Saliva as a diagnostic tool in oral cancer


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Abstract
Saliva testing, a non-invasive alternative to serum testing, may be an effective modality for diagnosis and for prognosis prediction of oral cancer, as well as for monitoring post therapy status, by measuring specific salivary macromolecules, examining proteomic or genomic targets such as enzymes, cytokines, growth factors, metalloproteinases, endothelin, telomerase, cytokeratin, mRNA’s and DNA transcripts. Salivary analysis has been shown to be useful diagnostic tool also for distant malignancies such as breast cancer. In recent years, significant alterations have been demonstrated in the saliva of oral cancer patients in the epithelial tumour markers – Cyfra 21-1, TPS and CA12, various oxidative stress-related salivary parameters as ROS and RNS, biochemical and immunological parameters as IGF and MMP’s and RNA transcripts of IL6, IL-1B, DUSP1, H3, OA21, S100P, and SAT. Collectively these accumulated data are predicted to alter the field of oral cancer diagnosis by employing highly sensitive new tools which will enable both medical professionals and the patients themselves to monitor their saliva for diagnosis and prognosis prediction, as they relate to oral cancer. At this point however, the aim of salivary analysis is mainly for screening which may be helpful in the future. This article reviews on role of saliva in detection of oral cancer.

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Salivary testing, a non-invasive alternative to serum testing, is an effective modality for diagnosis and for prognosis prediction of various diseases such as oral cancer, as well as for monitoring the patient’s post therapy status. Follow-up of patients who have undergone treatment for oral cancer (oral squamous cell carcinoma (OSCC)) is done routinely and often in order to detect recurrences soon after they occur. The development of salivary diagnostic tools for these patients is of paramount importance, especially for high-risk populations (patients with premalignant lesions, “cured” patients, patients with previous history of cancer in general, tobacco and alcohol consumers and others). Home testing kits would further facilitate salivary testing as a diagnostic aid, enabling patients, especially those who live far from their treatment centers, to monitor their own health at home. Salivary ‘tools’ are those focused on measuring changes of specific salivary macromolecules such as proteins or nucleic acids (as fatty acids are rather scarce in saliva, i.e. examining genomic or proteomic targets such as enzymes, cytokines, growth factors, metalloproteinases, endothelin, telomerase, cytokeratin, mRNA’s, DNA aberrations, etc. [1-12].

Studying various salivary ions and antibodies has also been suggested. Altered biological fields or clusters of common aberrations which affect various molecules at different levels could be concurrently analyzed, exploring, simultaneously, various epithelial tumour markers or various oxidative stress-related enzymes, peptides and nucleic acids or various NF-kB dependent cytokines, and the like (see Table 1). Salivary DNA promoter hypermethylation analysis has also been found to be an efficient tool for early diagnosis of oral cancer. [13-15]. Oral cancer is a common human malignancy with an increasing incidence (especially in younger people) and a 5-year mortality rate of approximately 50% [16-19], a statistic that has not changed significantly in more than 50 years. [20-26] Its location in the mouth, face, and/or neck generates a relatively high rate of related morbidity, as treatment often results in significant mutilation and compromised functions. OSCC includes both mobile (oral) and base of tongue cancer lesions. Most often an oral cancer lesion is located at the lateral border of the tongue, though one located at the base of tongue is considered especially lethal. Clinically, it is important to note that the therapeutic modality currently offered to patients is based on traditional stage-predicting indices (based mostly on the TNM criteria) and on histological grading. Unfortunately, these predictors are subjective and can be relatively unreliable, as two tumours with identical staging and grading often behave in a totally different manner, and while one responds to therapy, the other is lethal. Accordingly, there has been an ever-growing effort dedicated to the basic research of oral cancer, focusing on the identification of biological indicators for the diagnosis of its biological nature and aggressiveness.

