Recent Advances in Salivary Proteomics, Genomics and Transcriptomics: A Reliable Tool in Periodontal diagnosis – A Review

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ABSTRACT

Aim: This review intends to provide a highlight on the potential application of salivary proteomics in periodontal diagnosis and attempts to throw light on the emerging salivary diagnostic tools for periodontal disease detection. **Background:** Clinical parameters for detection of periodontitis such as probing depth, attachment level, bleeding on probing, plaque index, and radiographic assessment of alveolar bone loss provide information on the severity of periodontitis, but they do not measure disease activity. Ideally, development and application of rapid and simple diagnostic tests based on host salivary or immune factors may facilitate early detection of patients at risk for periodontal diseases, allow appropriate intervention, decrease the need for more aggressive treatment and improve the response to periodontal therapy. **Results:** Studies have shown that saliva could be used as a diagnostic fluid as it is one of the inexpensive, noninvasive and easy-to-use diagnostic methods. Salivary constituents that have been studied as potential diagnostic biomarkers for periodontal disease includes locally produced proteins of host and bacterial origin, genetic/genomic biomarkers such as DNA and mRNA of host origin, bacteria and bacterial products, ions, steroid hormones and volatile compounds. **Conclusion:** Periodontal oral diagnostic devices will enable screening of large populations, more quickly and effectively. Use of salivary biomarkers to sample large populations will help to identify at-risk groups more effectively and increase access to treatment for those most at need, thereby, improving public health.

Keywords: Biomarker, dietary intake, oral cancer, proteomics, saliva, β-carotene.

INTRODUCTION

Periodontal diseases affect 5-30% of adult population constituting one of the most common bacterial infections in humans (1). Methods for the detection of periodontitis and the identification of patients at risk for progressive disease are under active investigation. Although clinical parameters provide information on the severity of periodontitis, they do not measure disease activity.

Ideally, development and application of rapid and simple diagnostic tests based on host salivary or immune factors may facilitate early detection of patients at risk for periodontal diseases and allow appropriate intervention.

The purpose of this review is to highlight the recent advances in the potential application of salivary biomarkers in periodontal diagnosis.

Potential salivary biomarkers:

Major salivary gland secretion mediators associated with periodontal diseases include biomarkers like immunoglobulins (IgA, IgM, IgG), mucin, lysozyme, Lactoferrin, histatin, peroxidase and C-reactive protein (1).

IMMUNOGLOBULINS

Immunoglobulins play an important rolein the defense mechanism of saliva. IgA, IgM, IgGinterferes with the adherence of bacteria or by inhibiting bacterial metabolism. The preponderant immunoglobulin found in saliva is IgA. Major and minor salivary glands contribute all of the secretory IgA (sIgA) and lesser amounts of IgG and IgM (2). sIgA may control the oral microbiota by reducing the adherence of bacterial cells to the oral mucosa and teeth (3).

IgG is primarily derived from serum via gingival crevicular fluid (GCF) and is present in low concentration. IgG concentration increases in saliva during inflammation of the periodontal tissue which causes more severe vascular permeability resulting in increased flow of GCF (4). Guven reported that higher levels of IgA were present in whole saliva collected from patients with gingivitis and periodontitis when compared to healthy controls (5). Reiff found a decrease in the salivary IgA and IgG levels in periodontitis patients following treatment (6). Schenk *et al* measured salivary IgA levels against oral bacteria in an experimental gingivitis model (7). Patients with low mean number of bleeding gingival units demonstrated significantly higher levels of salivary IgA antibody reactive with *Streptococcus mutans*, *Aggregatibacter actinomycetemcomitans and Eubacterium saburreum*. The authors reported that high levels of salivary IgA against bacteria in dental plaque might protect against the development of gingivitis.

