adaptive quality, saliva is now considered as the blood stream of oral cavity.

Salivary markers for oral cancer detection

Several salivary markers are found to be significantly increased for oral cancer detection among the patients [14,35-38]. These Molecular markers for the diagnostic purpose can be determined in 3 levels: changes in the cellular Deoxyribonucleic acid (DNA) which results in altered mRNA transcripts followed by alterations in the protein levels [39]. The molecular markers for the diagnosis as given by Markopoulos and coauthors [39] shown in the **Table 1.**

Cellular DNA markers

DNA markers are the effective markers that can be used universally, since there can be no cell/tumor cell that does not contain genetic material. However, the specificity of DNA markers

Table 1 Markopoulos table for salivary markers.

Changes in cellular DNA	Altered mRNA transcripts	Altered Protein Markers
Allelic loss of chromosomes	Presence of IL-8	Elevated levels of Defensin 1
Mitochondrial DNA mutations	Presence of IL-β	Elevated CD44
P53 gene mutations	Dual specificity phosphatase 1	Elevated IL-6, IL-8
Promoter hypermethylation of genes	(DUSP-1) H3F3A (H3 Histone, Family 3 A)	Inhibitors of Apoptosis (IAP)
		Squamous Cell Carcinoma associated antigen (SCC-Ag)
Cyclin D1 gene amplification	Ornithine decarboxylase antizyme 1 (OAZ1)	
Increase in Ki67 markers		Carcino embryogenic antigen (CEA)
Microsatellite alterations of DNA	S1 Calcium binding Protein P (S100P)	Carcino antigen (CA19-9)
Presence of HPV(Human	Spermidine/spermine N-1 acetyl	CA128
Papilloma Virus) and EBV(Epstein	Transferase (SAT)	Serum tumor marker
Barr Virus) virus genome		Intermediate Filament protein (cytra 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

in the tissues is very low [35]. But there are enormous changes in the host DNA of tumor cells that cause chromosomal aberrations such as point mutation, deletion, translocation, amplification and methylation. In addition the cyclin D1, epidermal growth factor (EGFR), microsatellite instability can occur and HPV presence can be witnessed. Investigators have found that premalignant lesions with aneuploidy transform into malignancy more frequently than those lesions containing normal DNA irrespective of the having epithelial dysplasia graded in histopathological observations [36-37]. DNA aneuploidy is known to be associated with advanced stage carcinomas, Hence it may help in predicting the longevity of the tumor [38].

Loss of heterozygosity (LOH) is noted as loss of genetic material in one of the chromosomal pair. Literature studies have shown that LOH seen in the sites of a human suppressor gene is an early predictor of malignancy occurrence in precancerous lesion [39]. Similar studies have shown that repeated LOH in chromosome 3p, 9q, 13q and 17p are observed during early stage of oral carcinogenesis [40-42]. One of the studies found that allelic loss at 3p and 9q chromosome increased the risk of malignancy development by 3.8 fold and further it increased to [33] fold when chromosomes 4q, 8p, 11q, 13q and 17p exhibited LOH [43]. This helps the clinician to identify high and low risk lesions to maintain the treatment strategy.

Mitochondrial DNA mutations been identified in 46% of head and neck cancer among which 67% of patients were diagnosed with saliva samples by direct sequencing method [44] p53 allele mutation has been shown in 22% of pre-cancer and 20% of oral cancer patients [45-48]. In some of the studies tumor specific p53 mutations was observed in 71% saliva samples from patients with head and neck cancer by performing plaque hybridization technique [46]. Moreover genes such as p16, p27, p63, p73 have found to be altered in varying degrees in oral cancer [45].

A gene silencing mode attained through Promoter methylation that depends on the epigenetic factors is also known to be involved in oral cancer mechanism. The main genes that undergo methylation are CDKN2A, *CDH1*, *MGMT*, DAPK149 CDKN2A is included in the retinoblastoma pathway and it is found to be methylated in 23-67% of primary carcinogenic cells. *CDH1* is a gene responsible for cell adhesion, and undergoes metastasis during mutation. It has been seen that 85% of neoplastic cells have undergone promoter methylation in *CDH1* gene [49] Alsop16, *MGMT* and *DAP-K* gene underwent promoter hyper methylation in 65% of oral cancer patients diagnosed with saliva samples [50].

Amplification and over expression is a characteristic attributed to cancer cells. It is noted that c-MYCIN-MYC gene exhibited amplification in the elevated levels in 20-40% of oral cancers. High proliferating amplification of 11q13 chromosome with 1NT2, HST1 and Cyclin D oncogenes were observed in 30-50% of oral cancer patients [51]. Cyclin D1 showed poor amplification [52] whereas STAT [3] expression was observed up to 82% only when related to chewing tobacco. Some of the DNA markers like 8-oxoguanine DNA glycosylase, phosphorylated-Src and mammary serine protease inhibitor (Maspin) showed decreased levels of expression except Ki67 in the saliva of oral cancer patients [53]. The presence of virus genomes like HPV and EBV is one of the identified potential DNA markers in detecting oral cancers and also to predict tumor progression [54].

