Review Article

Saliva as a diagnostic tool

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Abstract

Salivary diagnostics is an emerging field that has progressed through several important developments in the past decade, including the publication of the human salivary proteome and the infusion of federal funds to integrate nanotechnologies and microfluidic engineering concepts into developing compact point-of-care devices for rapid analysis of this secretion. In this article, we discuss some of these developments and their relevance to the prognosis, diagnosis and management of periodontitis, as an oral target, and cardiovascular disease, as a systemic example for the potential of these biodiagnostics. Our findings suggest that several biomarkers are associated with distinct biological stages of these diseases and demonstrate promise as practical biomarkers in identifying and managing periodontal disease, and acute myocardial infarction. The majority of these studies have progressed through biomarker discovery, with the identified molecules requiring more robust clinical studies to enable substantive validation for disease diagnosis. It is predicted that with continued advances in this field the use of a combination of biomarkers in multiplex panels is likely to yield accurate screening tools for these diagnoses in the near future.

Key words

Acute myocardial infarction, Lab-on-a-chip, Periodontitis, Salivary diagnosis.

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. For example, in the case of ovarian cancer, which is the fifth most common malignancy and the fifth leading cause of cancer mortality in women in the US, the survival rate 5 years post diagnosis can reach 93% and 70% if tumors are detected in early stage I and II, respectively, but drops precipitously to 37% and 10% if the diagnosis is made in stages III and IV when the cancer is well

established and spreading [1] Type II diabetes, a serious metabolic disorder affecting more than 7% of American adults, could be well controlled solely by diet or changing lifestyle if symptoms were detected early enough [2]. People are aware of the importance of regular health check-ups; however, most systemic diseases are not diagnosed until morbid symptoms become apparent in the late phase. To overcome this challenge, medical researchers are devoted to finding molecular disease biomarkers that reveal a hidden lethal threat before the disease becomes complicated. These markers could be DNA, RNA, or protein molecules that act as indicators reflecting particular physiological states. In the past decade, scientists have demonstrated that human genetic alterations can be detected both intracellularly and extracellularly by molecular diagnostics [3]. In addition, abnormal nucleic acids and/or proteins have been identified in patients' bodily fluids such as blood, urine, and cerebrospinal fluid, and have been demonstrated to be effective biomarkers for diagnostic use [4-6]. Besides a significant impact on biological research, over the last decades molecular diagnostics have proved valuable in clinical applications [7, 8]. Most systemic diseases such as cancer, cardiovascular, metabolic, and neurological diseases are very challenging to diagnose without supplementary clinical evaluation. Even with a complete work up, the diagnosis usually remains uncertain due to complications of the disease. Currently three major limitations have prevented people from recognizing the full potential of disease detection, and have seriously hampered the development of clinical diagnostics, namely:

- Lack of definitive molecular biomarkers for specific diseases;
- Lack of an easy and inexpensive sampling method with minimal discomfort; and
- Lack of an accurate, easy-to-use, and portable platform to facilitate early disease detection.

Since 2002, the National Institute of Dental and Cranio-facial Research (NIDCR) created opportunities to overcome these limitations by exploring oral fluids as a diagnostic tool for assessment of health and disease status. Saliva, an oral fluid that contains an abundance of proteins and genetic molecules and is readi1y accessible *via* a totally noninvasive approach, has long been recognized as the potential solution to limitation number 2 [9]. Through the visionary investment by the NIDCR, the discovery of salivary biomarkers and ongoing development of salivary diagnostics technologies now provide promising solutions for limitations 1 and 3.

There is considerable excitement surrounding the application of saliva-based diagnostics for oral diseases, and we believe that this will soon be followed by application of highly informative salivary biomarkers to other high-impact systemic disorders because saliva is composed of various molecules that are filtered, processed, and secreted from the vasculature that nourish the salivary glands [10, 11]. This realization will enable scientists to bridge oral health research with systemic disease diagnosis. With the additional advantages of an easy, safe, cost-effective, and non-invasive diagnostic approach, saliva shows high potential for monitoring general health and disease, with enormous translational values, and unparalleled opportunities for clinical applications.