BACTERIA IN SALIVA

Saliva is widely regarded as a microbial reservoir and can serve as a carrier for bacterial transmission. Detection of certain species in saliva can reflect their presence in periodontal pockets (8). Therefore analysis of saliva may prove to be an important approach for detection of pathogenic oral bacteria,

and may replace other more complicated and more invasive sampling methods.

Saliva could serve as a growth medium for oral *Streptococcus* species and *Actinomyces viscosus*. Bowden (9) suggested that the number of bacterial cells for a given species in unstimulated saliva may indicate whether that microorganism is actively growing in plaque.

In addition to previously recognized species such as *A.actinomycetemcomitans, Porphyromonas gingivalis, Tannerella forsythia, Fusobacterium nucleatum, Prevotella nigrescens, Prevotella intermedia, Treponema denticola, and Mycoplasma species,* application of molecular methods rather than traditional cultural methods has dramatically extended the depth of knowledge about other periodontopathic microbes.

SERUM MARKERS IN SALIVA, CORTISOL

Emotional stress is a risk factor for periodontitis (10). Emotional stress is associated with increase in serum cortisol levels which has a repressive effect on the inflammatory process and immune response. Elevated salivary cortisol levels were observed in individuals with severe periodontitis, a higher level of financial stress, and emotion-focused coping, when compared to individuals with little/no periodontal disease, less financial strain, and low levels of emotion-focused coping (11). However, use of salivary cortisol levels as a biomarker for periodontitis is yet to be determined.

There is a lot of literature analyzing the use of lysozyme, lactoferrin, fibronectin, epithelial keratins, inflammatory cells, epidermal growth factor, vascular endothelial growth factor, platelet activating factor,histatin, mucin, β-glucoronidase, β-galactosidase, peroxidase, salivary ions as potential biomarkers for periodontitis. Some of the recent developments in the field of salivary diagnosis are discussed below.

SYSTEMIC MARKERS ASSOCIATED TO PERIODONTAL INFECTION

C-reactive protein (CRP) is a systemic marker released during the acute phase of an inflammatory response. High levels of C-reactive protein have been associated with chronic and aggressive periodontal diseases and with other inflammatory markers (12).

Studies have shown elevated levels in periodontitis patients.

It has recently been shown to be measurable from saliva using 'lab on a chip' method (13). Pederson *et al* first reported that CRP levels were elevated in the saliva of patients with periodontitis (14). CRP levels were 18.2-times higher in whole saliva of patients who had periodontal disease compared with those of healthy subjects (15). By contrast, another study reported lower CRP levels in chronic periodontitis patients compared with healthy controls (16). However, the majority opinion is that salivary CRP levels appear to be increased in periodontitis patients.

MARKERS OF PERIODONTAL SOFT TISSUE INFLAMMATION

Increased levels of **Interleukin-1β** (IL-1β) have been detected in GCF and have been associated with gingival inflammation, severity of periodontal disease and an absence of therapeutic effectiveness (17). In a study of whole expectorated saliva, they found that levels of IL-1β were significantly higher in the saliva of periodontitis patients than in healthy controls (18). IL-1β levels, positively correlated with several periodontal indices including: clinical attachment level, bleeding on probing, percentage of sites with pocket depths of at least 4 mm and overall periodontal diseases verity.

Interleukin-6 levels were directly proportional to bone loss scores of adult patients with chronic periodontitis (19).

Macrophage inflammatory protein (MIP)-1αis secreted by inflammatory cells. It is majorly associated with cell adhesion and migration. It stimulates osteoclast progenitor cells and/or monocytes to become active osteoclasts in a dose-dependent manner (20). MIP-1α has been detected at higher levels in saliva in a longitudinal study of adolescents with aggressive periodontitis compared with healthy controls (21).

