



Salivary Diagnostics for Oral Cancer

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ABSTRACT

Oral cancers annually strike 38,000 individuals in the United States and hundreds of thousands of others around the globe. Despite treatment advances, the disease's overall five-year survival rate has not improved in the past three decades and remains among the worst of all cancers. One factor behind oral cancer's high mortality is the challenge detecting it at its early stages. The use of saliva for the detection of oral cancer has been a historical goal that has yet to come to fruition. This review highlights translational research efforts in alignment with initiatives sparked by the National Institute of Dental and Craniofacial Research toward bringing saliva diagnostics to fruition and, in particular, for saliva-based oral cancer detection.

The ability to monitor health status, disease onset and progression, and treatment outcome through noninvasive means is a most desirable goal in the health care promotion and delivery. There are three prerequisites to materialize this goal: specific biomarkers associated with a health or disease state; a noninvasive approach to detect and monitor the biomarkers; and the technologies to discriminate the biomarkers. The author presents a roadmap to achieve these goals through the use of oral fluids (saliva) as the diagnostic medium to scrutinize the health and/or disease status of individuals. This is an ideal opportunity to bridge state of the art micro-/nano-electromechanical system (MEMS/NEMS) sensors to oral fluid for diagnostic applications. With oral fluid being the "mirror of body,"



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it is a perfect medium to be explored for health and disease surveillance. The translational applications and opportunities are enormous.

A growing number of proof-of-principle examples have been established for using saliva to monitor systemic diseases and conditions. The barriers to widespread implementation of saliva diagnostics, derived from technological problems such as sensitivity, miniaturization, high throughput, automation, portability, low cost, high functionality, and speed to enable detection and measurements of multiple disease markers in saliva, have largely been overcome. Techniques emerging from a combination of miniaturization technologies and discoveries in many different fields of biology, chemistry, physics, and engineering are leading to high throughput, automated, portable, low cost, more efficient, and rapid biochemical analyses. Miniaturized diagnostic technologies will be able, with minute amounts of body fluids, to yield critical patient information reflecting health and disease status. These “lab-on-a-chip” platforms will be able to perform multiple operations in parallel in nonlaboratory settings such as the field, factory, hospital clinic, or home. It is envisioned that such technologies will allow the simultaneous assessment of multiple conditions of health and disease and provide clinicians with prevention and therapeutic strategies to meet patient needs.

Oral Cancer

Oral cancers are the sixth-most common cancer in the United States, affecting 38,000 Americans yearly and killing 7,200. Worldwide, they annually affect an estimated 350,000 individuals. More than 90 percent of these cancers are squamous cell carcinoma. Despite treatment advances that have resulted in reductions in patient morbidity, the overall five-year survival rate for oral squamous cell carcinoma remains among the worst of all cancer death rates, approximately

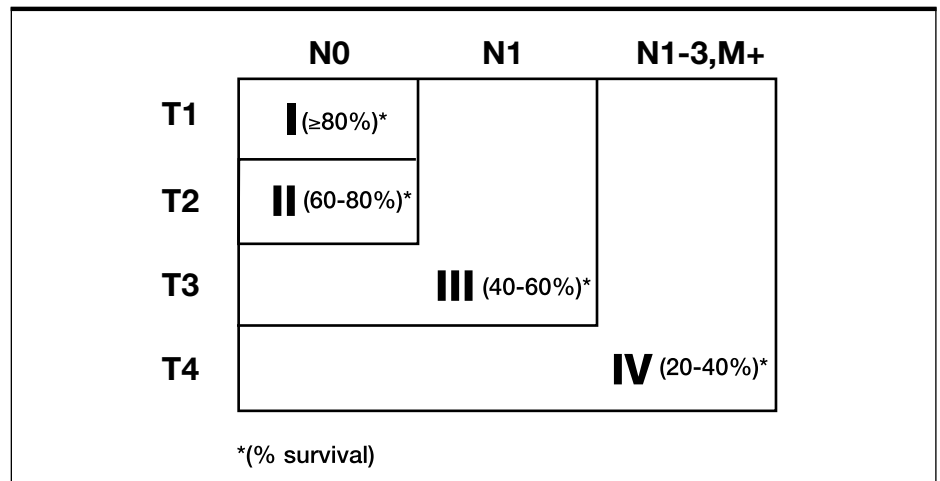


Figure 1. Clinical stage and survival rates of oral cancer. The survival rates have not changed in the past 30 years.

