

INVITED MEDICAL REVIEW

Saliva: diagnostics and therapeutic perspectives

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For the past two decades, salivary diagnostic approaches have been developed to monitor oral diseases such as periodontal diseases and to assess caries risk. 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 as most compounds found in blood are also present in saliva. Accordingly, saliva can reflect the physiologic state of the body, including emotional, endocrinal, nutritional and metabolic variations and acts as a source for the monitoring of oral and also systemic health. This review presents an update on the status of saliva diagnostics and delves into their applications to the discovery of biomarkers for cancer detection and therapeutic applications. Translating scientific findings of nucleic acids, proteins and metabolites in body fluids to clinical applications is a cumbersome and challenging journey. Our research group is pursuing the biology of salivary analytes and the development of technologies for detection of distinct biomarkers with high sensitivity and specificity. The avenue of saliva diagnostics incorporating transcriptomic, proteomic and metabolomic findings will enable us to connect salivary molecular analytes to monitor therapies, therapeutic outcomes, and finally disease progression in cancer.

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Introduction

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 crevice 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). 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.

Just as is the case with blood, saliva is a complex fluid containing a variety of enzymes, hormones, antibodies, antimicrobial constituents, and cytokines (Zelles *et al*, 1995; Rehak *et al*, 2000). Many of these enter saliva from the blood by passing through cells by transcellular, passive intracellular diffusion and active transport, or paracellular routes by extracellular ultra filtration within the salivary glands or through the gingival sulcus (Drobitch and Svensson, 1992; Haeckel and Hanecke, 1993; Jusko and Milsap, 1993). So, most compounds found in blood are also present in saliva. Accordingly saliva can reflect the physiologic state of the body, including emotional, endocrinal, nutritional and metabolic variations. 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. In this article, we will review the current status of saliva proteomics and transcriptomics and their applications to the discovery of biomarkers for cancer detection and therapeutic applications.

Saliva diagnostics

For the past two decades, salivary diagnostic approaches have been developed to monitor oral diseases such as periodontal diseases (Kornman *et al*, 1997; Socransky *et al*, 2000) and to assess caries risk (Baughan *et al*, 2000). 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. Large numbers of medically valuable analytes in saliva have been gradually unveiled that represent biomarkers for different diseases including cancer (Boyle *et al*, 1994; Li *et al*, 2004a; Zhang *et al*, 2010), autoimmune (Streckfus *et al*, 2001; Hu *et al*, 2007b), viral (Chaita *et al*, 1995;

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Ochnio *et al.*, 1997; El-Medany *et al.*, 1999; Pozo and Tenorio, 1999) and bacterial (Kountouras, 1998; Lendenmann *et al.*, 2000) diseases as well as HIV (Emmons, 1997; Malamud, 1997). Furthermore, a growing body of evidence is getting established for cardiovascular (Adam *et al.*, 1999) and metabolic diseases (Walt *et al.*, 2007) such as diabetes mellitus (Rao *et al.*, 2009; Sashikumar and Kannan, 2010). 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 personalized individual medicine applications including clinical decisions and post treatment outcome predications.

Technologies for discovery of salivary biomarkers

The term, biomarker, refers to measurable and quantifiable biologic parameters that can serve as indicators for health and physiology-related assessments, such as pathogenic processes, environmental exposure, disease diagnosis and prognosis or pharmacologic responses to a therapeutic intervention. Saliva is a unique body fluid for the development of molecular diagnostics as it contains not only components found in serum but also offers several advantages over serum. Saliva collection is cost-effective, safe, easy and non-invasive. However, previously available technologies did not allow the necessary screening of the complex constituents, which are present in saliva at low concentrations but in abundance and so its potential was frequently underestimated (Helmerhorst and Oppenheim, 2007). Promising new technologies with higher detection sensitivity have opened up new horizons, ultimately meeting the requirements for successfully screening saliva components, especially proteins and nucleic acids. Studies of the salivary proteome have shown that, in addition to the major salivary protein families such as alpha-amylase, saliva contains hundreds of minor proteins or peptides defining the first salivary biomarker alphabet. These salivary proteomic markers are present in low concentrations and can play an important role in the identification of diseases at the onset/premalignant stage itself. Saliva not only contains proteins but also nucleic acids, and thus the second salivary diagnostic alphabet, the saliva transcriptome, is currently a focus of interest. Our laboratory has performed a significant body of work and is dedicating considerable resources to investigate the transcriptomic approach in saliva, and thus spearheading the detection of messenger RNA (mRNA) and microRNA in human saliva.

