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New perspectives of human saliva as genetic biomarker in early disease diagnostics

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Review

ABSTRACT

Saliva is an easily obtainable biofluid rich in genetic biomarkers that can aid in predicting and diagnosing various diseases. Salivary DNA serves as a reliable diagnostic marker, with DNA yield quality from saliva comparable to that from blood and urine, making it suitable for genetic and molecular analysis. Beyond DNA, salivary RNA can also act as genetic biomarkers for conditions such as oral squamous cell carcinoma and other diseases. Currently, noncoding RNAs in saliva, including microRNAs (miRNAs), small nucleolar RNAs (snoRNAs), circular RNAs (circRNAs), and piwi-interacting RNAs (piRNAs), are recognized as potential disease markers. The small size and stability of these molecules in different body fluids, including saliva, provide advantages in molecular diagnostics. For example, salivary 8-OHdG can be used to measure DNA damage and assess disease progression, such as the transition from oral premalignant disorders to oral cancer. Salivary diagnostics is an emerging field, and its integration with genomics facilitates the early detection of various diseases. These genomic components of saliva offer a timely, cost-effective, and non-invasive diagnostic medium. This review aims to explore the genetic biomarkers present in saliva for various diseases.

Keywords: Saliva biomarkers, disease diagnosis, genetic biomarker, salivary RNA, salivaomics.

INTRODUCTION

Saliva, an exocrine secretion from the salivary glands, contains a variety of molecules including polypeptides, proteins, nucleic acids, electrolytes, hormones, and growth factors. These components dynamically contribute to maintaining a healthy oral cavity and overall systemic health. Research in detecting disease biomarkers has identified saliva as a valuable tool for biomarker identification. Liquid biopsy refers to the analysis of nonsolid biological tissues like blood, saliva, amniotic fluid, and other body fluids. Saliva is gaining prominence in diagnostics due to its non-invasive, easy-to-use, and cost-effective collection method. It does not require trained medical personnel, and samples can be collected multiple times at different intervals. Additionally, there are minimal risks of cross-contamination, and the shipping and storage of saliva samples are simpler compared to serum.

Biomarkers can be found in various forms, including DNA, coding and non-coding RNA, lipids, metabolites, and proteins. Due to the intricate interactions between salivary proteins, it is crucial to develop a comprehensive panel of biomarkers for disease detection.[1] Saliva is a vital biological fluid for identifying biomarkers. Analysing saliva can reveal new information about biomarkers, as it contains biomolecules from systemic sources that enter the oral cavity through various pathways. These biomolecules reflect tissue fluid levels of hormonal, immunological, and toxicological substances.[2] Salivary analysis has become important due to its origin, composition equivalent to serum, and interactions with other organs.[3] Saliva collection and processing criteria need to be standardized based on specific diseases. A significant limitation to the routine diagnostic use of saliva is that its component levels are lower than those in serum and other biological fluids.[4] Nonetheless, saliva tests can diagnose oral diseases such as caries, periodontal diseases, and oral malignant lesions.[5] They can also detect systemic illnesses like diabetes mellitus and cardiovascular diseases, as well as monitor drug use and assist in forensic studies. Saliva presents a vast potential for advancements in the medical field.[6]

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SALIVA AS A SOURCE OF GENETIC MATERIAL

The oral cavity could be a non-invasive source of genomic material. In recent years, saliva has emerged as a new tool for genetic testing due to its minimal invasive approaches.[7] In a genetic epidemiologic study of type 1 diabetes mellitus with Norwegian children, DNA was extracted from buccal swabs and human leukocyte antigen HLA-DQA1 and -DQB1 allelic polymorphisms were determined by polymerase chain reaction which resulted in comparable results with previous studies.[8] In another study conducted by Adriaanse *et al*., 2016, DNA isolation using buccal swabs yielded a good quality and quantity of DNA to perform HLA-DQ typing in children for celiac disease which could reduce the need for current venipuncture.[9] Saliva is also a source of extracellular or cell-free DNA which has the potential to be used in forensic case studies.[10] Not only DNA but RNA also could be isolated from saliva and salivary RNA analysis was done using microarray to understand neonatal development.[11] Salivary mRNA could be a potent biomarker for early oral squamous cell carcinoma (OSCC) diagnosis and a study done by Oh *et al*.,2020 showed that mRNA levels of six genes (NGFI-A binding protein 2 (NAB2), cytochrome P450, family 27, subfamily A, polypeptide 1 (CYP27A1), nuclear pore complex interacting protein family, member B4 (NPIPB4), monoamine oxidase B (MAOB), sialic acid acetyltransferase (SIAE), and collagen, type III, alpha 1 (COL3A1)) were significantly lower in the saliva of OSCC patients.[12] Salivary interleukin-6 (IL-6) mRNA expression was significantly higher in patients with OSCC and could be considered as a potential biomarker of OSCC.[13] Exosomes have been successfully isolated from saliva and salivary exosomes could be useful tools for omics analysis due to the presence of lipids, proteins, and nucleic acids in exosomes.[14] The study by Zhong *et al*., 2005 investigated the expression of telomerase in saliva and it was detected positively in 75% of patients with OSCC and suggested that the telomerase in saliva could be used as an assistant marker for the disease.[15] Mitochondrial DNA mutations are useful targets to detect head and neck cancer and by sequencing alone, the study by Fliss *et al*.,2000 was able to detect mtDNA mutations in 67% of saliva samples.[16]

