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Salivary Biomarker

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Abstract

Saliva, a multi-constituent oral fluid, has a high potential for the surveillance of general health and disease. It contains an abundance of proteins and genetic molecules and is readily accessible via a totally non-invasive approach, which has long been recognized as the potential solution to limitations over other fluids. The use of saliva as a Diagnostic fluid has become somewhat of a translational research success story. Technologies are now available enabling saliva to be used to diagnose disease and predict disease progression.

Keywords: Saliva, Diagnostic Fluid, oral fluid, salivary biomarkers.

Introduction

Early detection of disease plays a crucial role in successful therapy. In most cases, the earlier the disease is diagnosed, the more likely it is to be successfully cured or well controlled. Managing a disease, especially in the early stage, may dramatically reduce the severity of its impact on the patient's life, or prevent and/or delay subsequent complications[1]. Since 2002, the National Institute of Dental and Craniofacial Research (NIDCR) created opportunities to overcome the limitations by exploring oral fluids as a diagnostic tool for the assessment of health and disease status. Human saliva is a clear, slightly acidic (pH = 6.0–7.0) biologic fluid containing a mixture of secretions from multiple salivary glands, including the parotid, submandibular, sublingual and other minor glands beneath the oral mucosa as well as gingival crevicular fluid. This complex oral fluid serves the execution of multiple physiologic functions such as oral digestion, food swallowing and tasting, tissue lubrication, maintenance of tooth integrity, antibacterial and antiviral protection (Mandel, 1987)[2]. In addition to the important role of maintaining the homeostasis of the oral cavity system, the oral fluid is a perfect medium to be explored for health and disease surveillance[3].

One of the principal advantages of using saliva as a diagnostic media is that its sampling is easy and noninvasive, thus eliminating any discomfort and pain associated with blood collection while also avoiding privacy issues associated with urine collection. Additionally, compared with blood, saliva contains a smaller quantity of proteins, therefore decreasing any potential risk of non-specific interference and hydrostatic interactions[4].

Saliva Profile

Water is the most abundant component in saliva, representing 99% of saliva's total composition. The solid components soluble in the aqueous phase differ from person to person, and can even vary in the same individual at distinct times during a day. The inorganic species are mainly composed of weak and strong ions including Na^+, K^+, Cl^-, Ca^{2+}, HPO_2^{3-} , HCO^{3-} , Mg^{2+}, and NH_3. The organic species consist of body secretion products (urea, uric acid and creatinine); putrefaction products (putrescine and cadaverine); lipids (cholesterol and fatty acids), and more than 400 types of protein. Among those proteins, the most relevant ones are glandular in origin (alpha amylase, histatins, cystatins, lactoferrins, lysozymes, mucins, and proline-rich proteins (PRPs)) or are plasma-derivatives (albumin, secretory immunoglobulin A (sIgA), and transferrin)[5].

Properties of saliva as a diagnostic fluid

Saliva is a clear, slightly acidic (pH = 6.0–7.0) and complex biological fluid composed of secretions from major salivary glands: the parotid, submandibular, and sublingual glands, as well as multitudes of minor glands including labial, buccal, lingual, and palatal tissues. In general, human salivary glands produce about 1–1.5 L of serous and mucinous saliva daily by combining water, salts, and an abundance of molecules from the blood with a cocktail of salivary proteins in the oral cavity to give rise to the multi-constituent whole saliva[6].

Saliva provides biological materials, e.g., mammalian and microorganism proteins, DNAs, and cells for potential medical and law enforcement use. Dentists and oral biologists have utilized the culture counts of Streptococcus mutans and lactobacillus from saliva to predict caries risk[7]. It is well known that saliva samples have been used for forensic DNA testing. The development of salivary/oral fluid-based diagnostics has focused on testing hormones, drugs and antibodies with some success in the past few decades. For example, commercialized saliva based testing systems have been used for the detection of HIV antibodies with high specificity and sensitivity similar to blood testing [8]. Antibodies to hepatitis B, C, and several other infectious pathogens (e.g., rubeola and dengue) can also be detected in saliva[9].