30 percent to 40 percent for the past few decades, considerably lower than those for colorectal, cervix, and breast origin.^{1,2} One patient dies from oral cancer every hour in the United States. This high morbidity rate can be attributed to factors including nonresponsiveness to chemotherapy and radiation therapy, late presentation of lesions, and a lack of satisfactory biological markers for early lesion detection.³ According to The Oral Cancer Foundation, oral cancer is particularly dangerous because it has a high risk of developing second, primary tumors. Patients who survive a first encounter with the disease have up to a 20 times higher risk of developing a second cancer.

Current Oral Cancer Diagnostic/ Screening Approaches

The most definitive procedure for oral cancer diagnosis is a scalpel biopsy, followed by the careful histopathological evaluation by a qualified pathologist. For this to be an effective procedure, it requires three consecutive events: a visit to the dentist/physician’s office, the biopsy by the licensed health care provider, and a pathologist’s evaluation. When effectively administered and reim-

bursed, as is in the Scandinavian countries, this can lead to early detection of oral cancer lesions that otherwise would have progressed to later stage cancer, which carries a worse prognosis. **Figure 1** illustrates the prognostic difference of an oral cancer lesion as it advances down the slide of later stage lesion. Detection of an oral cancer at Stage I will carry a prognosis of 80 percent survival, while the same lesion, when it progresses to Stage III, will carry a 20 percent survival. This is a dramatic difference that will affect not only the quality of life for the patient, but a significant savings on the health care costs on the medical treatments of a Stage I versus Stage III oral cancer patient.

With this in mind, scientists have been searching for alternative approaches to biopsy, with the hope to come up with “the Pap smear” test for oral cancer detection, which has significantly improved the mortality of cervical cancer. Since most oral cancers arise as asymptomatic small lesions, only when the clinician or patient notes abnormal tissues do formal diagnosis procedures begin.⁴ Microscopic investigation of the progressive cancer is often conducted too late for successful intervention.⁵ It is also impractical to use

imaging techniques for cancer screening, since they are time-consuming and expensive. These techniques are typically used for confirmation because of their insensitivity for small lesions.⁶ Studies have demonstrated that a good, positive predictive value can be achieved by oral cancer tissue staining with Toluidine blue.^{7,8} However, extensive experience is required in applying this technique and in interpreting its results. Exfoliative cytology may be a less invasive method for oral cancer detection, but exfoliated cancer cells tend to correlate

with tumor burden, with lower rates of detection seen in those with minimal or early disease.⁹ A number of molecular-based diagnostic markers have been used to detect the presence of oral squamous cell carcinoma with varying degrees of sensitivity and specificity. DNA markers include TP53, microsatellite instability, and the presence of HPV and EBV genomic sequences.¹⁰⁻¹³ Cytokeratins have been used for RNA diagnostics while SCC, CD44, CYFRA and telomerase have been used for protein markers.^{14,15} None of these markers, however, universally identifies oral squamous cell carcinoma. Microsatellite markers are the most promising amongst these, where at least one of a panel of 23 markers can detect the presence of an oral cancer cell in the saliva of 79 percent of oral cancer patients.¹⁶⁻¹⁹ Microsatellite instability analysis, however, is not particularly sensitive and requires a large amount of cancer cell DNA, about one cancer cell among 200 normal cells. Further, it is difficult to perform on a large number of clinical samples because many markers are necessary for testing.¹⁶

Recently, a number of clinical diagnostic aids for oral cancer detection have emerged. They include the

OralCDx, ViziLite and Toluidine blue. The OralCDx is a brush biopsy, a non-invasive chairside procedure to determine if an oral lesion is benign or potentially harmful. Precancerous and early stage oral cancerous lesions can be determined. All suspicious lesions with abnormal cytology will be required for

DESPITE TREATMENT ADVANCES THAT HAVE RESULTED IN REDUCTIONS IN PATIENT MORBIDITY, THE OVERALL FIVE-YEAR SURVIVAL RATE FOR ORAL SQUAMOUS CELL CARCINOMA REMAINS AMONG THE WORST OF ALL CANCER DEATH RATES.

a biopsy follow-up. The ViziLite visual examine system is based on differential density of the nuclear content and mitochondrial matrix of abnormal cells is typically greater than normal cells. The increased molecular density is believed to reveal the increased proliferative rate and metabolic activity of precancerous cells. The ViziLite exam enhances the examiner's ability to see the difference in the nuclear/cytoplasmic ratio of dysplastic cells. After rinsing with a dilute acetic acid solution, the dense nucleus of abnormal squamous epithelium tissue will appear white when viewed under a diffuse low-energy wavelength light. Normal epithelium will absorb the light and appear dark. ViziLite can identify an abnormality, but a definitive diagnosis only can be made by biopsy.