Noncoding RNAs as potential disease biomarkers

In addition to mRNA, non-coding RNAs such as microRNAs (miRNAs), small nucleolar RNAs (snoRNAs), circular RNA (circRNA), and piwi-interacting RNAs (piRNAs) are present in saliva and are emerging as potential disease markers.[17,18] The short size of these molecules makes them stable in different body fluids including saliva and is less susceptible to degradation by ribonucleases (RNases).[19] In a study performed by Zahran et al., 2015, miRNA was isolated from saliva and three salivary miRNAs (miRNA-21, miRNA-184, and miRNA-145) were showed as possible markers for malignant transformation in oral mucosal lesions.[20] It was identified that miRNAs (mmu-miR-140-5p, hsa-miR-374, hsa-miR-222, hsa-miR-15b, hsa-let-7g, and hsa-miR-132) were differently expressed between saliva samples of patients with a malignant tumor and benign parotid gland tumor.[21] The differential expression of salivary miRNAs from Head and neck squamous cell carcinoma (HNSCC) in the Ecuadorian population was studied using PCR Arrays which identified miR-122-5p, miR-92a-3p, miR-124-3p, miR-205-5p, and miR-146a-5p were most associated [22]. Bahn *et al*., 2015 compared >90 RNA-sequence data sets of different origins and observed that piRNAs were higher in cell-free saliva compared to other body fluids and miRNA expression profiles were similar to those in serum and cerebrospinal fluid [23]. piRNAs are found to be highly exclusive to saliva with very low abundance in blood or cerebrospinal fluid and indicate that salivary piRNAs might have been generated from cells in the oral mucosa or salivary glands, rather than circulating from systemic organs via blood.[24]

Proteome of saliva

Protein components present in saliva include proline-rich proteins, α-amylases, mucins, salivary ("S-type") cystatins, histatins, statherin, lipocalin, and P-B peptide and are secreted from three major glands, parotid, sub-mandibular, and sub-lingual.[25] Proteins in the whole saliva have been identified using large-scale mass spectrometry-based technologies and many of these proteins are also found to be present in the human plasma proteome, indicating that salivary proteins may also circulate and be indicators of systemic health.[26] Using mass spectrometry analysis,[27] salivary proteome was analyzed and a set of 139 proteins along with their proteotypic peptides were identified which could serve as a reference of secretory markers for clinical applications in oral malignancies.[28] Another mass spectrometry analysis of the proteome of the saliva of chronic graft-versus-host-disease (cGVHD) revealed reduction of salivary lactoperoxidase, lactotransferrin, and several proteins included in the cysteine proteinase inhibitor family suggesting impaired oral antimicrobial host immunity in cGVHD patients [29]. To identify disease-related markers in type 1 diabetes, with and without microvascular complications, the salivary proteome and peptidome profile were carried out using iTRAQ-based quantitative approach which revealed that bactericidal/permeability-increasing protein-like 1, pancreatic adenocarcinoma, alpha-2- macroglobulin, defensin alpha 3 neutrophil-specific, leukocyte elastase inhibitor, matrix metalloproteinase-9, neutrophil elastase, plastin-2, protein S100-A8, and protein S100- A9 were related with microvascular complications such as retinopathy and nephropathy.[30]

