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Genomic Targets in Saliva

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Abstract

Saliva, the most accessible and noninvasive biofluid of our body, harbors a wide spectrum of biological analytes informative for clinical diagnostic applications. While proteomic constituents are a logical first choice as salivary diagnostic analytes, genomic targets have emerged as highly informative and discriminatory. This awareness, coupled with the ability to harness genomic information by high-throughput technology platforms such as genome-wide microarrays, ideally positions salivary genomic targets for exploring the value of saliva for detection of specific disease states and augmenting the diagnostic and discriminatory value of the saliva proteome for clinical applications. Buccal cells and saliva have been used as sources of genomic DNA for a variety of clinical and forensic applications. For discovery of disease targets in saliva, the recent realization that there is a transcriptome in saliva presented an additional target for oral diagnostics. All healthy subjects evaluated have approximately 3,000 different mRNA molecules in their saliva. 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. Data are also now in place to validate the presence of unique diagnostic panels of salivary mRNAs in subjects with Sjögren's disease.

Keywords

human saliva transcriptome analysis; mRNA biomarkers; noninvasive disease detection

Saliva is a mirror of oral health and also a reservoir of analytes from systemic sources that reach the oral cavity through various pathways. The molecular composition of saliva reflects tissue fluid levels of therapeutic, hormonal, immunological, or toxicological molecules. In addition, markers for diseases (e.g., infectious and neoplastic) can be present. Consequently these fluids provide sources for assessment and monitoring of systemic health and disease states, exposure to environmental and job-related toxins, and the use of abusive or therapeutic drugs. This is the basis of our vision to develop disease diagnostics and promote human health surveillance by analysis of saliva. Proteomics, genomics, and microbial analysis will be the driving forces for disease marker development.

Our group aims to expand the toolbox of oral-fluid-based diagnostics by developing proteomic and genomic “alphabets” of healthy individuals and disease-specific signatures. Here we present the rationale and progress with mRNA-based salivary biomarkers, illustrating the presence, integrity, and potential diagnostic utility of mRNA found in saliva.

Stable, cell-free circulating DNA in plasma was first observed almost 60 years ago.¹ Increased plasma DNA levels were shown in cancer patients² and several groups demonstrated that plasma DNA displays tumor-specific characteristics. These include somatic mutations in tumor suppressor genes or oncogenes, microsatellite alterations, abnormal promoter methylation, mitochondrial DNA mutations, and the presence of tumor-related viral DNA.^{3–9} Tumor-specific cell-free DNA in the circulation has been found in a wide range of malignancies. Genetic alterations and mRNA signatures can successfully be identified in body fluids that drain from organs affected by tumors.¹⁰ Thus, cell-free biomarkers derived from the tumor “travel” through the body and can be detected in blood and other body fluids. As studies of tumor-derived DNA detection in plasma of cancer patients were being pursued, Lo *et al.*¹¹ detected “fetal” DNA in the plasma of pregnant women. In the following years, the presence of placental and tumor-specific cell-free RNA in plasma was also demonstrated.^{12–15} Meanwhile many body fluids have been shown to contain cell-free nucleic acids of potential diagnostic value. Research in this area has demonstrated that these analytes are useful in noninvasive prenatal diagnosis and can detect cancer and other systemic diseases including diabetes, stroke, and myocardial infarction.¹⁶

The potential for saliva-based tests to detect oral cancer has been demonstrated in a number of studies utilizing the analysis of promoter hypermethylation,¹⁷ exfoliated cells,¹⁸ and even the microbiota.¹⁹ Our group recently established that studying the transcript levels of mRNA in saliva presents potential diagnostic opportunities for oral cancer.²⁰ We are addressing the need for early cancer detection by an extensive effort to develop and validate a diagnostic test based on mRNA profiles from saliva. Four signature RNA transcripts are elevated in saliva of oral cancer patients.²¹ These mRNAs were identified through microarray studies and validated by real-time quantitative polymerase chain reaction (qPCR). A distinction between patients with oral cancer and healthy control subjects demonstrated 91% specificity and 91% sensitivity, the area under the receiver operator characteristics (ROC) curve measuring 95%. These markers are being validated according to established guidelines.²² At the same time we are developing the optimal test set-up for multicenter studies and investigating methodologies for high-throughput and on-site testing. The examination of transcriptional changes provides clear advantages in comparison to genetic changes as the subtle genetic changes (loss of heterozygosity, point mutations, and methylation changes) are translated into high copy number messages of unique sequences.

