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Salivary Biomarkers-A Review of Powerful Diagnostic tool

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Abstract: *Early detection of any disease plays an important role in successful treatment. Early diagnosis and treatment reduces the severity and possible complications of disease activity. Human saliva is not just a fluid in our mouth, but it mirrors our body's health and well-being. Biomolecules that are circulating in the blood are also found in human saliva. It consists of approximately about 2,000 proteins, and 26% of these proteins are also found in blood, therefore it emphasizes saliva's importance as an added biological resource for disease diagnosis and monitoring, as well as an ultimate diagnostic medium to establish a person's response to treatment. It has been a great challenge in periodontologyto determine biomarkers for screening and predicting the early onset of disease (prognostic tests) or evaluating the disease activity and the efficacy of therapy (diagnostic tests). Traditional diagnostic measures, such as periodontal pocket depth, attachment level, plaque index, bleeding on probing and radiographic assessment of alveolar bone loss, are not necessarily the most efficient method for early diagnosis.Nowadays, clinical chairside tests are in use for more precise molecular diagnostics and treatments.A simple and non-invasive diagnostic tool that allows rapid screening, provides accurate predictive information and enables reliable evaluation of periodontal disease status would be of great value to both dentists and patients. The field of saliva diagnostics (SDs) began in the early 60s when salivary calcium levels were found to be elevated in cystic fibrosis patients, and 50 years since then how the field has unmitigated to an unpredicted distance due to the development of increasingly sensitive detection techniques. Hence, today in the era of nanotechnology and genomics, field of salivary diagnostics is promising a dramatic change in disease diagnosis and clinical monitoring. It has expanded into detection of cancer, heart and infectious diseases.*

Keywords: *Periodontal disease, Salivary Biomarkers, diagnostic – chairside tests.*

I. Introduction

The ability to monitor health status, disease onset, progression, and treatment outcome through noninvasive means is a highly desirable goal in health care promotion and delivery. Saliva is a perfect medium to be explored for health and disease surveillance[1-3].Saliva is said to be a "mirror of the body" because it is an indicator of health not just in the oral cavity but throughout the body[4]. The molecular composition of saliva includes therapeutic, hormonal, immunologic, and toxicological molecules, which can provide vital clues to systemic health[5].

Recently, several proteomics studies contributed to the partial elucidation of the salivary proteome (more than 2400 protein components have been characterized) both in terms of composition, contributions to whole saliva and genetic/physiological variability. On this basis, it is not too optimistic to believe that in the near future, human saliva could become a relevant diagnostic fluid[6].

A good diagnostic method should have the characteristics of high sensitivity, specificity, portability, and low cost for subsequent clinical application.

Now a days, the improved efficiency and accuracy of genomic and proteomic biomarker discovery technologies are turning salivary diagnostics into a clinical and commercial reality.

Saliva in comparison with serum[7]:

In diagnostics, saliva is an excellent alternative to serum since it contains sufficient quantities of disease biomarkers, ribonucleic acid (RNA), and deoxyribonucleic acid (DNA), and the collection method is noninvasive, safe, and easy. Saliva is also easier to handle during diagnostic procedures than blood because it does not clot

Saliva in comparison with gingival crevicular fluid (GCF)[7]:

Saliva collection is less technique-sensitive than GCF collection. This makes saliva a potentially valuable fluid for the diagnosis.

Salivary analysis can be done for the diagnosis of the following conditions[8]

1. Hereditary disease
2. Autoimmune disease
3. Malignancy
4. Infection
5. Monitoring of levels of hormones
6. Monitoring of levels of drugs
7. Bone turnover marker in saliva
8. Forensic Evidence
9. Oral diseases
10. Diagnosis of Oral Disease with Relevance for Systemic Diseases

Role of Salivary Biomarkers in diagnosis of Oral and Systemic diseases

Till date, most of the biomarkers have been identified from various body fluids. Among which blood and saliva are the most widely studied body fluids they contain reliable biomarkers for oral and systemic diseases. It is an informative body fluid containing an array of analyte (Protein, mRNA and DNA) that can be used as biomarkers for translation and clinical applications[9].

The salivary biomarkers have been classified into Proteomic, genomic and microbiological biomarkers[10].