The salivary proteome

The capability to identify proteins and to determine their covalent structures has been central to the life sciences. The amino acid sequence of proteins provides a link between proteins and their coding genes via the genetic code, and, principally, a link between cell physiology and genetics. The identification of proteins

provides an insight into complex cellular regulatory networks (Domon and Aebersold, 2006). Studying the proteome, the protein complement of the genome, in bodily fluids is valuable because of its clinical significance as a source of disease markers and thus, the proteome of human salivary fluid has the potential to open up new doors for the discovery of disease biomarkers. Salivary proteins not only play a role in maintaining oral and general health but may also serve as biologic markers to survey normal health and disease status. As a consequence, analysis and cataloging of the human salivary proteome will be of great interest to researchers within the fields of saliva-based diagnostics and oral biology (Hu *et al.*, 2007c).

Saliva proteome knowledge base

The complete catalog of the salivary proteome, including its stratification according to their parotid, submandibular/sublingual origins, has been generated using state-of-the-art, sensitive and high-throughput mass spectrometry (MS) proteomic technology combined with different protein separation methods (Karas and Hillenkamp, 1988; Wilmarth *et al.*, 2004; Hirtz *et al.*, 2005; Hu *et al.*, 2005, 2007a; Xie *et al.*, 2005a,b; Guo *et al.*, 2006; Walz *et al.*, 2006; Yates *et al.*, 2006; Denny *et al.*, 2008; Lee and Wong, 2009; Yan *et al.*, 2009). This systematic study of all salivary secretory proteome components, post translational modifications and protein complexes has been elucidated to make a description of their structure and function fully accessible and available to the public (<http://www.hspp.ucla.edu>). At this website, the Saliva Proteome Knowledge Base (SPKB) and the Salivary Proteome Wiki (SPW) are linked. SPW centralizes the acquired proteomic data and annotates the identified saliva proteins and is fully accessible to the public for queries. The National Institute of Dental and Craniofacial Research (NIDCR) will soon launch the SPW, which is a collaborative environment to share and annotate salivary proteomics data. An initial key step for saliva to be of practical use for disease diagnosis and health monitoring is the classification of its protein components. Recently, several reports aiming to comprehensively catalog the salivary proteome have been published with numbers of proteins identified ranging from hundreds to over thousands (Wilmarth *et al.*, 2004; Hu *et al.*, 2005, 2006, 2007b,d; Xie *et al.*, 2005a,b; Guo *et al.*, 2006; Ramachandran *et al.*, 2006). In 2008, an NIDCR-funded consortium study comprehensively identified and cataloged the human ductal salivary proteome and led to the compilation of 1166 proteins, out of which 914 were identified in the parotid and 917 in the submandibular and sublingual fluids, as well as 665 proteins that were common to all the glands (for further information go to: <http://www.hspp.ucla.edu>) (Denny *et al.*, 2008).

Human Plasma Proteome Project

It is important to compare the saliva protein composition with other established proteomes, such as plasma to understand the unique utility of the salivary proteome in the context of its function and potential diagnostic

value. Overlap in protein content between saliva and plasma may indicate that saliva could be used as a diagnostic alternative to blood tests. Numerous studies have uncovered how changes in the concentrations of specific plasma proteins have been associated with disease processes, leading to well-accepted clinical applications (Kasper, 2004). To date, the plasma proteome is perhaps the most extensively studied human proteome. The international HUPO Human Plasma Proteome Project, a collaboration of many laboratories using MS technology, compiled a core dataset of 3020 distinct proteins (with a minimum of two unique peptides per protein) (Adamski *et al*, 2005; Omenn, 2005; Omenn *et al*, 2005), out of which 889 proteins were confirmed as high-confidence identifications through a rigorous statistical approach adjusting for protein length and multiple comparisons testing (States *et al*, 2006).

