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Salivary mRNA targets for cancer diagnostics

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Abstract

Head and neck squamous cell carcinoma (HNSCC) affects almost 1 million people worldwide per year. Despite therapeutic advances the overall survival rate remains low because diagnosis often occurs only at advanced stages with poor prognosis. Like in most cancers, the implementation of an early detection scheme would have a positive impact on this disease. Similarly, as oral cancer has a very high recurrence rate, the early identification of recurrence or second primary tumors is an important challenge.

HNSCC detection is currently based on expert clinical examination of the upper aerodigestive tract and histologic analysis of suspicious areas, but it may be undetectable in hidden sites, and unfortunately visual screening for oral lesions is an often neglected part of dental healthcare. Our group is actively pursuing the assembly of a toolbox for the molecular analysis of oral fluid. Here we present our current status utilizing the salivary transcriptome for oral cancer diagnostics.

Background: History of saliva molecular analysis

Saliva is a mirror of the human health and a reservoir of analytes from systemic sources that reach the oral cavity through various pathways. The composition of saliva reflects levels of hormonal, immunological, toxicological and infectious disease markers. Consequently this fluid provides a source for the monitoring of oral and also systemic health. This is the basis of our vision to develop disease diagnostics and promote human health surveillance by analysis of saliva.

Saliva has been used for diagnostics over more than two thousand years. Doctors of ancient cultures considered saliva as part of the circulation and changes in saliva are indicative of certain aspects of the patients health. For example, the viscosity and odor, as well as the individual's gustatory sensation of their own saliva have been used. Over-secretion of saliva is linked to cold stimulation of the stomach or heartburn, and a sweet flavor of the saliva is correlated with spleen malfunctions. Such theories are historical landmarks of the earliest medical applications of saliva in diagnostics.

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For many years, salivary research was focused on the analyses of the basic biochemical and physiological aspects of saliva from healthy individuals. The intrinsic value of saliva as a potential indicator of systemic health was not recognized. In one of the earliest reports, it was observed that the salivary levels of thiocyanate ions could be used to differentiate smokers from non-smokers. The study surveyed blood, saliva and urine, and indicated saliva as the most sensitive. This observation was later applied as chemical indication of smoking in evaluating self-reports of cigarette usage.

The use of saliva in a diagnostic setting has obvious advantages to blood based testing [1,2]. Oral fluid is a perfect medium offering a non-invasive, easy to obtain means for patient specimen collection. Saliva testing potentially allows the patient to collect their own saliva sample at home, yielding savings in healthcare costs, convenience for the patient, facilitating multiple sampling and having a positive impact on patient compliance.

It is possible to measure the concentration of drugs, hormones, antibodies and other molecules in saliva. Drug monitoring includes therapeutic drugs like theophylline, lithium, methadone and cyclosporine as well as abusive drugs such as alcohol, cocaine, marijuana, opiates and methamphetamines. Virtually all natural steroid hormones of significance in routine endocrinology, estrogens, testosterone, DHEA, progesterone, cortisol and melatonin can be monitored in saliva. For example, salivary estriol testing is approved by the FDA to predict preterm birth [3]. In addition, the onset and severity of infectious diseases can be determined by monitoring the presence of antibodies to viruses (HIV, Hepatitis A, B, C and measles).

Nucleic acids in body fluids

Many body fluids have been shown to contain nucleic acids of potential diagnostic value. On the forefront of this research is the field of non-invasive prenatal diagnosis: Within ten years of its finding cell-free fetal DNA testing has become a clinical reality in several European countries [4]. The genomic applications of DNA also have led to the discovery of placental derived cell-free mRNA signatures of placental origin in the circulation of the pregnant woman. Increased levels of RNA and DNA are indicative of certain pregnancy associated complications, and these analytes also offer hope to find alternatives to invasive prenatal testing for fetal aneuploidies.

Currently, saliva is widely used as a convenient source of genomic DNA. Salivary DNA has been used in the forensic sciences for a long time as a reliable source of DNA evidence for applications such as identity testing (22); single nucleotide polymorphism (SNP) detection from saliva DNA has even been proposed to offer higher accuracy than blood based analysis (23, 24). It can be envisioned that, as technologies for affordable high-throughput genotyping become a reality, salivary genomic DNA will eventually be used for genomic scanning for risk prediction and other pharmacogenomic applications of personalized medicine. This will render saliva banking a widespread practice, which would in turn be a driving force for the advancement of its analytical promises.

Cancer Biomarkers in Saliva

The non-invasive nature of collection, the direct contact to the oral cavity and the relationship between oral fluid and blood levels make saliva a useful and promising specimen. The ability to observe disease onset, progression, recurrence or treatment outcome through non-invasive means is highly important to advance health care management. Several salivary biomarkers that provide diagnostic information about cancers in the oral cavity or in the head and neck region have been identified. Antibodies to, for example to p53 [5], and salivary soluble CD44 and EGF have been proposed as head and neck squamous cell carcinoma biomarkers [6,7].