Classification of Biomarkers[10]

Proteomic Biomarkers			Genomic Biomarkers	Microbial biomarkers	Other markers
Immunoglubilins	Calprotectin	Kininase	Cathepsin C gene mutation	*Aggregatibacter Actinomycetemcomitans*	Calcium
Acid phosphatase	Caprylate esteraselipae	Lactoferrin	Collagen gene mutation	*Campylobactr rectus*	Cortisol
Alkaline phosphatas	Cathepsin B	Lactotransferrin	IL-1 polymorphis	*Mycoplasmas*	Hydrogen sulfide
Aspartate Aminotransferase	CD14	Lactate dehydrogenase	IL-10 polymorphisms	*Porphyromonas Gingivalis*	Methyl mercaptan
Aminopeptidases	Cystatins	Lysozyme	TNF Polymorphisms	*Prevotella intermedia*	Picolines
Beta-galactosidase	Elastase	MMP 1, MMP 2, MMP 3		*Peptostrepto coccus Micros*	PMNs
Beta-glucosidase	Epidermal growth Factor	MMP-8, MMP-9 MMP-13		*Prevotella nigrescens*	Pyridine
Beta-glucuronidase	Esterase	ICTP		*Treponema denticola*	
CRP	Fibronectin	Myeloperoxidase		*Tannerella forzythia*	
Alpha-glucosidase	Gelatinase	Osteocalcin		*Treponema socranskii*	
Histatin	Kallikrein	Osteonectin			
Mucins	Peroxidase	Osteopontin			

Various Salivary Biomarkers Of Periodontal Disease

DENTAL BIOFILM	INFLAMMATORY	COLLAGEN BREAKDOWN	BONE REMODELLING
Immunoglobulins (IgA, IgM, IgG)	β-glucuronidase	α2-macroglobulin	Alkaline phosphatase
Mucins	C- reactive protein	MMP-8	Osteoprotegerin
Lysozyme	Il-1β	MMP-9	Osteocalcin
Lactoferrin	Il-6	Aspartate aminotransferase	SPARC/osteonectin
Histatin	MIP 1α	Alanine aminotransferase	RANKL
Peroxidase	Tumor necrosis factor-α	TIMPs	β C-terminal type I collagen telopeptide
			C-telopeptide pyridinoline cross-links of type I collagen

IL: Interleukin; MIP: Macrophage inflammatory protein; MMP: Matrix metalloproteinase; RANKL: Receptor activator of NF-κB ligand; SPARC: Secreted protein, acidic, rich in cysteine; TIMP: Tissue inhibitors of metalloproteinase.

The wide continuum of molecules present in saliva provides valuable information for clinical diagnostic applications in clinical utility for followings:
1. Proteomic analysis
2. Genomic analysis
3. Transcriptome analysis

Salivary Proteomic Analysis

Human saliva is a plasma ultra-filtrate and contains proteins either synthesized in situ in the salivary glands or derived from blood and contains biomarkers derived from serum, gingival crevicular fluid, and mucosal transudate. To date, researchers have identified 2,340 proteins in the salivary proteome, of which 20–30% are also found in blood[11], an encouraging indicator of the clinical utility of saliva as a diagnostic fluid. In contrast to the plasma proteome, in which 99% of the total protein content is contributed by 22 highly abundant proteins, the 20 most abundant proteins in WS constitute only 40% of the protein content[12]. This composition suggests that detecting biomolecules of clinical sensitivity and specificity in saliva should be practicable and easier than in blood. How molecules of blood transport in saliva may also be important for successful use of saliva as a diagnostic fluid. Lipophilic molecules such as steroid hormones passively diffuse into saliva, while water and electrolytes pass through the pores of acinar cells. Various peptides in blood move through protein channels, and large proteins are transported via pinocytosis[13].

Proteomic Biomarkers

Development of analytical technologies in the post-genomic era has allowed for large scale identification of proteins/peptides (proteome) and ribonucleic acids (RNA; transcriptome), and their functions/structures in cells and fluids. The high throughout proteomic studies have catalogued at least 1166 proteins in the major salivary gland secretions, of which 914 are recovered from parotid and 917 from submandibular/sublingual ductal saliva, with 57% of these proteins present in both glandular saliva[14]. The proteome of human minor salivary gland secretion showed 56 proteins[15]. More surprisingly, the salivary transcriptome (RNAs) has been discovered using microarray profiling in recent years and approximately 3000 messenger RNAs (mRNAs) are identified in cell-free WS. Most recently, the presence of microRNA (miRNA; ~50) was discovered in WS. Unlike mRNA, miRNA consists of 18–24 nucleotides transcribed from non-protein coding genes and regulates protein translation through an RNA-induced silencing complex (RIST)[16].