History of Using Saliva for Oral Cancer Molecular Detection

The use of saliva for oral cancer screening or diagnostics is still in its infancy. Its use began by a report of a small study in Taiwan by Liao et al. in 2000 claiming that exon 4 codon 63 of the p53 gene is mutated in salivary DNA from 5/8 (62.5 percent) of

oral cancer patients.²⁰ Five of 27 control subjects (18.5 percent) had similar mutations in their p53 gene. El-Naggar et al. in 2001 demonstrated genetic heterogeneity in saliva from patients with oral squamous carcinomas and suggested the use of epithelial cells in saliva from patients with head and neck squamous tumorigenesis for genetic analysis.²¹ More recently, Jiang et al. reported the increase of mitochondrial DNA content in the saliva of head and neck cancer patients.²² Another report from the same group reported that

quantitative analysis of HPV 16 DNA in salivary rinses allows for detection of HPV-related head and neck cancer.²³ However, the authors cautioned that specific limitations exist that prevent the application of this as a screening technique for a broad population.

The UCLA Approach to Saliva Diagnostic for Oral Cancer

The laboratory at the University of California, Los Angeles, utilizes research platforms toward the global identification of disease signatures in saliva. The premise of the author's approach is that since serum contents, such as disease biomarkers, will be largely present in saliva, oral fluid is a logical source to harness disease biomarkers. The lab employed both a proteome-wide as well as a genome-wide approach toward identification of disease biomarkers and signatures.

Human salivary proteome as targets for human disease diagnostics

UCLA is one of the three NIDCR-funded groups to comprehensively decipher human salivary pathogenesis. Three hundred and nine distinct proteins in human whole saliva using 2-DGE/MS and

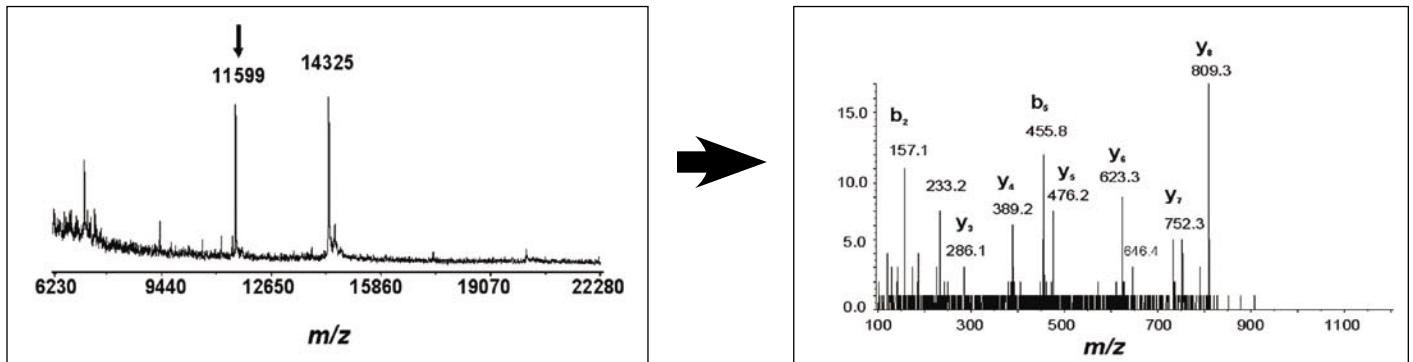


Figure 2. Identification of a potential saliva protein marker for oral cancer. Oral fluid sample was separated by LC (C4 column) and fractions collected. Panel A depicts the MALDI-MS spectrum of a LC fraction containing the protein of ~11600 Da. This fraction was digested for LC-MS/MS analysis. Panel B depicts the MS/MS spectrum of a tryptic peptide, VGEFSGANK, originated from thioredoxin.

