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Saliva As A Biomarker Tool

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ABSTRACT

Saliva is a readily accessible biofluid that contains numerous genetic biomarkers to predict and diagnose several diseases. Salivary DNA is a robust marker for diagnostics and the quality of DNA yield obtained from saliva is comparable with blood and urine and can be used for genetic and molecular analysis. DNA and RNA from saliva could be used as genetic biomarkers for oral squamous cell carcinoma and other diseases. Nowadays, noncoding RNAs such as microRNAs (miRNAs), small nucleolar RNAs (snoRNAs), circular RNA (circRNA), and piwi-interacting RNAs (piRNAs) found in saliva are considered as potential disease markers. The small size and stability of these molecules in various body fluids including saliva makes them advantageous in molecular diagnostics. Furthermore, salivary 8-OHdG can be used as a biomarker of DNA damage to assess disease progression, for example, from oral premalignant disorder to oral cancer. Salivary diagnostics is an emerging field along with the application of genomics aid in the early detection of different diseases. These genomic alphabets of saliva may serve as a timely, cost-effective, non-invasive diagnostic medium. This review aims to discuss the genetic biomarkers of saliva in various diseases.

Keywords: Saliva, biomarker tools, DNA damage, oral premalignant disorder

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INTRODUCTION

Saliva is an exocrine secretion of salivary glands with a wide array of molecules including polypeptides, proteins, nucleic acids, electrolytes, hormones, and growth factors that dynamically function to maintain a healthy oral cavity and in turn systemic health. Detecting biomarkers for the disease is an important field of research and saliva is an attractive tool for biomarker identification. Liquid biopsy analyzes nonsolid biological tissues such as blood, saliva, amniotic fluid, and other biological fluids. Saliva is an emerging tool for diagnostic purposes as the collection method is non-invasive, easy to use, and inexpensive. There is no need for trained medical staff and samples can be obtained multiple times at different time points. In addition, there are minimal cross-contamination risks and shipping and storage are more manageable compared to serum.

Biomarkers can exist in different forms, such as DNA, coding and noncoding RNA, lipids, metabolites, and proteins. As there is a complex interaction between salivary proteins, it is important to develop a panel of biomarkers for diseases (Buzalaf MAR et al., 2020). Saliva is an important biological fluid for biomarker identification. Studying saliva can provide novel information about biomarkers and it also consists of biomolecules from systemic sources that reach the oral cavity through various pathways and reflect tissue fluid levels of hormonal, immunological, and toxicological molecules (Zimmermannet al., 2007). Salivary analysis has become important due to its origin, composition equivalent to serum, and interactions with other organs (Lima et al., 2007). Saliva collection and processing criteria must be standardized according to the diseases. One main limitation that hinders the routine diagnostic use of saliva is that the levels of components of saliva are lower than in the serum and other biological fluids. Some of the oral diseases diagnosed with saliva tests are caries, periodontal diseases, and oral malignant lesions. Systemic illnesses such as diabetes mellitus and cardiovascular diseases can also be diagnosed and used to detect and monitor drugs and in forensic study. Saliva provides a massive opportunity for the medicinal field.

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. 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 (Witsø et al., 2002). 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 (Adriaanse et al., 2016). Saliva is also a source of extracellular or cell-free DNA that can be used in forensic case studies (Vandewoestyne et al., 2013). DNA and RNA could also be isolated from saliva and salivary RNA analysis was done using microarray to understand neonatal development (Maron et al., 2010). 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 (Oh SY (Maron et al., 2020). Salivary interleukin-6 (IL-6) mRNA expression was significantly higher in patients with OSCC and could be considered as a potential biomarker of OSCC (Márton et al., 2019). Exosomes have been successfully isolated from saliva and salivary exosomes could be useful tools for omics analysis due to lipids, proteins, and nucleic acids in exosomes (Adeola (Márton et al., 2020). 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 (Zhong *et al.,*., 2005). 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 (Fliss MS *et al.,*.,2000).

Noncoding RNAs as potential disease biomarkers

In addition to mRNA, noncoding 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 (Wong et al., 2015). The short size of these molecules makes them stable in different body fluids including saliva and is less susceptible to degradation by ribonucleases (RNases) (Majem et al., 2015). 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 (Zahran et al., 2015). 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 (Matse et al., 2013). 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 (Salazar-Ruales (Matse et al., 2018). 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 (Bahn et al., 2015). 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 (Lin et al., 2015).

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 (Castagnola et al., 2017). 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 (Griffin 2015). Using mass spectrometry analysis, 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 (Sivadasan et al., 2015).

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 (Bassim et al., 2012). 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 (Caseiro et al., 2013).

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. 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 (de Souza et al., 2015). 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 (Pedersen et al., 2018). 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 (Katsiougiannis et al., 2016). Sialadenitis and sialadenosis are common causes of submandibular gland swelling and include reduced salivary secretions and duct obstruction (Adhikari, Soni et al., 2020). 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 (Khurshid et al., 2018).

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 (Nandakumar et al., 2020). 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 (Cabras et al., 2013). 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 (Sanna et al., 2015).

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. 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 (Dickinson 2002).

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. 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. 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 (Subbiah et al., 2021).

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. 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 (Hernández-Arenas et al., 2021). 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 (van Veen et al., 2018).

Saliva as a diagnostic medium

Saliva is extensively being researched for diagnostic purposes. 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. 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 (Özlem , YARAT 2020). 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 (Czumbel et al., 2020).

Using salivary samples, it was possible to diagnose Dengue IgG antibody with high sensitivity and specificity (Banavar et al., 2014). HIV antibodies can be detected in saliva providing an alternative to blood to diagnose HIV infection (Balamane et al., 2020). 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 (Dhanya , Hegde et al., 2016).

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 (Kim et al., 2020). 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. 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 (Streckfus et al., 2000).

Conclusion

Saliva as a diagnostic tool to detect different genetic markers for various diseases offers a promising inexpensive, painless, and stress-free approach. In recent years, the genomic and proteomic technologies in clinical settings with the use of saliva in early diagnosis aids in the monitoring of disease management. Further analysis, research, and validation are imperative for the widespread use and development of point-of-care devices of salivary biomarkers for clinical applications.

Author contribution

Usha Subbiah, Harini Venkata Subbiah conceived of the presented idea Sumathi K Shenbaga Lalitha S encouraged and supervised the findings of this work. All authors discussed the results and contributed to the final manuscript.

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Conflict of interest .Nil

Study significance

Salivary diagnostics is an emerging field along with the application of genomics aid in the early detection of different diseases. These genomic alphabets of saliva may serve as a timely, cost-effective, non-invasive diagnostic medium. This review aims to discuss the genetic biomarkers of saliva in various diseases.

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