Tumor necrosis factor alpha (TNF-α) is a proinflammatory cytokine important in the pathogenesis of various inflammatory conditions (22). Frodge *et al* reported that TNF-α levels were detectable in all salivary samples from patients who had chronic adult periodontal disease and healthy controls (23). In addition, they found that TNF-α levels in saliva were significantly elevated in periodontitis

subjects compared with healthy controls. Elevated salivary TNF-α levels correlated with an increased number of sites with bleeding on probing, sites with pocket depth of at least 4 mm and clinical attachment levels of at least 2mm. Similar findings have been reported by Ng *et al* (19). These data suggest that salivary TNF-α levels maybe used as potential biomarker.

MARKERS OF ALVEOLAR BONE LOSS

Matrix metalloproteinases are host proteinases accountable for both tissue degradation and remodeling. Host cell-derived interstitial collagenases cleave the gingival and periodontal ligament collagens during progressive periodontal breakdown. MMP-8 is the most ubiquitous marker in GCF and diseased periodontal tissue. Recently, the level of MMP-8 was demonstrated to be highly elevated in saliva from patients with periodontal disease using a rapid point-of-care microfluidic device (24). It is also elevated in peri-implant sulcular fluid from peri-implantitis lesions (25). Salivary MMP-8 levels are elevated in patients with aggressive periodontitis compared with healthy controls (26). Mean levels of MMP-8 were found to be more than four times that of healthy controls in the saliva of patients with periodontal disease (18, 24).

Gelatinase, MMP-9, is produced by neutrophils. In a study done by Teng *et al*, patients were asked to rinse and expectorate, to collect subject based samples and to avoid individual site based GCF samples (27). When analyzed, the mean MMP-9 levels were found to be increased two fold, in patients with progressive attachment loss.

Collagenase-3, also referred as MMP-13 has also been connected with peri-implantitis. It was found that elevated levels of both MMP-13 and MMP-8 correlated with irreversible peri-implant vertical bone loss around loosening dental implants (28).

Pyridinoline crosslinks, such as **pyridinoline cross-linked carboxy-terminaltelopeptide of type I collagen (ICTP)** serves as one of the potential biomarker for periodontal disease. Various studies have investigated the role of pyridinoline cross-links to detect bone resorption in periodontitis and peri-implantitis as well as in response to periodontal therapy (29, 30). ICTP has been shown to be a hopeful predictor of both future alveolar bone and attachment loss.

Osteonectin is an acidic extracellular matrix glycoprotein that plays a vital role in bone

mineralization, cell-matrix interactions, and collagen binding. Ng *et al* analyzed salivary biomarkers associated with alveolar bone loss (19). They found thatseveral individual biomarkers were significantly correlated with bone loss score (IL-1β, IL-6, PGE_2, osteonectin, and osteocalcin). But, multivariate analysis revealed that IL-1β and osteonectin were the only two biomarkers studied that significantly correlated with bone loss score.

Osteocalcin is a 5.4 KD calcium-binding protein of bone and is the most abundant non-collagenous protein of the mineralized tissue. It chemotactically attracts osteoclast progenitor cells and blood monocytes. In addition, it is stimulated by vitamin D3, producing concentrations that inhibit collagen synthesis in osteoblasts, promote bone resorption and stimulate the differentiation of progenitor cells capable of bone resorption (31). Rapid bone turnover in turn causes increase in serum osteocacin levels. For all these reasons, osteocalcin has been suggested as a possible marker for bone resorption and hence periodontal disease progression.

Miricescu *et al* analyzed the relationship between oxidative stress and alveolar bone loss in chronic periodontitis (32). Significant positive correlations were observed between osteocalcin and clinical parameters of periodontal disease. Salivary levels of osteocalcin and osteonectin were found to be inversely correlated with bone-loss scores in patients with periodontal disease.

Osteopontin is a single-chain polypeptide and in bone matrix, OPN is highly concentrated at the clear zone attachment areas of the plasma membrane. Several investigations on OPN indicated that OPN concentrations in gingival crevicular fluid increased proportionally with the progression of disease; and OPN levels in GCF were significantly reduced following nonsurgical periodontal treatment (33, 34).