Various salivary biomarkers, which have been shown to be significantly altered in OSCC patients as compared with healthy controls. Salivary tumour markers and oral cancer Circulatory tumour markers for OSCC have been investigated in various studies [27-36] and have shown relatively moderate sensitivity and specificity values relative to diagnosis, prognosis prediction and treatment monitoring. For example, Kurokawa et al. [37,38] analyzed circulating CEA, SCC, IAP and Cyfra concentrations in OSCC patients, and found sensitivity and accuracy values of 81% and 77.8%, respectively.
<table>
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<th>Biomarker</th>
<th>Biological function</th>
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<tr>
<td>IAP</td>
<td>Apoptosis inhibitor</td>
<td>Kurokawa et al.37,38</td>
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<td>SCC</td>
<td>Squamous cell carcinoma associated antigen</td>
<td>Hoffmann et al.[36], Krimmel et al.[35], Nagler et al.[20,27,42], Heilner et al.[31], Zoller et al.[32,33]</td>
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<td>CEA</td>
<td>Carcinogenic embryonic carcinoma</td>
<td>Hoffmann et al.[36], Krimmel et al.[35], Zoller et al.[32,33], Nagler et al.[20,27,42]</td>
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<td>CA19-9</td>
<td>Carcino-antigen</td>
<td>Hoffmann et al.[36], Krimmel et al.[35], Nagler et al.[20,27,42]</td>
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<td>CA12B</td>
<td>Serum tumor antigen</td>
<td>Hoffmann et al.[36], Krimmel et al.[35]</td>
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<td>CA125</td>
<td></td>
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<td>Cyfra 21-1</td>
<td>Intermediate filament protein</td>
<td>Kurokawa et al.[37,38], Nagler et al.[20,27,42], Bhatavdekar et al.[28,29], Yin et al.[30], Nagler et al.[20,27,42], Bhatavdekar et al.[28,29], Yin et al.[30]</td>
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<td>TPS</td>
<td>Tissue polypeptide specific antigen</td>
<td>Nagler et al.[20,27,42], Bhatavdekar et al.[28,29], Yin et al.[30]</td>
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<td>RNS</td>
<td>Reactive nitrogen species</td>
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<td>8-OHdG</td>
<td>DNA damage marker</td>
<td>Bahar et al.[43]</td>
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<td>LDH</td>
<td>Lactate dehydrogenase – marker of tissue breakdown</td>
<td>Shpitzer et al.[45]</td>
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<td>IgG</td>
<td>Immunoglobulin</td>
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<td>Sec IgA</td>
<td>Mucosal immunoglobulin</td>
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<td>IGF</td>
<td>Growth factor</td>
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<td>LOH</td>
<td>Loss of heterozygosity – loss of specific chromosomal regions</td>
<td>El-Naggar et al.[46]</td>
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<td>DNA</td>
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<td>Viet et al.[49], Righini et al.[15,50], Rosas et al.[51], Franzmann et al.[4,11]</td>
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<td>IL8</td>
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<td>S100P</td>
<td>Calcium binding protein, cell cycle and differentiation regulator</td>
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<td>SAT</td>
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Table 1. Various salivary biomarkers, which have been shown to be significantly altered in OSCC patients as compared with healthy controls.
Their analysis of CEA, ACC and IAP yielded values of 69% and 90.3%, respectively. Hoffmann et al. [36] and Krimmel et al. [35] analyzed circulating levels of SCC, CEA, CA19-9 and CA125, finding correlation with the tumor burden for only the SCC antigen. They reported rather low sensitivity values for this antigen (except for patients with distant metastasis) and noted that the circulating SCC antigen had not been routinely used previously, as its reported sensitivity had been relatively low in other studies as well (15–40%), even though its specificity was quite high (70–90%). Hellner et al. reported that circulating SCC sensitivity in oral cancer patients was only 24% while much lower for CEA. Zoller et al. [32,33] reported that while CA19-9, CA125 and CA15-3 exhibited poor sensitivity, the sensitivity values for circulating SCC and CEA in oral cancer patients were 33% and 43%, respectively. Such a wide range was also found for other circulatory markers, such as CEA, CA19-9 or TPS, which were in the range of 25–96% and 65–75%, respectively. [27–30] One method suggested to improve the sensitivity and accuracy of such an analysis was concurrent examination of various circulatory markers (performing a “combination assay”). [37,38] Surprisingly, very few studies have examined tumor markers in the saliva of OSCC patients. Such an examination may be of great benefit because of the direct contact between saliva and the oral cancer lesion, particularly since salivary analysis has been shown to be a useful diagnostic tool for other distant malignancies such as breast carcinoma. [39] Moreover, these scarce salivary reports focused only on one commonly analyzed tumor marker, the CEA, [40,41] which did not prove to be sensitive or specific enough. In a recently published study [42] our group examined the saliva of OSCC (tongue) patients for the six most-often studied serum circulatory epithelial tumor markers: CEA, CA19-9, CEA, CA125, and CA125. The data obtained were then compared with other traditionally examined parameters used for the diagnosis and evaluation of disease severity. We found a significant increase of 400% in salivary concentrations of CEA, CA19-9, CEA, CA125, and CA19-9. The salivary concentrations of CA19-9, SCC and CEA were increased without statistical significance. A concurrent analysis of the three significantly increased markers revealed sensitivity, specificity and negative and positive predictive values of 71%, 75%, 71%, and 75%, respectively. These increases support the use of salivary tumor markers as a diagnostic tool, especially when a concurrent analysis for significantly increased markers is performed.