Physiology of saliva

Saliva is a complex oral fluid that includes secretions from three pairs of major salivary glands and from the minor salivary glands of the labial, buccal, lingual, palatoglossal, and palatal mucosa. Saliva can be considered as gland-specific saliva and whole saliva. Evaluation of the secretions from the individual salivary glands is primarily useful for the detection of gland-specific pathology, i.e. infection and obstruction [1]. However, whole saliva is most frequently studied when salivary analysis is used for the evaluation of systemic disorders. Whole saliva is a mixed fluid that is derived predominantly from

major and minor salivary glands. Whole saliva also contains gingival cervicular fluid, mucosal transudate, expectorated bronchial and nasal secretions, serum and blood derivatives from oral wound, bacteria, bacterial products, viruses, and fungi, desquamated epithelial cells, other cellular components, and food debris [12-17]. Saliva can be collected with or without stimulation. Stimulated saliva is collected by masticatory action or by gustatory stimulation. Unstimulated saliva is collected without exogenous gustatory, masticatory, or mechanical stimulation. The best two ways to collect whole saliva are the draining method, in which saliva is allowed to drip off the lower lip, and the spitting method, in which the subject expectorates saliva into a test tube [18]. The salivary glands are composed of specialized epithelial cells, and their structure can be divided into two specific regions: the acinar and ductal regions. All the salivary fluid is produced from the local vascular bed in the acinar region, and is transported through the duct system, where excess sodium and chloride are reabsorbed and some additional proteins are secreted, and then empties into the oral cavity [19]. The acini of salivary glands can be characterized as serous, seromucous, or mucous types. The acini of parotid gland are mainly serous and seromucous, those of submandibular gland are mainly seromucous, and those of sublingual glands are mainly mucous. The minor salivary glands mostly produce mucus type of secretion. About 99% of saliva is water and the other 1% is a complex of organic and inorganic molecules. Daily secretion rates range between 500 and 700 ml, and the average volume in the mouth is 1.1 ml. Saliva production is controlled by the autonomic nervous system. At rest, the secretion ranges from 0.25 to 0.35 ml/min and is mostly produced by the submandibular and sublingual glands. Sensory, electrical, or mechanical stimuli can raise the secretion rate to 1.5 ml/min. The greatest volume of saliva is produced before, during, and after meals, reaching its maximum peak at around 12 a.m., and falls considerably at night, while sleeping [20]. Saliva has major functions like digestion, protection, lubrication, buffering action, maintenance of tooth integrity, and perception of taste [21-24]. Salivary immunoglobulins take part in elimination of bacteria, fungi, and viruses through specific immune mechanisms and agglutination. Salivary cystatins S, C, and D show antiviral, antiparasitic, and antibacterial activities. Salivary amylase is proposed to perform inhibitory effect on growth of microorganism.

Saliva as a diagnostic fluid

Saliva diagnostics is a later bloomer, as only recently has there been a growing appreciation that saliva can reflect virtually the entire spectrum of normal and disease states [25]. These include tissue levels of natural substances and a large variety of molecules introduced for therapeutic, dependency or recreational purposes, emotional status, hormonal status, immunological status, neurological effects, and nutritional and metabolic influences. A major barrier to using saliva as a diagnostic fluid has been the fact that many informative analytes are generally present in lower amounts in saliva than in serum [26]. With new and highly sensitive technologies, the lower level of analytes in saliva is no longer a limitation. Almost anything that can be measured in blood can also be measured in saliva. Saliva has been reliably used to detect HIV-1 and -2, and viral hepatitis A, B and C. It can also be used to monitor a variety of drugs including marijuana, cocaine and alcohol .There are compelling reasons to use saliva as a diagnostic fluid to monitor health and diseases. As a clinical medium, saliva has many advantages over serum. Saliva is easy to collect, store and ship and can be obtained at low cost in sufficient quantities for analysis. For patients, the non-invasive collecting techniques dramatically reduce anxiety and discomfort and simplify procurement of repeated samples for longitudinal monitoring over time. For professionals, saliva collection is safer than venepuncture, which could expose health care providers to HIV or hepatitis virus. Saliva is also easier to handle for diagnostic procedures since it does not clot, lessening the manipulations required. Saliva-

based diagnostics are therefore less invasive, less expensive and present less risk to both the patient and the provider than current methodologies.

Developments of technologies for saliva-based diagnostics

Five years ago, in 2002, the NIDCR initiated a concerted research effort in the area of saliva diagnostics. NIDCR funded seven U01 awards to develop microfluidics and microelectro-mechanical systems (MEMS) for saliva diagnostics. The aim is to identify technologically viable systems and support their advance towards commercialization. MEMS are integrated systems consisting of sensors, actuators, and electronics on a common silicon substrate developed through microfabrication technology. Minute sample and reagent volumes are processed and analyzed with integrated detectors. The seven NIDCR-supported U01 awards focused on the development of microfluidic and MEMS technologies for measuring DNA, gene transcripts (mRNA), proteins, electrolytes and small molecules in saliva, as well as overall profile correlates of a particular disease state, such as cardiovascular disease [27, 28]. Despite laboratory progress in dental schools and engineering departments, none of the new technologies will become a practical and clinical reality without strong partnerships with industry, early in the development stage. The reasons include the many challenges that such technologies face at every stage including fabrication, integration of individual components, validation, regulatory approval and finally commercialization. This has sparked a new U01-level initiative for the 'Development and Validation of Technologies for Saliva-Based Diagnostics' in order for the currently developed academic saliva diagnostics groups to team up with industrial partners for further development of functional prototypes and testing their robustness for clinical applications. Four of the initial seven groups were recently renewed for 5 years for the second round.