The protein compositions found in human salivary and plasma fluids have been compared (Yan *et al*, 2009) and approximately 27% of the whole saliva proteins are found in plasma. Despite this apparently low degree of overlap, the distribution found across Gene Ontological categories such as molecular function, biologic processes and cellular components shows strong similarities (Loo *et al*, 2010). Nearly 40% of the proteins that have been suggested to be candidate markers for cancer, cardiovascular disease and stroke can be found in whole saliva. Several sources can contribute to the overlap of protein identifications in saliva and plasma: (1) leakage of plasma into saliva through intracellular and/or extracellular routes including outflow of gingival crevicular fluid; (2) plasma and saliva may share essential proteins needed to maintain their physiologic function as body fluids; and (3) proteins derived from cell debris may be in close contact with either fluid.

These comparisons and correlations should encourage researchers to consider the use of saliva for discovering new protein biomarkers and for detecting early signs of disease throughout the body. Moreover, following the diagnostic value of proteins in blood, proteomic analysis of saliva over the course of disease progression could reveal valuable biomarkers for early detection and monitoring of disease status in a non-invasive manner. Profiling the saliva proteins before and after pharmacologic interventions may provide important information on drug efficacy and toxicity.

Salivary transcriptome

Messenger (m) RNA is the direct precursor of proteins and in general the corresponding levels are correlated in cells and tissue samples (Zimmermann and Wong, 2008). Nucleic acids such as DNA and RNA are currently much easier to screen in an 'omic' manner, and candidate disease markers can be verified by sensitive and specific PCR-based methodologies allowing a relatively efficient level of throughput. Initially, as an attempt in linking IL-8 protein to IL-8 RNA in the same saliva supernatant in an oral cancer study, our group discovered that mRNAs are present in cell-free

saliva (St John *et al*, 2004). Following this finding, salivary RNA of healthy subjects was profiled on gene expression-based microarrays and more than 3000 species of mRNA were found, out of which a set of 185 mRNAs were consistently detected in healthy subjects [normal salivary core transcriptome (NSCT)] (Li *et al*, 2004b). The salivary transcriptome presented a second diagnostic alphabet in saliva and opened the avenue of salivary transcriptome diagnostics.

At the UCLA Dental Research Institute, we have established a robust platform to study salivary mRNA including automated extraction, purification, amplification, and high-throughput microarray screening. Moreover, we have developed statistical and informatics tools that are tailored for salivary biomarker discovery and validation. In the past years, the nature, origin and characterization of salivary mRNA have been actively pursued (Li *et al*, 2004a; Park *et al*, 2006, 2007; Walz *et al*, 2006; Hu *et al*, 2007b; Zhang *et al*, 2010). Early studies from our laboratory have demonstrated the feasibility of using salivary mRNA for detection of oral cancer (Li *et al*, 2004a; St John *et al*, 2004). In parallel, human mRNAs have been applied in various fields such as among forensic scientists who use a small set of saliva-specific mRNAs to identify body fluids (Juusola and Ballantyne, 2003; Nussbaumer *et al*, 2006), furthermore inflammatory mRNA markers were detected in whole saliva to monitor the status of periodontal disease in type II diabetic patients (Gomes *et al*, 2006).

Identification of salivary transcriptomic biomarkers

At present, the main strategy to identify salivary transcriptomic biomarkers is through microarray technology. After profiling the transcriptomic biomarkers by microarray, they are validated by quantitative (q)PCR, the gold standard for quantification of nucleic acids. The application of gene expression profiling to saliva samples is hampered by the presence of partially fragmented and degraded RNAs that are difficult to amplify and detect with the prevailing technologies. Moreover, the often limited volume of saliva samples is a challenge to qPCR validation of multiple candidates. This hurdle was overcome by a study performed in our laboratory providing proof-of-concept data on the combination of a universal mRNA-amplification method for the Affymetrix all-exon array for candidate selection and a multiplex pre-amplification method for validation (Hu *et al*, 2008b). At present, the low amounts of RNA in saliva do not hinder the reverse transcriptase (RT)-qPCR performance as a multiplex RT-PCR-based pre-amplification approach was developed allowing accurate qualification of over 50 targets from one reaction (Hu *et al*, 2008b). Moreover, performing simultaneously RT-reactions for different targets offers cost- and time-effectiveness and allows a small volume of pre-amplification product to be used for subsequent qPCR measurements.