“shotgun” proteomics have been identified. This work was recently published in *Proteomics* and highlighted in *Journal of Proteome Research* in 2005.²⁴ Using a similar approach, comparative proteome analysis of submandibular (SM) and sublingual (SL) saliva were conducted.²⁵

To date, two salivary proteins, IL8 and thioredoxin, which can discriminate saliva of oral cancer from control subjects, have been discovered. IL8 was discovered through previous tissue-based expression profiling effort.²⁶ IL8 is significantly elevated in saliva of oral cancer patients and is highly discriminatory of detecting oral cancer in saliva (n=64) with an receiver operator characteristic value of 0.95, sensitivity 86 percent, and specificity 97 percent at cutoff of 600 pg/ml.^{26,27} Of interest is that both IL8 protein and RNA are concordantly increased.²⁷ The concentration of IL8 protein in saliva of oral cancer patient and control subjects are 750 ± 236 pg/mL and 250 ± 130 pg/mL, respectively. Similarly, for salivary IL8 mRNA concentration, oral cancer patients are significantly higher than in control subjects. Due to the frequent inflammation association of this cytokine, it has been further demonstrated that the oral cancer elevation of salivary IL8 mRNA and protein is significantly higher than in advanced periodontitis patients.

These results allow for the conclusion that while severe inflammation in the oral cavity, as in advanced periodontitis patients, does elevate salivary IL8 protein and mRNA levels, it is not significant. Salivary IL8 protein and mRNA levels in oral cancer patients are elevated significantly above those of control patients as well as advanced periodontitis patients, supporting the use of salivary IL8 as a biomarker for oral cancer detection.²⁸

Thioredoxin was discovered as salivary oral cancer biomarkers by a proteomic approach using MALDI-TOF. It has been established as an integrated methodology to sequence candidate protein/peptide biomarkers. Using MALDI-MS profiling of saliva proteins, it was identified that a ~11600 Da protein was present at a significantly higher level in oral cancer saliva than matched control subjects ($p < 0.01$). To identify this candidate biomarker, an oral cancer saliva sample was fractionated by reverse-phase LC (C4 column) followed by MALDI-MS of the LC fraction containing the candidate biomarker of ~11600 Da (Figure 2a). This fraction was subsequently digested by trypsin for LC-MS/MS analysis. Figure 2b shows the tandem MS spectrum of a double-charged tryptic peptide, VGEFSGANK, originated from thioredoxin. Mascot

database searching indicated that, totally, 4 peptides were matched to this protein, with a sequence coverage of 31 percent. These results suggested that saliva thioredoxin is a validated biomarker for oral cancer detection.²⁹

Human salivary transcriptome as targets for cancer diagnostics

The UCLA research group recently found that there are approximately 3,000 human mRNAs in normal subjects’ cell-free saliva.³⁰ Further, there is a core signature of 185 mRNAs present in all normal subjects, which provides the rationale for the use the salivary transcriptome for disease diagnostics. The discovery that a large panel of human RNA can be reliably detected in saliva gives rise to the potential of this novel clinical approach. The diagnostic value of this approach was evaluated by using oral squamous cell carcinoma as the proof-of-principle disease and found that of the ~3,000 mRNAs, seven salivary RNAs were consistently elevated in saliva from oral cancer patients. Of these, four in combination (OAZ-1, SAT, IL8 and IL1- β), have the ability to discriminate saliva from oral cancer patients from that of control subjects, with an receiver operator characteristic value of 0.95, a specificity of 91 percent, and a sensitivity