The saliva counts of 3 oral bacteria species were found to be diagnostic indicators of OSCC [8].

Genetic alterations in the tumor tissue have been traced in the cellular component of oral fluid (Rosas, Koch et al. 2001). These can be epigenetic changes of methylation, loss of heterozygosity or point mutations of genomic or mitochondrial DNA. Also, elevated mitochondrial DNA was associated with HNSC and with advanced stage disease (Jiang, Masayesva et al. 2005).

Salivary transcriptome

While the genomic biomarkers are obtained from the cellular component of the oral fluid or mouth washings, our group's efforts have focused on the supernatant and thus cell free phase of whole oral fluid. We reasoned that salivary analytes would be more apt for diagnostic clinical utilization were they to be contained in the cell free fraction of saliva as the cellular pellet will comprise predominately normal oral epithelial cells and leukocytes. Sparked by the participation in the human salivary proteome project we have looked at the presence of an additional analyte in this body fluid: mRNA.

Messenger RNA is the direct precursor of proteins and in general the corresponding levels are correlated in cells and tissue samples. Nucleic acids such as DNA and RNA are currently much easier to screen in "omic" manner and candidate disease markers can be verified by sensitive and specific PCR based methodologies which allow a decent level of throughput.

In an attempt to link IL-8 protein, a putative biomarker for oral cancer, to IL-8 RNA in the same saliva supernatant, our group discovered that human messenger RNAs are present in cell-free form in saliva [9]. Upon this finding we profiled salivary RNA of healthy subjects on gene expression arrays, establishing the "normal salivary core transcripts" (NSCT), a set of 185 mRNAs which were detected in each saliva supernatant of the studied 10 healthy subjects [10]. The translational utility of salivary transcriptome analysis was established by the array based discovery of several oral cancer mRNA biomarkers [11]: 9 candidate transcripts were chosen from a comparison of ten early stage oral cancer patients and ten healthy matched control subjects for validation by quantitative "real-time" RT-PCR. In a validation cohort of 32 patients and 32 controls (including the samples used in the discovery) 7 transcripts were confirmed to be elevated in OSCC with statistical significance ($p<0.05$ with Wilcoxon's signed rank test): DUSP1, H3F3A, IL1B, IL8, OAZ1, SAT and S100P. Combinations of these biomarkers displayed a sensitivity and specificity of up to 91% in distinguishing patients from controls, which places them amongst the most discriminatory panels of cancer biomarkers from body fluids. In collaboration with the National Cancer Institute's Early Disease Detection Network (EDRN) we are currently validating the salivary transcriptome biomarkers for oral cancer detection. Targeting the initially validated 7 salivary oral cancer markers, over 150 additional patients have been tested.

As already discussed, mRNA in plasma has been widely accepted and the utilization of this related analyte recently peaked in the non-invasive determination of fetal aneuploidies from maternal plasma [12]. We have observed that similar to plasma the endogenous cell-free mRNA in saliva is protected from immediate degradation [13,14]. Meanwhile, the salivary mRNA finding has been challenged by others unable to detect mRNA in saliva supernatant and also not in whole saliva [15]. However, while the method used for RNA detection is mRNA specific, it has a reduced sensitivity and stands in direct conflict with the fragmented nature of salivary mRNA. More importantly, the reported protocol of RNA extraction reveals several flaws at a closer look - while no positive control for the extraction procedure is reported [16]. Additional technical inadequacies, the inability to extract RNA even from the cell pellet and the missing of positive extraction controls "suggest a problem with their experimental technique that puts

the validity of their conclusions about the nonpersistence of mRNA in the cell-free portion of saliva in doubt" [17]. In the past few years mRNA from saliva has been targeted in forensic applications to identify stains caused by body fluids [18,19]. In an extension of this undertaking, salivary RNA was also applied successfully to array analysis by an independent group to identify new saliva identifying mRNAs [19]. Lastly, salivary amylase mRNA (and protein) were shown to be highly correlated with sleep deprivation [20].

Since our initial reports we have published and accumulated an abundance of additional supporting evidence, ranging from the characterization of salivary RNA [13], over assessing salivary RNA integrity by cDNA library analysis [21], to identifying and supporting the commercialization of an effective stabilizer of RNA in whole saliva, RNAprotect® Saliva (QIAGEN) [22]. Salivary cDNA library analysis showed that none of the 117 cloned mRNAs from saliva supernatant are from a pseudogene of contaminating genomic origin.