Exosome discovery in human saliva

Since our initial report, we have published and accumulated an abundance of additional supporting

evidence such as the characterization of salivary RNA (Li *et al*, 2004b) including the finding that RNAs in saliva, similar to cell-free RNA in plasma, are protected from environmental degradation by their association with macromolecules (Park *et al*, 2006). Sequencing results from a saliva cDNA library demonstrated the fragmentation and the consequently missing 3' poly-A tail of most saliva RNAs (Park *et al*, 2007). This is a key study as it impressively demonstrated that the detected 117 cloned salivary mRNAs did neither originate from contaminating genomic DNA nor from pseudo-genes. Conversely, all sequences were proven to be RNA from human origin. The surprising stability of the partially degraded mRNAs in saliva may be conferred by a similar mechanism as is the case in plasma. In both body fluids the RNA is associated with some macromolecule or subcellular body. As per a more recent study, salivary mRNAs were localized inside salivary exosomes, and these nucleic acids were protected against ribonucleases in saliva (Palanisamy *et al*, 2010). Moreover, saliva exosomes have been discovered to regulate the cell-cell environment by altering their gene expression allowing us to understand the molecular basis of oral diseases better. These findings amongst others (Ratajczak *et al*, 2006; Valadi *et al*, 2007; Skog *et al*, 2008) open up a new research perspective on the use of exosomal transfer of mRNA to target another cell type and to activate or modulate gene expression in oral keratinocytes. Currently, exosomes have elicited intense focus of interest as they might be the key to protecting salivary RNA with their lipid bi-layers and shuttling RNA from the system (the tumor site) into saliva to target sites.

Salivary microRNA

MicroRNAs (miRNAs) are encoded by genes but are not translated into proteins. They are non-coding RNAs, instead each primary transcript (a pri-miRNA) is processed into a short stem-loop structure called a pre-miRNA and finally into a functional miRNA. Mature miRNA molecules are partially complementary to one or more mRNA molecules, and their main function is to down-regulate gene expression. Initially, the miRNAs lin-4 and let-7 were discovered in *C. elegans* (Grosshans and Slack, 2002), categorized according to mass (Lee and Ambros, 2001) and since then, the mechanism of miRNA production and mode of action has been well characterized (Zeng, 2006). Hundreds of miRNAs from various organisms have been discovered, and functional assays have established that miRNAs serve important functions in cell growth, differentiation, apoptosis, stress and immune response as well as glucose secretion (Lu *et al*, 2005; Taganov *et al*, 2007; Stadler and Ruohola-Baker, 2008). In saliva, miRNA are present in both whole saliva and saliva supernatant out of which 314 of the 708 human miRNAs registered in the most recent release of miRBase version 12.0 were profiled (Park *et al*, 2009; Michael *et al*, 2010), and so the third diagnostic alphabet in saliva was established.

Proteomic and transcriptomic approaches will allow us to comprehensively profile these two salivary ana-

lytes. Moreover, combining biomarkers from both compartments could reveal whether these two classes of biomarker can be synergistic for early detection, disease progression, and monitoring of disease status. Similarly, profiling the saliva proteins, mRNAs and microRNAs before and after pharmacologic interventions may provide important information on drug efficacy and toxicity in the context of therapeutic responsiveness.