The balance between **receptor activator of NF-κB ligand (RANKL) and Osteoprotegerin** (OPG) is essential to bone remodeling. Salivary RANKL levels are significantly higher in untreated non-smoking, periodontitis patients than those who received maintenance therapy (35). However, in the study done by Frodge *et al*, levels of soluble RANKL were hardly found in the saliva of periodontitis patients (23). It has been suggested that soluble RANKL levels may be difficult to detect in saliva because this fraction may be bound to OPG or is degraded within saliva. Salivary OPG levels were elevated during periodontitis and correlated positively with bleeding on probing, probing depth, and clinical attachment level (18). However, long term studies are needed to suggest RANKL and OPG as potential salivary biomarkers for periodontal disease.

AMINOTRANSFERASES

Aminotransferases (aspartate aminotransferase [AST] and alanine aminotransferase [ALT]) are enzymes related to periodontal disease diagnosis. They are cytoplasmic enzymes needed for various amino acid production and serve as diagnostic analytes of cellular injury. They are detected in saliva, periodontal tissue, GCF, and in the enamel pellicle (36).

In one study, salivary AST activity was significantly increased compared with controls, but the salivary ALT activity was not significantly altered in patients with periodontal disease (37). Salivary AST levels were significantly higher in a group of patients who had more severe periodontal disease than controls (38). In another study, AST levels significantly increased with increasing severity of periodontitis and ALT levels increased, but not to a significant level, above the healthy controls (39). It has also been demonstrated that salivary levels of AST and ALT in patients with periodontitis decrease significantly after scaling (40).

GENOMIC APPROACHES FOR THE DISCOVERY OF SALIVARY BIOMARKERS

A number of studies have examined links between polymorphisms within host response factors and aggressive periodontitis. These include genes encoding inflammatory cytokines such as TNF-α and IL-1, the anti-inflammatory cytokine IL-10 and Fc-gamma receptors (41, 42).

In a study done by Yoshie *et al*, the examination of levels of several salivary enzymes after scaling and IL-1 genotypes in Japanese patients with chronic periodontitis showed that salivary alanine aminotransferase, aspartate aminotransferase, and lactate dehydrogenase levels reflected inflammation and destruction of periodontal tissue (40). It was found that one of the IL-1α polymorphisms (IL-1α+4845 alleles) may influence post-scaling values of salivary aspartate aminotransferase and alanine aminotransferase.

Reactive oxygen species, originating predominantly from polymorphonuclear leukocytes,

stimulate tissue damage through DNA damage, lipid peroxidation, protein disruption and stimulation of inflammatory cytokine release. 8-hydroxy-deoxyguanosine, a product of oxidative DNA damage, has been explored as a biomarker for detecting periodontitis (43).

CURRENT SALIVARY DIAGNOSTIC TESTS FOR PERIODONTAL DISEASE

There are currently two salivary diagnostic tests available in the American market for the detection of periodontal diseases (44). One test identifies the type and concentration of specific periodontal pathogenic microorganisms in patient's saliva samples. The laboratory report gives an idea of the pathogenic properties of the detected pathogens, which allows the clinician to determine the most appropriate antimicrobial therapy, and also the ability to customize a treatment plan.

Another salivary test detects genetic susceptibility to periodontitis, which analyzes genetic variation in individuals that affects the production of the inflammatory cytokines interleukin-1 α and β. It allows the clinician to identify patients who are at a greater risk for the development of severe periodontal destruction, but they lack the ability to specifically predict the periods of disease activity.

Shimazaki *et al* analyzed the effectiveness of **salivary occult blood test** (SOBT) as a screening method for periodontal status (45). They reported that sensitivity and specificity of the SOBT in screening for poor periodontal status were 0.72 and 0.52, respectively. In a multivariate logistic regression analysis, the results of the SOBT were significantly associated with the proportion of teeth with BOP and teeth with pocket depth ≥4mm.