The surprising stability of RNA in saliva may be conferred by a similar mechanism as is the case in plasma, which is still not known. In both body fluids the RNA is associated with some macromolecule or subcellular body. For example, apoptotic bodies have been suggested. In saliva, we have found a common AU-rich element (ARE) containing sequence motif in many of the NSCT transcripts (V. Palanisamy, D. T. Wong, unpublished). These ARE typically exists in the 3′-UTR of transcripts and recruit ARE binding proteins to control stability.

Over the past three years our group has been actively developing the application of patient-based transcriptome wide technologies to identify RNA biomarkers in saliva. The current "working" knowledge base of saliva mRNA analysis is outlined in box 1.

- Microarray technology was applied to identify RNA profiles in cell free saliva [10] and to define a normal***Salivary core transcriptome (NSCT)***
- Salivary ***mRNA biomarkers for oral cancer*** were identified through microarray technology as DUSP1, H3F3A, OAZ1, S100P, SAT, IL8 and IL1B [9–11].
- ***Sources:***The three major salivary glands, gingival crevice fluid, and dismantled oral epithelial cells contribute to the mRNA levels of whole saliva [13].
- Stability: We showed that RNAs in saliva, similar to cell-free RNA in plasma, are ***protected from immediate degradation*** by their association with macromolecules [13].
- Integrity: Sequencing results from a saliva cDNA library demonstrated the fragmentation and the consequently ***missing 3′ poly-A tail of most salivary RNAs***. This work also demonstrated the unequivocal conclusion that the salivary RNA signals detected can neither originate from contaminating genomic DNA nor from pseudogenes, as none of the 117 sequences obtained matched the sequences of known pseudogenes or could originated from the DNA sequence of the gene [21]. Also importantly all transcripts identified by sequencing are from***human origin***.
- Next to oral cancer, the diagnosis of other diseases of the oral cavity may benefit greatly from saliva-based RNA analysis. We reported salivary mRNA and protein ***biomarkers for Sjögren's Syndrome,*** an autoimmune disease of the salivary glands [23]. Array-based analysis identified 26 potential mRNA markers that discriminate between healthy and diseased individuals. This preliminary analysis may lead to the establishment of clearer diagnostic process and definition of the autoimmune disorder affecting the salivary glands. This would be an important

step towards the development of successful treatment or repression of disease progress.

- We developed a sequence specific ***RT-PCR preamplification with enzymatic cleanup*** that enables the unbiased quantitative PCR analysis of over 50 transcripts from one reaction. This method allows the inclusion of ***several normalizer genes and novel spike controls*** (Zimmermann et al, manuscript submitted).
- We identified a method for global and unbiased ***amplification of fragmented RNA*** for the analysis on the all exon array (Hu et al, manuscript submitted).
- Micro RNA was detected and quantified in whole saliva and supernatant.

Two recent technological advances will allow us to undertake future projects with increased efficiency:

First, we have identified a poly-A independent method to amplify salivary RNA fragments for microarray analysis, making it possible to utilize the Affymetrix all exon array (AEA). In this manner, the saliva expression profile can be determined with a resolution down to individual exons, overcoming the 3′ end bias of previous analyses on the Affymetrix U132 array. Currently we have defined the Salivary Exon Core Transcriptome (SECT). In comparison to the earlier NSCT of 185 genes it contains 1370 probe sets representing 851 unique genes expanding the number of genes detected considerably (manuscript pending review).

Second, we have developed a method for the multiplex RT-PCR based preamplification of multiple RNA sequences. After an enzymatic clean-up individual targets can be quantified by real-time PCR using SYBR green dye as detector. This convenient, robust and affordable solution overcomes the constriction previously imposed by the sample, and now it is possible to examine a large number of mRNAs from one droplet of saliva without exhausting the precious patient sample (manuscript pending review).

While the oral cancer markers are validated according to established guidelines we also strive to develop technology for the point-of-care measurement of salivary analytes. In collaboration with the engineering department we are currently engaged in the development of electrochemical protocols for the simultaneous detection of salivary protein and mRNA cancer markers. By devising a novel hairpin probe design for the on-chip detection of specific nucleic acid sequences, we have been able to lower the limit of detection down to femtomolar levels in only 4 μl of sample (about 2500 copies) (manuscript pending review). At the same time, industrial vendors are developing instrumentation and test cartridges for the “chair side” implementation of electrochemical saliva analysis.

Outlook

Translating scientific findings of nucleic acids in body fluids to clinical application is a cumbersome journey. In the rarest cases has the final destination (i.e. a clinically applied test) been reached. For the utilization of cancer biomarkers it is of utmost importance to understand the basic mechanisms underlying the appearance of biomarkers in order to be able to define clear analytical tests with high specificity and sensitivity. Our group continues to pursue the biology of salivary analytes and the development of technology and disease markers. In addition to the detection and monitoring of disease in the oral cavity we are convinced that our efforts will ultimately result in the ability to harness molecular analytes for the diagnosis and management of systemic diseases.

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