ISSN: 2581-5989
PubMed - National Library of Medicine - ID: 101738774

International Journal of Dental Science and Innovative Research (IJDSIR)

IJDSIR : Dental Publication Service

Available Online at: www.ijdsir.com

Volume – 4, Issue – 2, April - 2021, Page No. : 380 - 387

Saliva as a Biomarker

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Citation of this Article: T Pavan Kumar, Kezia Blessy, Sujatha S Reddy, N Rakesh, Shwetha V., "Saliva as a Biomarker", IJDSIR- April - 2021, Vol. – 4, Issue - 2, P. No. 380 – 387.

Type of Publication: Original research Article

Conflicts of Interest: Nil

Abstract

Saliva is a complex biological fluid which serves as a diagnostic tool in health and disease. It reflects metabolic, immunological, hormonal and nutritional state of a person. Further saliva testing is considered as the appropriate, non-invasive and reasonable testing for scientific investigations. Saliva chemistry is an evolving field and generally applied in forensic medicine and toxicology for analysis and detection of drug addiction and alcohol abuse by plotting blood and saliva data. Saliva testing fundamentally helps in the diagnosis, prognosis and monitoring of disease along with its ease of collection properties. As it is the era of genomic technologies and –omic research, collection of saliva has increased. Currently, several sensitive analytical techniques permit the detection and quantification of a large number of biomarkers in saliva such as mass spectrometry (MS), reverse transcription-polymerase chain reaction (RT-PCR), microarrays, nanoscale sensors, Western blot, immunoassay techniques or enzymatic assays. Recent advances in proteomic techniques have brought the discovery of a large number of biomarkers and therapeutic targets in a large number of oral diseases and systemic pathologies with impacts in the oral cavity. In this review

we have discussed about the immunological and analytical techniques used for the detection of salivary biomarkers.

Keywords: Saliva, Biomarkers, Proteomics

Introduction

Biomarkers are by definition objective, quantifiable characteristics of biological processes. According to the National Institute of Health (NIH), "a biomarker is a characteristic that is objectively measured and evaluated as an indicator of a normal biological process, pathogenic process or pharmaceutical response to therapeutic intervention". Biomarkers help us to understand the relationship between exposure to various environmental chemicals, development of diseases and identification of groups that are at increased risk for disease(Ilyin, Belkowski and Plata-Salamán, 2004)

The modern high-throughput genomic and proteomic approaches have been extensively used to observe the altered expressions of gene and protein in a variety of cancers including OSCC. It may be helpful to facilitate the identification of potential biomarkers for OSCC(Koch et al., 2008)(Brinkman and Wong, 2006). To generate the genomic or proteomic expression profiles, specimen collection is required. Tumor tissue and body fluid such as saliva or blood (serum and plasma) can potentially carry whole cells as well as protein, DNA, and RNA species that allow for detection of cellular alterations related to cancer. Compared to the tissue biopsy, body fluids have garnered much more attention for biomarker identification(Good et al., 2007) Examples of using body fluids for tumor detection include sputum for lung cancer diagnosis(Good et al., 2007), urine for urologic tumors(Hoque et al., 2004), saliva for OSCC, breast fluid, as well as serum or plasma for almost all types of cancer(Zimmermann, Noh and Wong, 2007).

The most popular body fluids which may contain reliable biomarkers for detecting OSCC include blood and saliva. Considering the complexity of the body fluids, saliva has the advantages of easily accessible in a non-invasive manner, low background of normal material (cells, DNR, RNA, and proteins) and inhibitory substances and less complex than blood. The fallen cells in oral cavity allow saliva to be the first choice of screening and the identification for the potential biomarkers of OSCC, which is also helpful in monitoring its development.

Salivary proteomics

Saliva is preprogrammed to have a certain composition in response to events in oral cavity. The first biomarker for cancer to be found in saliva was HER2/neu, a biomarker for breast cancer(Streckfus and Dubinsky, 2007). In 2008, 1,166 salivary proteins were initially identified in a National Institute of Dental and Craniofacial Research-funded project that sought to catalog and annotate the human salivary proteome(Denny et al., 2008). This project was an essential first step for saliva to be clinically useful in disease diagnosis and health monitoring. The majority of the proteins are synthesized and subsequently secreted into the oral cavity by the salivary gland acinar cells(Loo et al., 2010). This observation suggests that proteomic constituents of saliva are products of the salivary glands, which may be subject to internal and external factors. Consequently, the salivary proteome has been useful for identifying biomarkers for both local and distant diseases(Koch et al., 2008).

Some of the conventional methods used in the analysis of salivary proteomes are Chromatography techniques

Chromatography techniques

a) Ion exchange chromatography (IEC)

The IEC is a versatile tool for the purification of proteins on the basis of charged groups on its surface. The proteins vary from each other in their amino-acid sequence; certain amino acids are anionic while others are cationic. The net

charged contain by a protein at physiological pH is evaluated by equilibrium between these charges. Initially, it separates the protein on the basis of their charge nature (anionic and cationic), further on the basis of comparative charge strength. The IEC is highly valuable due to its low cost and its capacity to persist in buffer conditions(Jungbauer and Hahn, 2009)

b) Size exclusion Chromatography (SEC)

SEC separates the proteins through a porous carrier matrix with distinct pore size on the basis of permeation; therefore, the proteins are separated on the basis of molecular size. The SEC is robust technique capable of handling proteins in diverse physiological conditions in the presence of detergents, ions and co-factors or at various temperatures. The SEC is used to separate low molecular weight proteins and is a powerful tool for purification of non-covalent multi-meric protein complexes under biological conditions(Diederich et al., 2011)