Dabra *et al* studied the activity of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), acidic phosphatase (ACP), and gamaglutamyltransferase (GGT) in the saliva from patients with periodontal disease, before and after periodontal treatment and in the saliva from healthy subjects (46). The results showed statistically significant increased activity of AST, ALP, GGT, ALT, and ACP in the saliva from patients with periodontal disease, in relation to the control group. After conventional periodontal therapy, a significant decrease in the enzyme levels was seen.

EMERGING SALIVARY DIAGNOSTIC TOOLS

Recently, RNA was found to exist in saliva which can be used for the diagnosis of oral diseases, such as sjogren's syndrome and oral cancer, where salivary proteome and transcriptome are the emerging toolboxes for early detection, disease progression and therapeutic monitoring (47).

SALIVARY PROTEOME

Human salivary proteome analysis is important for understanding oral health and disease pathogenesis. So far, by using both two-dimensional gel electrophoresis / mass spectrometry and 'shotgun' proteomic approaches, 309 distinct proteins in human whole saliva has been identified by researchers (48). Collectively, 1,166 salivary proteins have been identified: 914 from the parotid gland and 917 from the combined submandibular and sublingual fluids (49).

The University of California at Los Angeles is the data-centralization site, harboring the entire database of the human salivary proteome known as the 'Salivary Proteome Knowledge Base'.

Haigh *et al* studied the alteration in salivary proteome associated with periodontitis. Increase in the abundance of the S100 proteins S100A8/A9/A6 was the chief alteration observed (50). Other proteins associated with host defence with altered abundance were haptoglobin, prolactin inducible protein and parotid secretory protein. This emphasized the primary involvement of S100 proteins in the host response during periodontitis and suggested a new potential biomarker for periodontitis to monitor the disease activity.

Range *et al* evaluated the modifications in salivary proteome associated with periodontitis in obese patients (51). Periodontal examinations and whole saliva samples were obtained from 38 obese patients including 13 periodontitis and 25 non-periodontitis subjects, and 19 healthy controls. Surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) mass spectrometry (MS) was used to compare the whole saliva polypeptide profiles. The SELDI-TOF-MS analysis detected eight putative markers. Six of them (albumin, α and β haemoglobin chains, α-defensins 1, 2 and 3) were increased and identified in obese subjects versus controls. Alpha-

defensins were less abundant in saliva of periodontitis obese patients (36.47 ± 19.84 µA) versus non-periodontitis obese patients (43.44 ± 30.34 µA), whereas α-defensins were more abundant in obese patients (40.99 ± 26.66 µA) versus controls (27.1 ± 23.98 µA). These results show that periodontal status modifies the salivary proteome in obese patients.

P. gingivalis is one of the highly invasive intracellular pathogen in patients suffering from periodontitis, which commonly resides along with *streptococcus gordonii* and *fusobacteriumnucleatum*. In the studyconucted by Kuboniwa *et al..* (52), whole cell quantitativeproteomics, along with mutant construction and analysis were conducted to investigate how *P. gingivalis* adapts to this three species community. The results showed that 403 proteins were down regulated and 89 proteins were upregulated. In another study, whole-cell proteomic analyses were conducted to investigate the changes from an extracellular to intracellular lifestyle for *Porphyromonasgingivalis* and found that a total of 385 proteins were over expressed in internalised *P. gingivalis* relative to controls (53). In another study, there was a shift in the production of cytotoxic fatty acids by intracellular *P. gingivalis*, which suggested that the interior of host cells provided a more energy rich environment compared to the extracellular milieu (54). These results showed that an adaptation to an epithelial cell environment induces a major shift in the proteome of the organism.