Molecular oncology detection based on salivary biomarkers

Multiple molecular alterations occur during cancer development. In order to be able to get a proper insight in to these processes, we need to characterize and integrate these components. Deciphering the molecular networks that distinguish progressive disease from non-progressive disease will shed light into the biology of cancer as well as lead to the identification of biomarkers that will aid in the selection of affected patients. Advances in mass spectrometric techniques have made quantitative proteomic profiling possible in tissues and fluids. However, transcript and protein abundance measurements may not always be concordant as cells have adopted elaborate regulatory mechanisms at the levels of transcription (e.g. binding of transcription factors, chromatin structure modification), post transcription (e.g. nucleocytoplasmic export or splicing of messenger RNA, differential ribosomal loading), and post translation (e.g. protein degradation or export) (Chan, 2006). In this regard, quantitative transcript and protein abundance measurements serve as yardsticks for each other. While similarities between protein and RNA levels increase our confidence in novel biomarkers discovery, differences may result in additional post transcriptional regulatory mechanisms as candidates for the design of therapeutics (Chan, 2006).

Salivary biomarker detection for oral squamous cell cancer

Early detection is a key question that needs to be addressed in almost all types of cancer. In oral squamous cell cancer (OSCC), if the cancer is detected at T1 stage, the 5-year survival rate is 80% compared to 20–40% if the cancer is diagnosed at later stages (T3 and T4). Saliva has been used as a diagnostic medium for OSCC, and saliva analytes such as proteins (Chai and Grandis, 2006), mRNA (Li *et al*, 2004a) and DNA have been used to detect OSCC (Brinkman and Wong, 2006; Chai and Grandis, 2006; Hu *et al*, 2008b). Using proteomics and genomics technologies, we have previously discovered and validated salivary OSCC markers in American patients (Li *et al*, 2004a,b; St John *et al*, 2004).

Salivary mRNA and microRNA biomarkers for oral squamous cell cancer

The translational utility of salivary transcriptomic analysis was established by the discovery of a panel of oral cancer mRNA biomarkers, using an expression-

based microarray (Li *et al*, 2004a). Nine candidate transcripts were chosen from a comparison of 10 early stage OSCC patients and 10 healthy matched control subjects and validated by RT-qPCR. In a validation cohort of 32 patients and 32 controls, seven 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% (ROC = 0.95) in distinguishing patients from controls placing them amongst the most discriminative panels of cancer biomarkers from a body fluid (Li *et al*, 2006). 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 seven salivary OSCC markers, over 220 additional patients have been tested (manuscript in preparation).

Investigating miRNA in saliva, two miRNAs, miR125a and miR-200a, were found to be differentially expressed in the saliva of the OSCC patients compared with controls. These findings suggest that the use of this third diagnostic alphabet in saliva can be helpful as a non-invasive and rapid diagnostic tool for the diagnosis of oral cancer (Park *et al*, 2009).

Proteomic salivary biomarkers for oral squamous cell cancer

After using laser-capture micro dissection, we have identified the expression of two cellular genes that are uniquely associated with OSCC: interleukin (IL) 6 and IL-8 (Alevizos *et al*, 2001). In saliva of newly diagnosed OSCC patients, IL-8 was detected at a higher concentration ($P < 0.01$) while IL-6 was at a higher concentration in serum of OSCC patients ($P < 0.01$). This finding indicated that IL-8 in saliva and IL-6 in serum hold promise as biomarkers (St John *et al*, 2004). Furthermore, our laboratory has developed and validated a panel of salivary biomarkers that is highly discriminative for oral cancer detection, showing that five salivary proteins (M2BP, MRP14, Profilin, CD59 and Catalase) were discriminative for oral cancer with a clinical accuracy greater than 90% (Hu *et al*, 2008a).