These advances have provided a large number of salivary molecular targets, e.g, proteins and RNAs, for disease biomarker discovery. Several investigators have already attempted to use high technologies and current salivary proteomic and transcriptomic knowledge for biomarker discovery in the areas of oral and breast cancer[17], periodontal diseases[18], [19] cardiovascular disease and Sjögren's syndrome.[20], [21]

Traditional biochemical techniques such as LC, gel electrophoresis, capillary electrophoresis, nuclear magnetic resonance, MS, immunoassay, and lectin probe analysis have been widely used in saliva proteome work for identifying the proteins present in glandular saliva.[22], [10]

In the past few years, multiplex biomarker detection systems have emerged through remarkable progress in the development of lab-on-a-chip (LOC) and point-of-care (POC) technologies.[23]The goal of these efforts is to build automated, miniaturized, and multiplexed platforms for rapid assays and readout. In general, the principles of conventional ELISA and/or nucleic acid hybridization is applied often with either electrochemical sensors or a microbead reactor.[24, 25]

The Texas/Kentucky Saliva Diagnostics Consortium is at the forefront of developing 3-D bead saliva/oral fluid diagnostics for cardiovascular, cancer, and periodontal diseases.[19, 24, 25]

Salivary proteomics for existing periodontal disease (PD)

Interlekin-1β (IL-1β) is a proinflammatory cytokine that stimulates the induction of adhesion molecules and other mediators which in turn facilitate and amplify the inflammatory response. Its levels correlated significantly with periodontal parameters after adjusting for the confounders. Moreover, combined levels of IL-1β and matrix metalloproteinase (MMP)-8 increased the risk of experiencing PD by 45 folds.[10]

MMPs, MMP-8, a key enzyme in extracellular collagen matrix degradation, derived predominantly from PMNs during acute stages of PD. Its presence significantly increased the risk of PD (odds ratios in the 11.3-15.4 range). MMP-1 (interstitial collagenase) also appeared to be activated in periondontitis[26]. Additionally, higher levels of other MMPs, including MMP-2, MMP-3 and MMP-9, were also reported in the saliva of periodontitis patients.[10]

Salivary proteomics for dental caries (DC)

Salivary proteins play a significant role in maintaining the oral health and prevent caries as stated by Mazengo*et al*[27].A significant amount of salivary phosphopeptides (PRP1/3, histatin-1 &statherin) were associated with the absence of DC, emphasizing the importance of these peptides in the maintenance of tooth integrity.[26,28]

In a recent study on early childhood caries, it was found that, a higher number of proline-rich protein bands significantly correlated among caries free subjects, substantiating the protective role of this protein, also a higher number of glycoprotein bands were observed in the WS of subjects with early childhood caries[10].

Salivary proteases as biomarkers for premalignant and malignant oral lesions

Unfortunately, clinicians now lack tests which easily and reliably distinguish pre-malignant oral lesions from those already transitioned to malignancy. Bioinformatics analysis of exfoliated epithelial cells from subjects saliva revealed increased myosin and actin abundance in malignant lesions as confirmed by western blotting. These findings provided a promising starting point for the development of non- invasive and inexpensive salivary tests to reliably detect oral cancer at an early stage[29].

The role of proteomics in salivary gland neoplasm has been studied by Nakashima et al. Investigated the adenoid cystic carcinoma and detected 4 up-regulated and 5 down-regulated proteins[30], one study showed that there is an important relationship between some proteins, such as transketolase, dim1p, v-ha-ras oncogene, type I collagen pro alpha, tumor necrosis factor (ligand) superfamily member 4, Pirin and tumor metastasis[31].The same Authors also investigated the differential expression of proteins in adenoid cystic carcinoma with lung metastasis and found that transketolase, modulator recognition factor 2, dim1p homolog, splicing factor (arginine/serine- rich 9) and v-ha-ras l oncogene were all hypoexpressed in poorly metastatic tumors and significantly upregulated in highly metastatic tumors.

Oral Fluid-based Lab-on-a-chip testing for detection of Acute myocardial infarction (AMI)

Coronary artery disease (CAD), a major component of cardiovascular diseases, causes 1 of every 5 deaths in the US in 2004. The survival by AMI is dependent on early diagnosis and emergency intervention and it is key for good prognosis[3].