Salivary Diagnostics

Saliva diagnostics for the past two decades, have been developed to monitor oral diseases such as periodontal diseases[10,11] and to assess caries risk [12]. Recently, the combination of emerging biotechnologies and salivary diagnostics has extended the range of saliva-based diagnostics from the oral cavity to the whole physiologic system.For example, commercialized saliva based testing systems have been used for the detection of HIV antibodies with high specificity and sensitivity similar to blood testing [8]. Antibodies to hepatitis B, C, and several other infectious pathogens (e.g., rubeola and dengue) can also be detected in saliva[9]. Large numbers of medically valuable analytes in saliva have been gradually unveiled that represent biomarkers for different diseases including cancer[(13,14)], autoimmune[15,16]viral[17,18,19,20] and bacterial[21,22] diseases as well as HIV[23,24]. Furthermore, a growing body of evidence is getting established for

cardiovascular[25] and metabolic diseases[26] such as diabetes mellitus[27,28]. These advances have widened the salivary diagnostic approach from the oral cavity to the whole physiologic system, and thus point towards a promising future for saliva diagnostics for clinical decisions and post-treatment outcome predications.

The Salivary Proteome

The proteome is the protein complement of the genome, and proteomics is analysis of the portion of the genome that is expressed. The proteomes in bodily fluids are valuable due to their high clinical potential as sources of disease markers. In principle, a global analysis of the human salivary proteomes can provide a comprehensive spectrum of oral and general health. Furthermore, analysis of salivary proteomes over the course of complications may unveil morbidity signatures in the early stage and monitor disease progression.

Proteome-based approaches have been applied over the last three decades to monitor changes in protein expression. Generally, protein expression is primarily analyzed by one or two-dimensional polyacrylamide gel electrophoresis (PAGE). To resolve the complex composition of saliva, 2-D PAGE allows separation not only of different molecules with similar molecular weights, but also of different modification patterns or isoforms of the same protein. Along with the development and introduction of mass spectrometry (MS), the PAGE-separated proteins can be more accurately characterized and identified, leading to a wider range of applications for proteomic assays. Proteins that are primarily identified by MS can be further characterized by ionization methods such as electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI). Moreover, coupling ESI and MALDI with mass analyzers, such as quadrupole/linear ion trap, time-of flight (TOF), quadrupole TOF (QTOF), Fourier transform ion cyclotron resonance (FT-ICR) and the OrbiTrap, may improve the sensitivity, resolution, accuracy, and efficiency of protein sequence determination. To date, MS technology has yielded advanced insight into the characteristics of salivary proteomes, and provided strong evidence supporting the use of saliva as a diagnostic tool. [30]

In some cases, however, simply discriminating up and/or down regulation of the expression of specific proteins may not directly reflect the circumstances of physiological states or disease progression. This is because biological functions of proteins may change due to posttranslational modifications that occur without alteration of protein level [31 32]. It has been demonstrated that many functional alterations of proteins result from posttranslational modifications such as phosphorylation, glycosylation, acetylation, and methylation[33 34]. These post-translationally modified proteins may represent signatures in some diseases such as autism spectrum disorder[35] and cervical cancer [35] To evaluate the potential of post translationally modified proteins as diagnostic biomarkers, dendrimer-associated MS/ MS, MALDI-MS, and targeted HPLC-ESI-MS/MS provide comprehensively analytical methodologies for proteins with different types of posttranslational modifications [36 37].