The transcriptome analysis of saliva performed by our group provides clear evidence for the clinical utility and potential of analyzing human mRNA in saliva.²³ While being easily accessible in a noninvasive manner, saliva has the advantage that the background of normal material (cells, DNA, RNA, and proteins) and inhibitory substances is much lower and less complex than in blood. This feature may prove to be an important advantage in certain instances, as our laboratory has recently shown with a comparison study of oral cancer mRNA markers.²¹

In the past decade, the potential for the use of saliva for the detection of oral cancer has been laid out by the analysis of genetic changes in the cellular compartment. However, low target concentrations, the subtleness of the genetic changes and the heterogeneity of early events in cancer development and progression have as yet prevented the translation of these findings to concepts for the early noninvasive detection of oral cancer. As such, the genomic information in saliva is mainly applied for the genetic banking for pharmacogenomic and

epidemiologic studies,²⁴ and for a variety of identity testing situations, as, for example, in forensics, by the military for purposes of identification, and tests with possible home-based sample collection in paternity testing and genetic ancestry testing (available from <http://Genetree.com>).

In forensics, a molecular-based test allows the identification of body fluid stains, demonstrating the utility and validity of salivary mRNA testing: On the basis of panels of body-fluid-specific transcripts, Juusola *et al.* demonstrated the distinction of stains originating from different body fluids such as blood, urine, semen, and saliva.^{25–28} It is probable that the predominant part of the RNA preserved in salivary stains is cellular.

The finding of cell-free mRNA in whole saliva has allowed us to establish a salivary core transcriptome of 185 genes present in all 10 samples analyzed, based on Affymetrix U133 array analysis.²³ An average of 3,143 probe sets on each array were assigned, representing approximately 3,000 different mRNAs per individual.

The finding of cell-free mRNA in saliva may seem surprising. Similar to cell-free RNA in plasma it is protected from degradation by association with macromolecules.²⁹ Since this discovery, we have pursued investigations addressing the source, integrity and stability of RNA in saliva. According to our findings from polymerase chain reaction (PCR)-based experiments, the RNA is generally fragmented. This is supported by constructing a cDNA library from salivary RNA.³⁰ The sequencing of the cloned fragments showed that most of the RNAs are not full length, and they stem from both nucleus and mitochondria. The sequence analysis excludes the possibility that genomic DNA contamination is a problem (or even the main contributor) in salivary RNA studies: None of the 117 sequences obtained matches the sequences of known pseudogenes or could originate from the DNA sequence of the gene. Other indications that genomic DNA does not confound our analyses of the RNA include the absence of amplification with no-RT controls³⁰ and the complete removal of a signal in the electrophoretic analysis of salivary RNA by RNase treatment (Fig. 1).