Discussion

mRNA transcripts as biomarkers

For years it was thought that RNA gets degraded in the saliva due to the presence of various RNAses [55-59]. However cell-free an RNA molecule exists in saliva both intact and as fragmented molecules [60]. These salivary mRNA remains protected in the apoptotic bodies [55,56] or in the gets released in the form of exosomes or microvesicles [57,58] Later to this, microRNAs, a small RNA molecules of 18-24 base pairs in length were discovered in saliva which could regulate transcription [61,62]. Studies have reported that certain mRNA molecules up-regulated in the saliva samples of patients suffering from Oral cancers [63]. The seven most commonly studied mRNA transcript molecules that get elevated in saliva of oral cancer patients are given in the **Table 2.**

Protein biomarkers

Salivary protein markers have shown moderate sensitivity and specificity towards prediction during prognosis. The protein defensins are peptide molecules found in the azurophil granules of polymorph nuclear leukocytes which possess antimicrobial and cytotoxic properties [64]. It has been reported that higher concentrations of defensin-1 saliva is an indication for the presence of tumerogenic cells as it was detected similarly in patients with oral cancer compared with healthy controls [65]. Many similar studies were performed on different protein molecules and their potential outcomes are given in the **Table 3.**

miRNA as biomarkers

miRNAs are [19-25] nucleotides short RNA transcripts. The RNA inducing silencing complex signal miRNAs to function during post-transcriptional regulation mechanism. They are present in saliva which has wide role in performing cellular functions. These Salivary miRNAs are available in the form of stable exosomes and they are known to express differentially varying from 10-100 fold in different cancer types. They are also been termed to have more specificity than mRNA to differentiate tumors [66-80].

Table 2 List of mRNA transcripts that undergoes alterations.

mRNA Transcripts	Functions
IL8 (Interleukin 8)	Plays a role in angiogenesis; replication; calcium-mediated signaling pathway; cell adhesion; chemotaxis; cell cycle arrest; immune response
IL1B (Interleukin 1)	Takes part in signal transduction; proliferation; inflammation and apoptosis
DUSP1 (Dual specificity phosphatase 1)	A role in protein modification; signal transduction and oxidative stress
H3F3A (H3 histone, family 3A)	Has a DNA binding activity
OAZ1 (ornithine decarboxylase antizyme 1)	Takes Part In Polyamine Biosynthesis
S100P (S100 calcium binding protein P)	A role in protein binding and calcium ion binding,
SAT (spermidine/spermine N1-acetyltransferase)	Takes part in enzyme and transferase activity

Table 3 List of protein biomarkers.

Protein Biomarkers	Potential outcome
Defensins [65]	Higher concentrations of defensin-1- presence of cancer cells
soluble CD44 [66]	Elevated, distinguishes cancer from benign disease with high specificity very high sensitivity due to methylation
IL-6 and/or IL-8 [67]	IL-8 at higher concentration in saliva and IL-6 higher concentration in serum are termed as promising biomarkers
IL1 [68]	Higher level of expression using LuminexxMAP technology
MMP1 and MMP3 [69]	Highly elevated
TNF-a70	Higher concentrations in saliva
P53 Autoantibody [70]	Presence in saliva
human telomerase reverse transcriptase (hTERT) [70]	75% positive expression of telomerase in saliva
Tetranectin [82]	Downregulated protein present in saliva
Inhibitors of apoptosis (IAP) [71]	Altered in comparison to the normal levels
Squamous cell carcinoma associated antigen (SCC-Ag) [72-74]	
Carcino- embryonic antigen (CEA)	
Carcinoantigen (CA19-9), CA128 [72,74]	
Serum tumorarker (CA125) [75]	
Intermediate filament protiens (Cyfra 21-1) [76-78]	
Tissue polypeptide specific antigen (TPS) 78-[79]	
Reactive nitrogen species (RNS) and 8OHdG	
DNA damage marker [76]	
Lactate dehydrogenase (LDH) and immunoglobulin (IgG) [77]	
s-IgA,	
Insulin growth factor (IGF)	
Metalloproteinases MMP-2 and MMP-11 [80]	

Tumor suppressing miRNAs, miR-125a and miR200a were reduced in the saliva of oral cancer patients in comparison to the normal controls was reported by Park and coauthors. In addition, salivary miR-31 was observed in highest levels than in the blood during all the stages of cancer [81-84].

Conclusion

Cancer screening using well-developed and time tested tools are the critical techniques which is been used to detect malignancies. Nevertheless, the emerging field of salivary diagnostics has an enormous potential in cancer screening. Due to its feasibility, specificity, sensitivity and non-invasive procedure, it has better compliance over the blood markers. Though there is no single marker that can confirm the presence of cancer, a group of

salivary biomarkers would be more beneficial. Advances in recent technologies in the field of biology, new saliva based markers (DNA, RNA and protein markers) are in force; hence we can look forward for saliva to perform as a diagnostic medium.

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