As of January 2009, over a thousand salivary proteins have been identified from major salivary glands[30]. For most of these proteins, their expression in saliva is quite distinct from that in serum or tear, and have already demonstrated clinical diagnostic values for diseases manifested in the oral cavity. For example, Sjögren's syndrome (SS), a chronic autoimmune disorder that is clinically recognized by dry mouth (xerostomia) and dry eyes (keratoconjunctivitis sicca), is associated with

changes in specific salivary constituents, such as an increase in inflammatory proteins (e.g. α-enolase, carbonic anhydrase I and II, salivary α-amylase fragments) and decrease in acinar proteins (e.g., lysozyme C, polymeric) Immunoglobulin receptor (pIgR), calgranulin A) compared with the profile in non-SS individuals [38 39]. Other research efforts showed that saliva is an important tool for the detection of oral squamous cell carcinoma (OSCC). Three tumor markers (Cyfra 21-1, tissue polypeptide antigen (TPA), and cancer antigen CA125) are significantly elevated in saliva when compared to the patients' sera [40].

Recently there has been discoverer and validation of a highly discriminatory panel of salivary biomarkers for oral cancer detection. Five salivary proteins (M2BP, MRP14, profilin, CD59, and catalase) were shown to be able to discriminate oral cancer with greater than 90% clinical accuracy [41]. Besides SS and OSCC, salivary proteomic constituents are also capable of detecting high-impact systemic disorders. For example, measurement of antibodies to HIV in saliva has been shown to be as accurate as measurement in serum, and the salivary assay has been commercialized as a product called OraQuick. Moreover, early studies suggest that measurement of salivary CA125 and epidermal growth factor may have diagnostic potential for ovarian cancer [42] and breast cancer [43], respectively. Current efforts to elucidate the proteomes from whole saliva or individual glandular (e.g., parotid, submandibular and sublingual) saliva have progressed rapidly along with development of MS and protein separation techniques. A central salivary protein database has been established by the University of California, Los Angeles (UCLA) research team (www.hspp.ucla.edu) in which we have assembled acquired proteomic data and exchanged research results with groups worldwide. The integration of up-to-date information is ongoing, and extensive comparisons between proteins in saliva and other bodily fluids are under construction. This comprehensive categorization of salivary proteomes will be an important resource for researchers who are studying protein chemistry, especially in the fields of oral biology and salivary diagnostics, and will be helpful for analyzing how the expression of salivary proteomes changes with different diseases and hence identifying corresponding disease-related salivary biomarkers.

Salivary Transcriptome

In addition to salivary proteome, in 2004 we discovered the salivary transcriptomes (RNA molecules) that are unusually stable in saliva [44]. They included mRNA molecules that cells use to convey the instructions carried by DNA for subsequent protein production. This discovery presented a second diagnostic alphabet in saliva and opened a door to another avenue of salivary transcriptomic diagnostics. Although the salivary transcriptome is an emerging concept, we have established a robust platform at UCLA for salivary RNA studies including automated extraction, purification, amplification, and high-throughput microarray screening. Importantly, we have also developed statistical and informatics tools that are tailored for salivary biomarker discovery and validation. Also, Early Disease Research Network (EDRN), an entity within the National Cancer Institute (NCI), has just completed an independent validation study of salivary RNA biomarkers for oral cancer detection. This investigation confirmed a clinical translational value of salivary RNA for oral cancer detection. In the past 5 years, research into the nature, origin and characterization of salivary mRNA has been actively pursued [45 46 47]. At present, the main strategy for identification of salivary transcriptomic biomarkers is through microarray

technology. Although it has been demonstrated that the 3′-based array employing poly-dT priming and two rounds of in vitro transcription (IVT) amplification works well for profiling salivary transcripts, some pitfalls still need to be overcome. For instance, much information is lost because approximately 50% of salivary RNA molecules are fragmented,(50) therefore they do not carry the poly-A tail and are not protected against degradation. Furthermore, the random priming approach in the RNA amplification may cause an additional shortening of the fragments resulting in further loss of RNA molecules during the procedure. To address these issues, we have recently made a significant improvement to saliva transcriptomic screening using an emerging 3′-poly(A)-independent amplification technology to recover all salivary RNA fragments (ExpressArt TRinucleotide mRNA Amplification Kita), followed by profiling all fragments on the Affymetrix All Exon Array (AEA) platform. This novel approach allows investigation of the salivary transcriptome at a higher resolution level via detection of individual exons. Theoretically, the increased resolution could detect more genes and hence increase the opportunities to discriminate disease markers. So far we have defined the salivary exon core transcriptome (SECT), which contains 1,370 probe sets representing 851 unique genes that are present in more than 85% of the tested saliva samples [48].