Diagnostic molecular targets in saliva: the proteome and the transcriptome

The salivary proteome

To fully utilize the diagnostic potential of saliva, one needs to comprehensively decipher and catalogue the informative components. In fiscal year 2003, NIDCR funded three U01 awards aiming to identify and catalogue human salivary proteins from the three major salivary glands. It is envisioned that the human salivary proteome (HSP) will be a resource to help elucidate disease pathogenesis and evaluate the influence of medications on the structure, composition and secretion of all salivary secretory constituents. Saliva Proteome Consortium in order to collectively decipher the HSP. In general, a 'divide and conquer' bottom-up strategy is used. The proteins from whole or ductal saliva (parotid and SM/SL) are initially fractionated with a variety of separation techniques including reversed-phase liquid chromatography (LC), strong cation exchange (SCX) LC, gel filtration LC, Zoom isoelectric focusing (Zoom IEF), and ultrafiltration. Further, the collected protein fractions are digested with a proteolytic enzyme, e.g. trypsin, and then analyzed with 1-D or 2-D LC-MS/MS. Finally, the acquired MS data are processed and submitted for database searching using Mascot database search engine. We are also comprehensively cataloguing saliva glycoproteins using LC-MS/MS and glycoprotein pull-down method based on hydrazide chemistry. Similar to plasma/serum counterparts, many proteins (e.g. mucins and amylases) in human saliva are glycosylated. In the glycoprotein pull-down approach, glycoproteins are coupled on to a hydrazide resin. The proteins are then digested and formerly *N*-glycosylated peptides are selectively released with the enzyme PNGase F and analysed by LC-MS/MS. Employing this method, coupled with in-solution isoelectric focusing separation as an additional means for pre-fractionation, we identified 84 formerly *N*-glycosylated peptides from 45 unique *N*-glycoproteins [29]. The multiplexed proteomic platforms have clearly deepened the HSP analysis. As the multiplexed proteomic platforms have clearly deepened the HSP analysis. As the analysis of parotid and SM/SL saliva nears

completion, we have catalogued in whole saliva (WS) more than 1000 proteins [30]. We have also developed a saliva proteome knowledge base (SPKB) to centralize the acquired proteomic data and annotate the identified saliva proteins. The SPKB is fully accessible to the public for queries regarding the identified proteins, which are linked to public protein databases. Elucidation of the normal salivary proteome is only the first step on a road with many forks. Comparison of such a normal protein catalogue with that of a diseased population will reveal diagnostic signatures that can discriminate between normal and diseased individuals. We have started making translational discoveries into the salivary proteome for oral cancer [31] and Sjögren's syndrome patients . Comparative analysis of HSP and human plasma proteome (HPP) suggests that extra-cellular proteins are predominant in HSP, whereas the membrane proteins are predominant in HPP. HSP proteins have significant binding and structural molecular activities whereas the HPP proteins show significant activities of nucleotide/nucleic acid binding. In terms of 'biological processes', a significant percentage of serum proteins are involved in cell cycle or signal transduction whereas a significant percentage of saliva proteins are involved in physiological or response-to-stimulus processes.

Conclusion

Saliva is a complex biological fluid that can be used to diagnose diseases or to detect the evolution of certain pathological situations in the human body. It can also be used to monitor the therapeutic levels of the drugs as well as to detect the illicit drug use. Saliva, as a diagnostic tool, clearly meets the demands for an inexpensive, noninvasive, and easy-to-use screening method. Initially, salivary diagnostics have been developed to monitor oral diseases such as periodontal diseases and to assess dental caries. With the help of molecular diagnostics and nanotechnology, a large number of salivary biomarkers for different diseases including cancer, autoimmune diseases, viral diseases, bacterial diseases, cardiovascular diseases, and HIV have been developed. These developments have extended the range of saliva-based diagnostics from the simple oral cavity to the whole physiological system. Thus, saliva-based diagnostics is on the cutting edge of diagnostic technology and can act as an alternative for clinicians to use in the near future to make clinical decisions and to predict post-treatment outcomes.

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