Table 1: Important salivary proteins and their functions

Salivary Proteins	Function
Mucins	Glycoproteins that protect tooth surface from demineralization, aids in lubrication and prevents bacterial adhesion
Lysozyme	Antibacterial enzyme that lyse bacterial cell wall
Lactoferrin	Iron binding glycoprotein that has bacteriostatic and bactericidal activity
Peroxidase	Eliminates hydrogen peroxide
Histatin	Inhibits bacterial enzymes
Defensins	Small cationic proteins with antimicrobial activity
Immunoglobulins Predominant is IgA	Inhibition of bacterial adherence, inactivation of bacterial enzymes and toxins

Metalloproteinases	Breakdown proteins such as collagen
Proline rich proteins	Calcium homeostasis
Statherin	Inhibits precipitation of calcium phosphate in saliva and also inhibit the growth of anaerobic bacteria
Cystatin	Protease inhibiting proteins
carbonic anhydrase VI	pH control

SALIVARY SECRETIONS AND ASSOCIATED DISEASES

Salivary analysis has become one of the important resources for monitoring health and the disease state due to its origin, composition similar to serum, and interactions with other organs.[3] The main innate defense factors present in saliva are the peroxidase systems, defensins, lysozyme, lactoferrin, and histatins and the interactions between these factors result in synergistic inhibitory effects on bacteria and prevent the development of bacteria mediated oral diseases such as dental caries and periodontitis. There was an increase in sodium, total protein, albumin, immunoglobulin (Ig)A, IgG, IgM, amylase, lysozyme, IL-2, IL-6, and neural growth factor (NGF) in the saliva of burning mouth syndrome patients and these salivary changes were found to be associated with inflammation, dry mouth, and taste alterations in burning mouth syndrome.[31] Xerostomia occurs when the unstimulated whole saliva flow rate falls by 40-50% of its normal value and may result from changes in salivary composition or function, particularly of lubricating mucins.[32] Sjögren's syndrome is characterized by dysfunction and destruction of the salivary and lacrimal glands and their secretory fluids, saliva and tears, reflect the pathophysiology of the disease. The protein signature of this syndrome comprises secretory proteins, enzymes, calcium-binding proteins, abundantly expressed immune-related molecules such as β-2-microglobulin, cathepsin-D, α-enolase, cystatins, defensins, and Ig γ-light chain.[33] Sialadenitis and sialadenosis are common causes of submandibular gland swelling and include reduced salivary secretions and duct obstruction.[34] Various cytokines such as IL-6, IL-8, IL-1a, IL-1b, TNF-a were found to be higher in oral cancer and these cytokines are proinflammatory and proangiogenic, which could be indicators of carcinogenic transformation from premalignant oral disorders (PMOD) to oral cancer.[35,36] The levels of salivary 8-hydroxydeoxyguanosine (8-OHdG) as a potential DNA damage biomarker in PMOD and OSCC were assessed and salivary 8-OHdG levels showed significant differences between cases and healthy controls indicating that salivary 8-OHdG can be used as a novel biomarker of DNA damage to assess disease progression from PMOD to OSCC.[37] When saliva of Down Syndrome patients was analyzed, the concentration of acidic proline rich proteins and S cystatins were found significantly reduced and levels of the antimicrobial α-defensins 1 and 2 and histatins 3 and 5 were significantly increased in the whole saliva of older Down syndrome subjects whereas S100A7, S100A8, and S100A12 levels were significantly increased in the whole saliva of Down syndrome subjects.[38] SAPHO syndrome is a rare disease characterized by synovitis, acne, pustulosis, hyperostosis, and osteomyelitis and there was a significant reduction in salivary proteins cystatin S1 and SN, histatins, the major acidic proline rich proteins, P-C and P-B peptides in SAPHO subjects.[39]