Quantitative real-time PCR (qPCR) is currently the gold standard for quantification of nucleic acids. It is perfectly appropriate for validation of transcriptomic biomarkers after profiling by microarray, and it is not restricted by the length of the RNA, even for fragmented RNA. However, low amounts of RNA in saliva tremendously hinder their performance in qPCR. To overcome this problem, a new multiplex reverse transcriptase-PCR-based pre-amplification approach was developed that allows accurate quantification of over 50 targets from one reaction. This method dramatically increases the capacity of quantitative analysis that it extends approximately six-fold for the magnitude of target input[49] and is tailored to the short nature of salivary RNA. It also offers good time- and cost-effectiveness by performing simultaneous reverse transcriptase reactions for different targets, allowing a small volume of pre-amplification product to be used for subsequent qPCR measurement.

The studies of salivary mRNA biomarkers from patients with primary T1/T2 OSCC showed promising results and demonstrated the diagnostic and translational potential of the salivary transcriptome[49] . Data combining microarray profiling and qPCR validation showed seven mRNA whose expression levels in patients were elevated at least 3.5-fold compared with matched healthy counterparts. These mRNAs are transcripts of DUSP1, H3F3A, OAZ1, S100P, SAT, IL-8, and IL-1β. In the initial study, the combination of these biomarkers presented 91% sensitivity and specificity , displaying a high credibility for discrimination of OSCC. To further validate the salivary transcriptomic biomarkers for oral cancer detection, they compared saliva and blood transcriptomes from the same patients with respect to their capability for disease discrimination. The study showed that a group of five transcriptomic biomarkers in serum can be consistently validated and distinguished OSCC with 91% sensitivity and 71% specificity (ROC = 0.88)[49] . The salivary transcriptome is a more discriminatory tool for oral cancer detection than the serum transcriptome. So far, over 220 additional oral cancer patients have been tested and the clinical accuracy of the salivary mRNA biomarkers holds up at > 82% (Wang et al., unpublished

data), indicating they are among the most discriminatory panels for OSCC screening to date.

Salivary Diagnostics – A New Industry in A Prospective Future

The value of saliva as a diagnostic tool has long been disregarded until the advantages of saliva-based approaches were recognized in the past decade, and led to an evolution from treating saliva as a diagnostic worthlessness to promoting salivary diagnostics. Regarding diagnostic capability, the gap between saliva and other bodily fluids, such as blood, urine, and cerebral spinal fluid, is closing, primarily due to rapid technology development, scientific validation of diagnostic analytes, and advocacy by the National Institute of Dental and Craniofacial Research (NIDCR). Salivary diagnostics would enable clinicians to monitor diseases frequently and easily and would have impact on the future medical research and therapy. In addition to previously mentioned oral cancer and Sjögren's syndrome, systemic disorders may be reflected diagnostically in saliva as well. At present, we have promising preliminary results showing that saliva can be used to detect lung cancer, pancreatic cancer, breast cancer, and type II diabetes; however, for each disease, we need further scientific validation, as well as to benchmark the diagnostic capacity of saliva against other bodily fluids. These studies are ongoing and will undoubtedly remain a major focus of investigation in the future. Based on the abundance of promising research efforts and the fact that research into salivary diagnostics is currently a priority at NIDCR, saliva-based diagnostics present unparalleled opportunities for research and commercialization opportunities. With the current rate of progression, salivary diagnostics can become a key player in routine health monitoring in the near future and enable the early detection of disease using a simple and effective assay. Thus, salivary diagnostics will not only save lives, but also preserve the quality of lives that have been saved.[50]

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