Studies on salivary proteome gains importance by the fact that these information can be used to develop potential new drugs for the treatment of periodontitis. Virtual screening (VS) is a computational technique used in drug discovery to search libraries of small molecules in order to identify those structures which are most likely to bind to a drug target, typically a protein receptor or enzyme. Studies on modifications in salivary proteome could play a vital role in virtual ligand screening.

SALIVARY TRANSCRIPTOME

Recently thousands of human mRNAs in saliva have been identified, using expression microarrays. Four saliva mRNA biomarkers (OAZ-1, SAT, IL1β, and IL8) collectively exhibit a 91% sensitivity and specificity for human oral cancer (13).

It has been found that RNA molecules elevated in oral cancer tissues are also elevated in saliva. High-density oligonucleotide microarrays were used to profile salivary mRNA and revealed that there are ~3,000 human mRNAs in the cell-free saliva supernatant of healthy subjects. Two recent studies demonstrated that fetal cell-free RNA crossed the placenta and was detected in maternal serum (55, 56).

Most recently Dutch forensic group was able to perform a gene-expression profile on saliva stains from crime scenes, leading to the identification of five saliva RNA markers (SPRR3, SPRR1A, KRT4, KRT6A and KRT13), stable for up to 180 days, which can be used for the identification of blood and saliva stains in forensic practice (57).

Hidayat *et al..* (58) studied salivary transcriptome biomarkers associated with periodontitis. Using an Oragene® RNA kit total RNA was purified from the saliva of 10 chronic periodontitis patients and 10 with healthy or only mildly inflamed gingivae (the health/gingivitis group). The quantity and quality of the total RNA was determined, and a measure of gene expression via cDNA was undertaken using the Affymetrix Microarray system. The microarray profiling result was further validated by real-time quantitative PCR. The results showed that there was acceptable quality and quantity of total RNA from saliva but a high proportion of it was of microbial origin and there was insufficient human salivary transcriptome for expression studies. Human salivary transcriptome detection was difficult as it was mostly partially fragmented and degraded in saliva. Further research is needed to enhance the extraction process of human mRNA from saliva.

RAPID POINT-OF-CARE DIAGNOSTICS FOR PERIODONTAL DISEASE

Prospective technologies such as 'lab on chip' have the potential to deal with oral fluids such as saliva and gingival crevicular fluid, and to provide a determination of a patient's periodontal disease-risk profile, current disease 'activity' and response to therapeutic interventions. This technology helps to speed up the clinical decision–making and to monitor the disease progression in a chronic infectious disease such as periodontitis.

Herr *et al..* (13) reported on a clinical point-of-care diagnostic test that enables rapid (<10 mins) measurement of levels of collagen cleaving enzyme MMP-8 in saliva and this can be achieved using 20µl of saliva. Based on this, a portable diagnostic device called the 'Integrated Microfluidic Platform for Oral Diagnostics' was developed. Lab-on-a-chip system

can be used for the concomitant measurement of the salivary biomarkers C-reactive protein, MMP-8 and interleukin-1β as related to the clinical expression of periodontitis. The results achieved by the lab-on-a-chip approach are in agreement with those acquired by standard enzyme-linked immunosorbent assay. The lab-on-a-chip assay procedure demonstrates a detection limit at 5 fg/ml and a useful range between 10fg/ml and 10 pg/ml (59).

Ideally diagnostic tests should demonstrate high specificity and sensitivity. Due to the complex nature of periodontal disease, it is unlikely that a single marker will prove to be both sensitive and specific. A combination of two or more markers may provide a more accurate assessment of the periodontal condition. Because of the simple and non-invasive method of collection, salivary diagnostic tests appear to hold promise for the future. The use of optimized point-of-care devices for periodontal surveillance will probably require less training and fewer resources than current diagnostic tests.

CONCLUSION

A combined analysis of proteomic, genomic, microbial and other indicators is required to identify the set of biomarkers with the most favorable combination of sensitivity, specificity, reproducibility and correlations with established disease diagnostic criteria, and reproducibility.

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