Currently, electrocardiogram (ECG) is standard equipment in emergency medical services (EMS) and is used as a diagnostic standard for emergency triage of patients with chest pain and/or unconsciousness. A typical ECG abnormality for an AMI is an ST segment elevation. Unfortunately, ECG alone only identifies ~35 % of all AMI cases admitted to the emergency department (ED) and misses the remaining 65%, that do not exhibit the characteristic ECG changes. The triage of potential AMI cases in the ED depends on supplemental blood testing that often includes cardiac troponins T and I (cTnT, cTnI), creatine kinases-MB (CK-MB), total CK and myoglobin (MYO)[24].However, these tests are, invasive and limited to the clinical laboratory setting and the few that have been developed for POC testing lack the analytical and clinical sensitivity and specificity to efficiently diagnose AMI[32].So there is need to have a non-invasive test with the required analytical and clinical performance that could be used in an ambulance setting to minimize the time from diagnosis to treatment of AMI patients. Saliva presents itself as an ideal fluid in this situation.

A study has been done to evaluate the potential use of AMI biomarkers in saliva by collecting unstimulated whole saliva within 48 hours from more than 80 patients with a definitive diagnosis of AMI and

compared with 80 healthy controls and assayed for 21 cardiac related proteins using conventional methodologies, such as LUMINEX, ELISA and Beckman Access instrumentation. Data gathered to demonstrate cardiac biomarkers/proteins such as C-reactive protein (CRP), myeloperoxidase (MPO), interleukins, matrix metallo-perteinase-9 (MMP-9), and cellular adhesion molecule-1 (sICAM-1), can be detected in saliva samples but, most importantly, demonstrated a capacity to differentiate healthy controls from AMI patients. Strikingly, it was showed, that AMI diagnosis was greatly improved with a combination of the ECG and AMI proteins in saliva[24].

Salivary Transcriptome analysis:

The Salivary Transcriptome (ST) offers an additional valuable resource for disease diagnostics. The first report of the ST demonstrated that the normal ST consists of about 3,000 mRNAs. Of particular importance is that of the 3,000 mRNAs, 180 are common between healthy subjects, constituting the normal salivary transcriptome core (NSTC)[33].

To demonstrate the diagnostic and translational potential of the ST, the UCLA group profiled and analyzed saliva from patients with oral cancer. Four genes from the NSTC (IL-8, ornithine decarboxylase, spermidineacetyltransferase and IL-1) were able to discriminate and predict, whether the saliva sample was from a patient with cancer or from a healthy subjects, with a sensitivity and specificity of 91%. The behavior of these ST biomarkers is consistent and their levels are significantly higher in saliva of patients with oral cancer compared to control subjects.

Development of Technologies for Saliva-Based Diagnostics

In 2002, NIDCR initiated a research effort in the area of salivary diagnostics, and progress is being made toward developing technology viable systems that are suitable for commercialization. NIDCR funded seven awards for the development of microfluidics and microelectromechanical systems (MEMS) for salivary diagnostics.

NIDCR*-funded salivary diagnostic technology development and salivary proteome research groups.

SALIVARY DIAGNOSTIC TECHNOLOGY DEVELOPMENT	SALIVARY PROTEOME
University of Texas at Austin Eric Anslyn, PhD "www.cm.utexas.edu/directory/eric_anslyn/"	**University of California, San Francisco** Susan Fisher, PhD "www.salivarium.ucsf.edu/"
New York University, New York City Daniel Malamud, PhD, MA "www.nyu.edu/dental/research/faculty/malamud.html"	**The Scripps Research Institute, La Jolla, Calif.** John Yates, PhD "fields.scripps.edu/public/project/saliva/"
Sandia National Laboratories, Livermore, Calif. Anup Singh, PhD "www.ca.sandia.gov/chembio/microfluidics/staff/singh.html"	**University of California, Los Angeles** David Wong, DMD, DMSc "www.hspp.ucla.edu"
University of Washington, Seattle David Stahl, PhD "www.stahl.ce.washington.edu/index.html" Paul Yager, PhD "http://faculty.washington.edu/yagerp/"	
Tufts University, Medford, Mass. David Walt, PhD "chem.tufts.edu/faculty/walt/"	
University of California, Los Angeles David Wong, DMD, DMSc "www.saliva.bme.ucla.edu/"	

* NIDCR: National Institute of Dental and Craniofacial Research.

Development of microfluidics and microelectromechanical systems (MEMS) for salivary diagnostics

Commercially Available Saliva Tests

Two U.S. companies were early pioneers of oral diagnostics: Epitope, Inc. and Saliva Diagnostic Systems, Inc. They both commercialized saliva collection devices in the early 1990s, and in 1996 the Food and Drug Administration (FDA) approved Epitope's Orasure HIV test, the first test that used oral fluid to test for an infectious disease. The OraQuick HIV test, which takes only 15 minutes to detect the HIV antibodies in saliva via mouth swab.