Transcriptomic salivary biomarkers for pancreatic cancer

A milestone in salivary diagnostics was recently achieved with the identification of significant differential salivary transcriptome profiles between patients with early stage resectable pancreatic cancer and healthy controls (Zhang *et al*, 2010). Currently, pancreatic cancer consistently leads to a typical clinical presentation of incurable disease at initial diagnosis because of a lack of detection technology and therefore new strategies and biomarkers for early detection are desperately needed. Zhang *et al* succeeded in linking the profiles of molecular signatures in saliva with their changes between disease and controls opening up the possibilities of detection of the pancreatic cancer much before it

reaches an incurable stage. Based on microarray profiling, 49 transcripts were up-regulated and 21 down-regulated out of which a total of seven up-regulated and five down-regulated genes were validated. These 12 mRNA biomarkers were discovered and validated showing significant differences between pancreatic cancer patients and healthy controls ($P < 0.05$, $n = 60$), yielding receiver-operating characteristics (ROC)-plot area under the curve (AUC) values between 0.682 and 0.823. The logistic regression model with the combination of four mRNA biomarkers (KRAS, MBD3L2, ACRV1 and DPM) could differentiate pancreatic cancer patients from non-cancer subjects including chronic pancreatitis and healthy controls, yielding an AUC value of 0.971 with 90% sensitivity and 95% specificity. Among the 12 validated mRNA biomarkers, several genes, e.g. MBD3L2, GLTSCR2, and TPT1, have been linked to carcinogenesis (Smith *et al*, 2000; Maehama *et al*, 2004; Okahara *et al*, 2004; Kim *et al*, 2008).

In addition to OSCC and pancreatic cancer, our laboratory is currently targeting several high-impact diseases such as breast-, lung- and ovarian cancers using expression-based microarray analysis and RT-qPCR. Sjögren's syndrome, an autoimmune disease that affects 4 million Americans, primarily women (9:1) is also discriminated via salivary biomarkers (Hu *et al*, 2007b, 2009). Furthermore, xenograft mouse models are being used in order to provide functional data aiming to provide a systemic proof-of-concept for salivary biomarkers showing that human tumor markers are trafficking from the tumor site into the mouse saliva (Gao *et al*, 2009).

Pharmacogenomics

One of the most promising research areas for the pharmaceutical industry that emerged in the postgenomic era is pharmacogenomics. Pharmacogenomics, broadly defined, is the study of the impact of genetic variation on the efficacy and toxicity of drugs, or the study of how a patient's genetic makeup determines the response to a therapeutic intervention (Peet and Bey, 2001). The context within which both, basic and translational pharmacogenomic sciences have developed will be a revolution in drug therapy. These advances could offer ways to maximize drug efficacy, minimize toxicity and select responsive patients. Among many factors influencing the drug response, it became clear that inheritance can be a very important factor (Vesell and Page, 1968a,b). Now, it is becoming equally clear that the union of transcriptomic, proteomic and metabolomic with genetic data will accelerate the process of understanding the mechanisms that are responsible for variable response to the powerful therapeutic agents used currently in medicine (Wang, 2009). Evolved from a single gene approach, pharmacogenomics now incorporates pathway-based and genome wide approaches.

Metabolomic profiles in cancer patients

Metabolomics is the study of the metabolome, which describes the repertoire of small molecules present in

cells, tissues, organs and biologic fluids (Fiehn *et al*, 2000; Aharoni *et al*, 2002; Dettmer and Hammock, 2004). The concentration and fluxes of these compounds result from a multifaceted interplay among gene expression, protein expression, and the environment- an environment that includes drug exposure. Metabolomics generates quantitative data for a large number of metabolites in order to understand metabolic dynamics associated with conditions such as disease or drug exposure (Kristal *et al*, 2007). This approach is in contrast to classical biochemical approaches, which often focus on single metabolites, single biochemical reactions, their kinetic properties, and/or defined sets of linked reactions. Metabolomics can complement data obtained from genomics and transcriptomics adding another 'layer' of data making a system approach possible to study individual variation in a disease state or during drug therapy and response.

Recently, our laboratory obtained and compared comprehensive salivary metabolic profiles of patients with oral, breast or pancreatic cancer, or periodontal disease, and healthy controls (Sugimoto *et al*, 2010). In this study, 57 principal metabolites that can be used to accurately predict the probability of being affected by each individual disease were identified. In all three cancer groups (oral, breast and pancreatic), the profiles of quantified metabolites manifested at relatively higher concentrations for most of the metabolites as compared with those in people with periodontal disease and control subjects. This body of evidence suggests cancer-specific signatures are embedded in saliva metabolites. AUCs were calculated to discriminate healthy controls from each disease. The AUCs were outstanding: 0.865 for oral cancer, 0.973 for breast cancer, 0.993 for pancreatic cancer, and 0.969 for periodontal diseases.