Salivary oxidative profile in oral cancer patients

In yet another study [43] our group looked at various oxidative stress-related salivary parameters, and found that oxidative and nitrative stress altered the salivary composition in OSCC patients. They analyzed the salivary reactive nitrogen species (RNS) were substantially higher (NO = 60%, NO2 = 190%, NO3 = 35%), while all salivary antioxidants were substantially lower. The 8-OHdG marker (a widely used indicator of DNA oxidation) increased by 65% and salivary carboxylation level was significantly higher. Aside from their importance at the diagnostic and prognostic levels, these salivary alterations may shed further light on the pathogenesis of oral cancer as well, since such an increase in both reactive oxygen species (ROS) and RNS may have been the event leading to the consumption and reduction of salivary antioxidant systems. This may well explain the oxidative damage to the DNA and proteins, and possibly the promotion of OSCC. The oxidized proteins and DNA found in the saliva of cancer patients seems to be the first demonstration of a direct link between salivary free radicals, antioxidants and OSCC. These accumulated data gain further credence from the study published by Almadori et al. [44] who evaluated the concentrations of glutathione and total ascorbic acid, low molecular weight antioxidants, in the saliva of OSCC patients, in order to identify differences compared to normal subjects and to obtain information about biochemical alterations of human saliva during carcinogenesis. The researchers found that high salivary glutathione may be an epidemiological marker for identification of subjects with increased risk for development of OSCC, to submit to strict follow-up and chemoprevention. They concluded that metabolic alterations of saliva could be both an epidemiological marker and a target for chemoprevention of oral and oropharyngeal carcinogenesis.

A comprehensive salivary analysis for oral cancer diagnosis

A recently published study by our group [45] utilized a comprehensive salivary analysis to evaluate biochemical and immunological parameters in the saliva of oral squamous cell carcinoma (OSCC) patients. In this study saliva was collected from OSCC patients and compared with saliva collected from healthy, age- and gender-matched individuals. All OSCC lesions were located at the lateral aspect of the mobile tongue. Salivary parameters analyzed included: sodium (Na), potassium (K), calcium (Ca), inorganic phosphate (P), magnesium (Mg), total protein (TP), albumin (Alb), lactate dehydrogenase (LDH), amylase (Amy), total immunoglobulin G (IgG), secretory immunoglobulin A (Sec. IgA), epidermal growth factor (EGF), insulin growth factor I (IGF-I) and metalloproteinase MMP-2 and MMP-9. We found that the salivary median total protein concentration was significantly higher by 26% (p = 0.01) in cancer patients, as were concentrations of Na, Ca, P and Mg, by 14% (p = 0.05), 59% (p = 0.05), 39% (p = .), and 28% (p = 0.12), respectively. Amylase and K concentration were lower by 25% (p = 0.12) and 15% (p = 0.03). Albumin was 108% higher (p = 0.0007), as were salivary LDH (88%, p = 0.002) and total IgG (125%, p = 0.01), while Sec. IgA was lower by 45% (p = 0.001). Concentrations of IGF, MMP-2 and MMP-9 were significantly higher by 117% (p = 0.03), 79% (p = 0.0003) and 35% (p = 0.05), respectively. Accordingly we concluded that a comprehensive salivary analysis revealed an overall altered salivary composition in OSCC patients, indicating a compromised oral environment in these patients, and suggesting salivary analysis as an attractive diagnostic tool for oral cancer.

Salivary loss of heterozygosity (LOH) in oral cancer

Recent studies show that loss of specific chromosomal regions (loss of heterozygosity, LOH) that contain known or putative tumor suppressor genes is an early predictor of subsequent progression of oral cancer and oral premalignant lesions. LOH was shown to occur more frequently than gene mutations in oral cancer.
The cell cycle gene, TP53 was the only gene with similar incidences of LOH and mutation. Site-specific activation was apparent in the cell signaling mitogen-activated protein kinase HRK-G in oral cancer. In oral cancer, the TP53 GC>TA transversion frequency increased with tobacco smoke exposure. El-Naggar et al. [46] found that 49% of the saliva samples of oral cancer patients had loss of heterozygosity in at least one of the 25 markers studied and concluded that: (1) epithelial cells in saliva from patients with head and neck squamous tumorigenesis provide suitable material for genetic analysis; (2) combined application of certain markers improves the detection of genetic alteration in these patients; (3) clonal heterogeneity between saliva and matching tumor supports genetic instability of the mucosal field in some of these patients; and (4) LOH at certain chromosomal loci appears to be associated with smoking and alcohol consumption. [46-48]