GENETIC VARIANT ANALYSIS OF SALIVARY SECRETIONS

The study by Badea *et al*.,2013 analyzed the genetic polymorphism of the IL-1 gene from oral swabs and the salivary level of the 8-OHdG biomarker and demonstrated that IL-1 gene polymorphism and level of 8-OHdG can be used in the evaluation of the oro-dental status of patients with aggressive periodontitis.[40] Cystatin 3 has two common haplotypes located at three sites, two in the promoter region and one in the signal peptide domain that causes A to T substitution and a mutation with the substitution L68Q has been shown to cause rare autosomal-dominant disease, hereditary cerebral hemorrhage with amyloidosis.[41] In a study conducted by Peres *et al.*, 2009, there was a positive association between higher buffer capacity and the rs2274327 (C/T) polymorphism of Carbonic anhydrase VI and the allele T and genotype TT were significantly less frequent in individuals with the highest buffer capacity.[42] A systematic review by Lips *et al*., 2017 showed an association between genetic polymorphisms and risk of dental caries for most of the salivary proteins and found a consistent association between salivary proteins related to the antimicrobial activity (beta defensin 1 and lysozyme-like protein), pH control (carbonic anhydrase VI), and bacterial colonization/adhesion (lactotransferrin, mucin, and proline-rich protein).[43] rs11362 and rs1799946 gene polymorphisms of 5' UTR of beta defensin 1 gene were found to be associated with the increased risk of dental caries.[44] A study conducted by Kuchler *et al.*, 2017 found that genetic variations in Amelogenin(AMELX), Ameloblastin (AMNB), and Estrogen-related receptor β (ESRRB) were associated with the calcium levels in saliva and genetic variation in Enamelin (ENAM) was associated with phosphorus in saliva.[45] In a study by Hernández-Arenas *et al*., 2021, the salivary detection of DNA repair gene, X-ray repair cross-complementing group 1 (XRCC1), rs25487 single-nucleotide polymorphism was carried out which showed that the SNP appeared to not modulate the risk of PMOD and OSCC in a Colombian population but showed significant association with clinicopathological characteristics in OSCC, and synergistic interaction between aging and smoking/alcohol consumption and might play a role in the etiopathogenesis of these two diseases.[46] Salivary samples were used to determine whether a panel of 18 SNPs (SNP18) may be used to predict breast cancer in combination with risk factors and mammographic density and SNP18 was found to likely aid risk-stratified screening and prevention strategies.[47]

SALIVA AS A DIAGNOSTIC MEDIUM

Saliva is extensively being researched for diagnostic purposes.[5] Saliva is used by clinical laboratories for the detection and determination of secretory IgA antibodies, salivary cortisol, hormones and genetic purposes including detection of microbial DNA, mRNA, siRNA, and miRNA.[48] Proteins like statherin, cystatin, histatins, and proline-rich proteins play an important role in enamel's structural integrity and are important biomarkers in caries diagnosis.[49] Saliva could also be used for diagnosing

infectious diseases. Saliva tests could be a promising alternative to nasopharyngeal swab tests for COVID-19 diagnosis but several factors should be considered which might affect the detectability of viral RNA in the saliva, such as the timing and method of sample collection, the choice of transport medium, storage, and transport temperatures.[50] Using salivary samples, it was possible to diagnose Dengue IgG antibody with high sensitivity and specificity.[51] HIV antibodies can be detected in saliva providing an alternative to blood to diagnose HIV infection.[52,53] However, the viral load could be lesser compared to blood but methods are being carried out to increase the accuracy of detection. The examination of the saliva of oral cancer patients has gained interest because of the direct contact with cancer lesions and also contains fallen cells making it a prime choice for screening. A study conducted by Dhanya & Hegde 2016 showed an increase in the level of fasting salivary glucose and a correlation between salivary glucose and serum glucose in diabetic patients and the study concluded that fasting salivary glucose level could be used as a noninvasive diagnostic and monitoring tool to assess the glycemic status of type II diabetes mellitus patients.[54] Saliva could be used to monitor drug levels. Salivary therapeutic drug monitoring was investigated and levels of antiepileptic drug, perampanel, in saliva was studied which showed that perampanel concentration in saliva correlated with that in plasma.[55] A meta-analysis by Rapado-González *et al*., 2020 showed that salivary biomarkers may be potentially used for the non-invasive diagnosis of malignant non-oral tumors and several biomarkers detected in saliva were able to discriminate cancer patients from healthy individuals with a significant degree of sensitivity and specificity [56]. Higher levels of *c-erb-2*, a receptor tyrosine kinase, were found in the saliva of patients with breast cancer when compared with patients with benign lesions.[57]

CONCLUSION

In conclusion, the use of saliva as a diagnostic tool to detect various genetic markers for diseases provides a promising, cost-effective, painless, and stress-free method. Recently, advancements in genomic and proteomic technologies have enabled the use of saliva in clinical settings for early diagnosis and disease management monitoring. Further analysis, research, and validation are essential for the widespread adoption and development of point-of-care devices utilizing salivary biomarkers for clinical applications.

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