The application of genome-wide techniques to perform 'pharmacogenomic' studies is in its infancy. Genomics, transcriptomics and even metabolomics are beginning to be applied to integrate studies of drug response, which clearly highlights the need for a system biology approach. These developments promise to substantially enhance our functional and mechanistic understanding of causes of variation in drug-response phenotypes and to help moving towards individualized drug therapy and personalized medicine (Wang, 2009).

Saliva ontology

The rapid development and maturity of the genomics field has led to the emergence of other 'omics' studies, such as proteomics, transcriptomics and metabolomics, which are now being widely implemented in studies of human disease. Furthermore, mining the data from multiple 'omics' studies can give deeper insight into biologic systems than can be obtained from any single 'omics' study and thus 'omics' databases such as PharmGKB (the Pharmacogenomics Knowledge Base, <http://www.pharmgkb.org>) and EPO-KB (the Empirical Proteomic Ontology Knowledge Base, <http://www.dbmi.pitt.edu/EPO-KB>) serve as important resources for emerging disciplines of system biology.

Saliva has not been widely integrated in ontology and terminology resources yet (Lisacek *et al*, 2006). Just recently, the Salivaomics Knowledge Base (SKB) has been created by aligning the salivary biomarker discovery and validation resources at UCLA (Figure 1) with the ontology resources developed by the OBO (Open Biomedical Ontologies) Foundry (<http://www.obofoundry.org>), including the new Saliva Ontology (SALO) (Ai *et al*, 2010). The SKB is a data repository, management system and web resource constructed to support human salivary proteomics, transcriptomics, miRNA, meta-

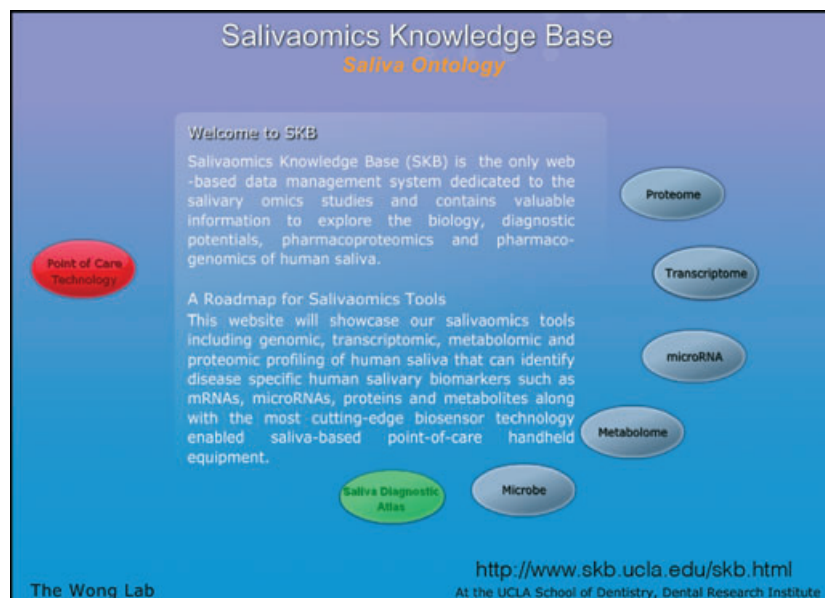


Figure 1 The Salivaomics Knowledge Base (SKB) has been created by aligning the salivary biomarker discovery and validation resources at UCLA with the ontology resources developed by the OBO (Open Biomedical Ontologies) Foundry (<http://www.obofoundry.org>), including the new Saliva Ontology (SALO). The SKB is a data repository, management system and web resource constructed to support human salivary proteomics, transcriptomics, miRNA, metabolomics and microbiome research

bolomics and microbiome research. The SKB will provide the first web resource dedicated to salivary 'omics' studies. It will contain the data and information needed to explore the biology, diagnostic potentials, pharmacoproteomics and pharmacogenomics of human saliva (SKB; <http://www.skb.ucla.edu/>). Overall, the SKB will allow a systems approach to the utilization of salivary diagnostic technology for personalized medicine applications.