While salivary RNA is surprisingly stable, the accurate quantitative analysis warrants immediate sample processing or stabilization. Sample collection and processing on ice halt the “degeneration” of transcriptional patterns for several hours, and in the laboratory setting, immediate freezing of the specimen is effective. However, this is not always possible in clinical or home-based sampling. The identification of suitable stabilization reagents fitting the needs and constraints of the test is an important step toward practicability of molecular profiling. We thus compared the stability of RNA from whole saliva at room temperature with three stabilizing reagents. The preservation of RNA profiles was compared by array analysis from freshly processed sample against the RNA using samples stored at room temperature for one week either with no stabilizer, with Suprase Inhibitor (20U/mL, Invitrogen [Carlsbad, CA, USA]), RNAlater (Ambion Inc., Austin, TX, USA; 1:1 mix with saliva) or the RNeasy Protect Saliva reagent (RPS, Qiagen Inc., Valencia, CA, USA; 1 volume sample plus 5 volumes RPS). The samples were analyzed using Human Genome U133 Plus 2.0 GeneChip Arrays from Affymetrix (Santa Clara, CA, USA) (Fig. 2). Comparison of samples between 0 and 7 days shows a twofold decrease in signal intensity for 159 transcripts with unstabilized samples, but for only 38 transcripts with RPS-stabilized samples. The comparison clearly shows that RNAlater, which is very good for the stabilization of blood and tissue RNA, results in more salivary RNA degradation than any of the other preservatives, even worse than the control sample without any stabilizer. Suprase Inhibitor is second best in this group, next to RPS, in salivary RNA stabilization. Thus it seems that RNAlater is not suited for the stabilization of salivary RNA. However, the overall expression profile is well preserved by the RPS reagent. This reagent has recently been made commercially available (Qiagen) for the stabilization of RNA in whole saliva.

Even storage of up to 1 week at room temperature did not affect the expression profile of seven oral cancer markers compared to the fresh sample (Fig. 3). Consequently, for the analysis of mRNA from whole saliva, RPS is the preservation reagent of choice.

The clinical potential of saliva mRNA analysis has been demonstrated by providing an oral cancer-specific detection model of mRNA biomarkers.²¹ The validation of the salivary oral cancer biomarkers was benchmarked against the use of serum-based prediction established with the same methodology and with samples from the same patient and control cohorts. In this comparison, the panel of four saliva-specific biomarkers possessed a slight edge over the serum-derived marker panel (ROC values of 0.95 vs. 0.88, respectively).³¹ These results show that the analysis of saliva may, at least in certain instances, not only be less invasive, but also provide a better value than blood, the commonly targeted body fluid in diagnostic investigations.

Besides oral cancer, the diagnosis of other diseases of the oral cavity may benefit greatly from saliva-based RNA analysis. Sjögren's syndrome is an autoimmune disease of the salivary glands. Their secretory fluids comprise the predominant part of what we call whole saliva or oral fluid. Not only is the treatment of Sjögren's syndrome in its infancy, but also even the diagnosis and studies of the disease are inhibited by unclear, variable definitions.

Array-based analysis identified 26 potential mRNA markers that discriminate between control and individuals with Sjögren's disease. This preliminary analysis may lead to the establishment of clearer diagnostic process and definition of this autoimmune disorder affecting the salivary glands. This would be an important step toward the development of successful treatment or repression of the disease progress. Our laboratory is currently exploring the utility of the salivary transcriptome (and proteome) in other oral and systemic diseases.

In conclusion, information related to oral and systemic health and disease is embedded in a single drop of saliva. It is our intention to establish the rationale, methodology and targets to harness the potential of genomic and proteomic information for the improvement of health care and patient well-being. By moving the time of detection to an earlier stage of the disease, health costs may be decreased dramatically, while patients will be the main beneficiaries through increased health and quality of life.

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FIGURE 1.

RNA extractions from saliva supernatant were analyzed with the Agilent Bioanalyzer Pico RNA Chip (Agilent Technologies, Palo Alto, CA, USA). Extracts were left untreated (Red), or digested with RNase (Green).

FIGURE 2.

Whole saliva was left untreated or stabilized with RNAlater, Suprase or RPS. RNA was extracted immediately or after incubation at room temperature for one week. The transcriptomes of fresh and stored aliquots for each stabilization reagent are compared on a scatter plot and transcripts with significantly different levels are highlighted in red. Expression profiles were established with the Human Genome U133 Plus 2.0 GeneChip Array.

FIGURE 3.

Whole saliva was stabilized with RPS and incubated for up to one week. The expression levels of seven transcripts were determined by quantitative RT-PCR, and cycle threshold values (C_T) are indicated on the y-axis.