Several companies outside the U.S. have commercial tests to detect drugs-of-abuse in a spit sample, including Cozart Biosciences, Securetec, and Mavand. Some of these companies send their kits via regular mail to customers, allowing individuals to collect their own saliva either in a cup or with a swab and then send the sample to a laboratory for analysis. Other tests target DNA in saliva. Canada-based DNA Genotek was the first company to commercialize a broad range of saliva collection tools for genotyping based on PCR, microarrays, and sequencing. Oral DNA Labs, a subsidiary of Quest Diagnostics, also offers two salivary tests in the U.S. in its CLIA-approved testing facility. My PerioPath is a DNA test that determines the risk of periodontal infections by detecting bacterial pathogens in saliva. OraRisk HPV is a salivary test that determines an individual's risk of developing HPV-related oral cancers. It identifies various HPV genotypes, including HPV 8, 11, 16, and 18.[34]

OraQuick, ADVANCE/Rapid HIV-1/2, Orasure HIV1, Periogard, Micro-plate EIA, ZRT Saliva Test, SALIVASCREEN 5 Professional, Q.E.D. Saliva Alcohol Test are a few of the examples of such commercially marketed chair side kits.[35]

Various Products and Their Uses for Measuring Salivary Biomarkers

PRODUCT NAME	PURPOSE
MyPerioID	identifies the type and concentration of the specific bacteria that cause periodontal diseases.
My PerioPath	determines the cause of periodontal infections.
Oral Fluid NanoSensor Test	simultaneous and precise detection of multiple salivary proteins and nucleic acids.
Electronic Taste Chips	detects multiple biomarkers for early diagnosis of periodontal disease
OraQuick	an antibody test that provides results in 20 minutes, usually detects HIV 1 and HIV 2
Integrated Microfluidic Platform for Oral Diagnostics	rapidly (3–10 min) measures the concentrations of MMP-8 and other biomarkers in small amounts (10 ml) of saliva

Commercially Available Chairside Diagnostic Kits

ASSAY	KIT	MANUFACTURER/SUPPLIER	FUNCTION
Bacterial enzymes & host enzymes	BANA periodontal test	Ora Tec Corporation Manassas (USA)	It utilizes the BANA test for bacterial trypsin like proteases.
	Periocheck (ASTech)	CollaGenex Pharmaceuticals, Newtown, PA	Detects presence of neutral proteinases i.e. Collagenase
	PerioScan	Oral B Laboratories	Detects enzymatic activity of*Aggregatibacter actinomycetemcomitans, T forsythus, P gingivalis*
Immunological detection	Evalusite	Kodak Eastman Company (Switzerland)	Immunological detection of antigens of *Aggregatibacter actinomycetemcomitans, P intermedia, P gingivalis*using antibodies (ELISA)
Biochemical Identification	Prognostic	Dentsply	Aids in detection of serine proteinases and elastases
	Biolise	SLT-Labinstruments, Crailsheim, Germany	Aids in detection of elastase
	Periogard	Colgate	Detects the presence of AST
	Pocket watch	SteriOss®, San Diego, CA, USA	Detects aspartate aminotransferase through colorimetric detection
	TOPAS	Affinity Labelling Technologies (USA)	Detects toxins derived from anaerobic metabolism and measures GCF protein level

II. Conclusion

Diagnostic tests are routinely used in evaluation of many diseases. Saliva-based diagnostics present incomparable opportunities for research and commercialization opportunities because of increased understanding of genomics, transcriptomics and proteomics. At this stage it seems to be an extremely important possible tool for regular screening of larger populations. it can be concluded that in the coming future, there are rich possibilities that salivary diagnostics can not only be used as a powerful tool for saving life but also to preserve those, which already have been saved. It will be a very helpful tool for population-based screening programs, confirmatory diagnosis, risk stratification, prognosis determination, and therapy response monitoring. Screening an entire population for a certain type of disease will be made possible in the near future by employing saliva diagnostics.

The use of proteomics and gene expression will advance the diagnosis and treatment of various oral pathological conditions. Current proteomics analysis has the capacity to provide new insights into the repertoire of expressed proteins and some inkling of their interactions,at a more global level than previously considered. Moreover, new diagnostic technologies such as nucleic acid and protein microarrays and micro fluids are under development for risk assessment and comprehensive screening of biomarkers.

These recent advances are leading to the development of more powerful diagnostic tools for practitioners to optimize their treatment predictability.

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