SALO is a consensus-based controlled vocabulary of terms and relations dedicated to the salivaomics domain and to saliva-related diagnostics following the principles of the OBO Foundry. SALO was defined based on cross-disciplinary interaction between saliva and protein experts, diagnosticians, and ontologists (SALO; <http://www.skb.ucla.edu/SALO/>). In order to improve development and testing of SALO, a corpus of saliva-relevant literature was incrementally developed in SKB to assist in identifying core terms, synonyms and definitions for inclusion within the ontology, and to provide examples of usage and links between SALO content and the corresponding items through their PubMed identifiers. A growing body of semantically enhanced web-enabled literature will be created within the SKB to support future research.

Point-of-care technology

Early detection is a key question that needs to be addressed in almost all types of diseases. The analysis of body fluids offers a great opportunity to detect diseases at an earlier stage. Saliva diagnostics are very attractive because of non-invasive sample collection and simple sample processing. For patients, the non-invasive saliva collection procedure reduces anxiety and discomfort and is easily accessible as compared with tissue biopsies. Therefore, developing highly sensitive and accurate assays for salivary mRNA/protein biomarkers makes saliva a valuable diagnostic fluid (Gormally *et al*, 2006; Herr *et al*, 2007).

Single biomarker detection is not effective enough for accurate diagnosis and medical decisions because of the complexity of the human biologic system and the high possibility of false positive and false negative rates. The combination of multiple biomarkers could include highly discriminative nucleic acids (Cagir *et al*, 1999), proteins (Behnisch *et al*, 2001; Srinivas *et al*, 2002) and small molecules such as metabolites (Behnisch *et al*, 2001). However, to date no technology has been reported addressing the multiplexing mode including the measurement of RNA, protein and small molecules.

Recently, a study conducted in our laboratory reported an electrochemical (EC) sensor for oral cancer detection based on the simultaneous detection of two salivary biomarkers: interleukin (IL)-8 mRNA and IL-8 protein (Wei *et al*, 2009). Thereby, the limit of detection of salivary IL-8 RNA reached to 3.9 fM in saliva while the limit of detection for IL-8 protein was 7.4 pg ml⁻¹ in saliva. Applying the IL-8 multiplex assay in 28 cancer and 28 matched controls showed a significant clinical discrimination with a 90% sensitivity and specificity for

both markers. Measuring both markers simultaneously, showed a better accuracy than individually indicating that EC sensor is not only an alternative method to PCR/ELISA, but also it provides fast, effective and accurate multiplexing measurements for real clinical diagnostics. This report is the first to show multiplexing detection of RNA and protein salivary biomarkers and based on this data the EC sensor promises to be a promising miniaturization system with a high clinical application impact.

Outlook

Translating scientific findings of nucleic acids, proteins and metabolites in body fluids to clinical applications is a cumbersome and challenging journey. Rarely, has the final destination of the clinical implementation of a test been reached. In order to utilize disease detection biomarkers, it is of utmost importance to understand the basic mechanisms (e.g. exosomes) underlying the rationale of salivary biomarkers. Our group continues to pursue the biology of salivary analytes and the development of technologies in order to detect distinct biomarkers with high sensitivity and specificity to achieve compliance for the clinical setting at the FDA level. We are convinced that our efforts in salivary diagnostics will ultimately result in the detection of diseases, including cancer, optimally at the premalignant stage, supporting the management of cancerous diseases by enhancing the survival rate. Moreover, the avenue of saliva diagnostics incorporating transcriptomic, proteomic and metabolomic findings will enable us to connect molecular analytes to monitor therapies, therapeutic outcomes, and finally disease progression in cancer.

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Conflict of interest

Both authors claim that there is no conflict of interest regarding this manuscript.

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