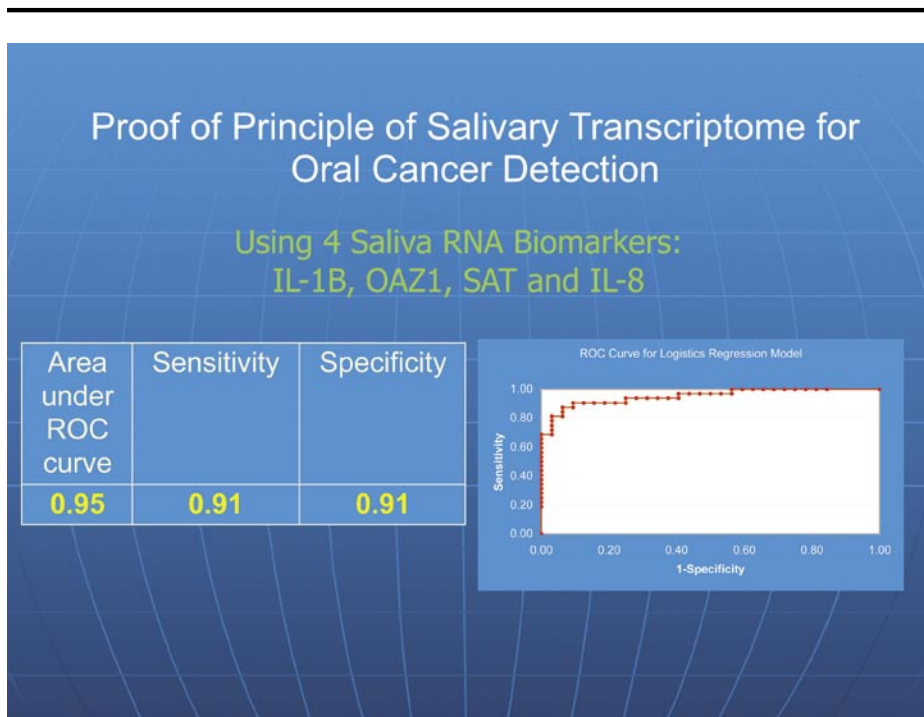


Figure 3. Receiver operator characteristic curve analysis for the predictive power of combined salivary mRNA biomarkers. The final logistic model included four salivary mRNA biomarkers, IL1B, OAZ1, SAT and IL-8. Using a cut-off probability of 50 percent, a sensitivity of 91 percent and specificity of 91 percent by receiver operator characteristic was obtained. The calculated area under the receiver operator characteristic curve was 0.95.

of 91 percent³¹ (Figure 3). While the initial study was done on 64 subjects, three additional independent clinical detection studies with 272 subjects have since been carried out, and found that the seven saliva mRNA biomarkers behaved very consistently with an overall accuracy rate of 85 percent.³⁰ The discovery of RNA biomarkers in saliva that can have oral cancer discriminatory ability is a novel finding. This is now being explored of its translational potential and value.

One often wonders which bodily fluids (blood, saliva, urine, cerebral spinal fluid) are more clinically diagnostic for a specific disease entity. The author recently made that comparison for oral cancer. The same patients identified with a saliva RNA signature for oral cancer detection were examined for serum RNA signatures for oral cancer detection. Similar to saliva, four RNA

biomarkers collectively can mark saliva of individuals with oral cancer with an receiver operator characteristic value of 0.88.³² While this is very good, the salivary RNA biomarkers have an receiver operator characteristic value of 0.95.³¹ Thus for oral cancer detection, saliva RNA biomarkers have a slight edge over serum RNA biomarkers.

Saliva diagnostics for other high-impact systemic diseases

Saliva has been examined for the detection of a number of systemic diseases ranging from infectious diseases, including HIV to Alzheimer's.³³⁻³⁷ The author's laboratory has begun to explore a number of efforts to identify high-impact systemic diseases and explore their diagnostic signatures in saliva. Breast cancer is the first systemic disease to be explored of the presence of proteomic and genomic signatures

in saliva of breast cancer patients. It should be noted that Charlie Streckfus reported that Her-2 and CA15-3 levels are elevated in cancer versus control subjects' saliva.^{38,39} It is also the intent of the UCLA group to carry out rigorous proteome- and genome-wide discovery efforts to identify and validate salivary proteomic and genomic biomarkers in breast cancer patients. The research will follow guidelines from the NCI Early Disease Research Network for biomarker validation, similar to the ongoing oral cancer biomarker validation.⁴⁰

Future Perspectives

While it is clear there is a national agenda to turn saliva diagnostics into a clinical and commercial reality, much work needs to be done before this vision can be realized. There remains the need to identify definitive disease-associated salivary biomarkers (proteins and genetic) that can be use in conjunction with the technology platforms for saliva diagnostics. The UCLA group is set to develop and validate the Oral Fluid NanoSensor Test, OFNASET, as a point-of-care chairside, portable and multiplexible device to be used for saliva diagnostics. In addition to the research infrastructure, the UCLA School of Dentistry can fully harness and validate proteomic and genomic biomarkers in saliva for human disease diagnostics. Collectively, technology platform advancement and the identification and validation of robust and discriminatory suites of salivary biomarkers for disease diagnostics represent the necessary marriage to propel saliva diagnostics into a clinical and commercial reality. At the same time, we are building the scientific foundation toward the use of saliva as a diagnostic fluid.⁴¹ Questions have arisen, such as where do salivary biomarkers, proteins, and RNA come from? Control mechanism of salivary RNA turnover and the fate of these RNA are currently being addressed.

This is a perfect example of translational research in reverse, based on a highly relevant clinical observation that saliva contains proteomic and genomic biomarkers for oral cancer detection, and building a scientific foundation toward the mechanistic background so as to allow us to better exploit the full clinical potential of saliva diagnostics. **CDA**

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