Salivary DNA methylation in oral cancer

Aberrant promoter hypermethylation is common in head and neck cancer and may be useful as a marker for cancer cells. Detection of aberrant promoter hypermethylation patterns of cancer-related genes in saliva of head and cancer patients is feasible and may be potentially useful for detecting and monitoring disease recurrence, since promoter DNA hypermethylation is a critical step in oral carcinogenesis and has a number of significant advantages over genetic and protein diagnostic markers. Methylation array analysis of saliva can produce a set of cancer-related genes that are specific and can be used as a composite biomarker for the early detection of oral cancer. Hence, methylation analysis in saliva is a very promising approach for early cancer detection in high-risk patients or for the post treatment follow up and rapid diagnosis of relapse. The methylation signature might also reflect the tumor prognosis and complete the histology to define the diagnosis. Finally, DNA methylation is reversible with demethylating agents, a new avenue for cancer therapy in association with conventional chemotherapy. [11,13,49-51]

Salivary proteomics and genomic targets analysis for oral cancer biomarker discovery

A leading group of researchers which has contributed substantially in recent years to the research in saliva for cancer diagnosis by using new and sophisticated approaches is that of DT Wong et al. from the UCLA saliva proteome consortium funded by the National Institute of Dental and Craniofacial Research (NIDCR). [52-67] These researchers have examined proteins and nucleic acids in the saliva of OSCC patients and, by using various highly sensitive methods, significantly increased the sensitivity and specificity indices of the salivary analysis of various candidate biomarkers, thus increasing its appeal and its likelihood of being accepted as a practical diagnostic tool in the clinical set-up. The combination of these candidate biomarkers yielded a receiver operating characteristic value of 93%, sensitivity of 90%, and specificity of 83% in detecting OSCC. The various methods employed by the group have included: Luminex Multianalyte Profiling (xMAP) technology, shotgun proteomics based on C4 reversed-phase liquid chromatography, capillary reversed-phase liquid chromatography with quadruple time-of-flight mass spectrometry, a surface immobilized optical protein sensor utilizing a confocal optics based sensor, matrix-assisted laser desorption/ionization–mass spectrometry (MALDI–MS), reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) or PCR (RT-PCR), in vitro translation, and the construction of a salivary cDNA library, humanGenome-wide U133A microarrays for profiling human salivary transcriptome etc. Comparison samples from oral cancer and control subjects have demonstrated that oral fluid contains proteomic signatures that may serve as biomarkers for OSCC, as 46 peptides/proteins were found at significantly different levels between the two groups. Furthermore, the fact that researchers found that a large panel of human mRNAs can be reliably detected in saliva gives rise to a novel clinical approach, salivary transcriptome diagnostics. Seven cancer-related mRNA biomarkers that exhibited at least a 3.5-fold elevation in OSCC saliva (p < 0.01) were consistently validated. These potential salivary RNA biomarkers are transcripts of IL8, IL1B, DUSP1, HA3, OAZ1, S100P, and SAT. The combinations of these biomarkers yielded sensitivity (91%) and specificity (91%) indistinguishable from the OSCC from the controls. It was found that all healthy subjects evaluated have approximately 3000 different mRNA molecules in their saliva and almost 200 of these salivary mRNAs are present in all subjects. Exploration of the clinical utility of the salivary transcriptome in oral cancer subjects shows that four salivary mRNAs (OAZ, SAT, IL8, and IL1b) collectively have a discriminatory power of 91% sensitivity and specificity for oral cancer detection, as previously mentioned. Additionally, microarray analysis showed that there are 1679 genes exhibited at significantly different expression levels in saliva between cancer patients and controls (p < 0.05). Collectively these accumulated data are predicted to alter the field of oral cancer diagnosis by employing highly sensitive new tools which will enable both medical professionals and the patients themselves to monitor their saliva for diagnosis and prognosis prediction, related to oral cancer.

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62. Wong DT. Towards a simple, saliva-based test for the detection of oral cancer 'oral fluid (saliva), which is the mirror of the body, is a perfect medium to be explored for health and disease surveillance'. Expert Rev Mol Diagn 2006; 6: 267–72.