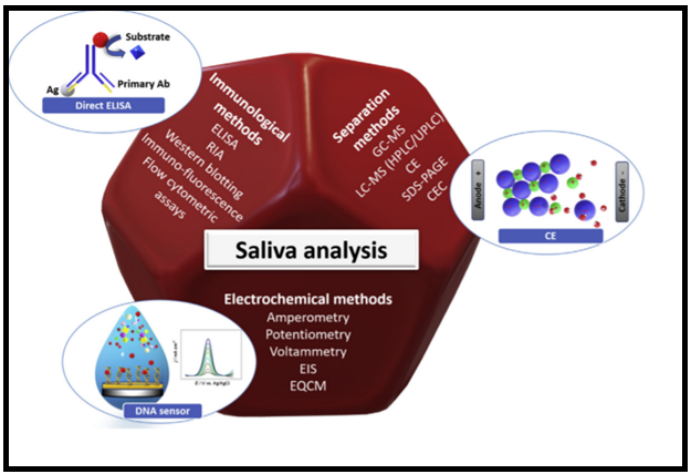
c) Affinity Chromatography

The affinity chromatography was a major breakthrough in protein purification that enables the researcher to explore protein degradation, post-translational modifications and protein–protein interaction. The basic principle behind the affinity chromatography is the reversible interaction between the affinity ligand of chromatographic matrix and the proteins to be purified(Hage et al., 2012)

The affinity chromatography has a wide range of applications in identification of microbial enzymes principally involved in the pathogenesis. Homodimer and heterodimer of HIV-I reverse transcriptase were rapidly purified by metal chelate affinity chromatography.

Other methods used to detect biomarkers and metabolites from saliva include immunological techniques, separation methods and lately, electrochemical methods through the use of biosensors

Figure 1: Schematic representation of immunological and analytical techniques for the detection of salivary biomarkers



Immunological techniques

The immunological techniques are used for the investigation of tumoral and cellular immune responses to oral organisms both in local oral tissues and fluids. The most representative immunological techniques used in salivary investigation and analysis are: enzyme-linked immunosorbent assay (ELISA), which quantify specific antibody and cytokines in gingival crevicular fluid, and saliva; flow cytometry, used especially for the characterization of T cells from peripheral blood and gingival tissues; immunohistological analysis of the inflammatory cell infiltrate in gingival tissues; RIA; and western blotting(Ngamchuea et al., 2018)

Enzyme Linked Immuno Sorbent Assay (ELISA)

In 1971, Engvall and Pearlmann published the first paper on ELISA and quantified the IgG in rabbit serum using the enzyme alkaline phosphatase. The ELISA is highly sensitive immunoassay and widely used for diagnostic purpose. The assay utilizes the antigen or antibodies on the solid surface and addition of enzyme-conjugated antibodies to measure the fluctuations in enzyme activities

that are proportional to antibody and antigen concentration in the biological specimen(Lequin, 2005)

Wheat proteins causes allergic reactions in susceptible individuals that have been traced in foods to protect wheat-sensitive individuals using commercially available ELISA kits. Sandwich ELISA was used for the detection of Cry1Ac protein of Bacillus thuringiensis from transgenic BT cotton as their release adversely affect the environment(Wang et al., 2007) Indirect competitive ELISA was developed to detect Botrytis cinerea in tissues of fruits. B. cinerea is a phyopathogenic fungus responsible for gray mold and often present as latent infection and deteriorate the healthy fruits(Fernández et al., 2011) Digital ELISA is capable of detecting single molecule in the blood. The assay was able to detect prostate-specific antigen (PSA) in the serum at low concentration of 14 fg/ml. This assay was capable to detect 1, 1-Dichloro-2, 2-bis (p-chlorophenyl) ethylene (p,p′-DDE); a metabolite of insecticide and persistent organic pollutant that accumulates in food chain and environment

Western Blotting

Western blotting is an important and powerful technique for detection of low abundance proteins that involve the separation of proteins using electrophoresis, transfer onto nitrocellulose membrane and the precise detection of a target protein by enzyme-conjugated antibodies(Lee, 2007) Western blotting is a dominant tool for antigen detection from various microorganisms and is quite helpful in diagnosis of infectious diseases. The seroprevalence of Herpes Simplex Virus type 2 (HSV-2) in African countries was investigated by measuring the specific immunoglobulin G in the sera of patients(Kaur and Kaur, 2013) Leishmania donovani is responsible for visceral leishmaniasis, which is classically diagnosed by the presence of HSP83 and HSP70 antigens in the bone marrow, spleen and liver

Western blotting was carried out by Li et al. for identification and validation of 10 rice reference proteins. Elongation factor 1-α and heat-shock proteins were the most expressed proteins in rice (Zhang, Sun and Wang, 2012). Kollerova et al. identified the Plum Pox Virus (PPV) capsid proteins from infected Nicotiana benthamiana(Kollerova, Glasa and Subr, 2008). The expression of PfCP-2.9 gene of Plasmodium falciparum in tomato was confirmed through western blot analysis. Specific IgE against Ara h1, Ara h2 and Ara h3 was determined in peanut allergic patients through western blotting(Koppelman et al., 2004)

Electrochemical methods

A large variety of electrochemical techniques are available for the salivary detection of biomarkers which are relevant from medical point of view. Despite all these advantages, electrochemical assays are relatively less common in biomedical and diagnostic applications, since in many cases, the developed assays and the optimized methods have not been tested in biological samples. This is mainly due to the lack of validation related with the difficulty of electrochemical measurement in real samples, to poor specificity as there are potential interferences. If the proposed methods are confirmed by validation, there will be more of these procedures used in diagnostic field.

Further, many studies have reported that the proteomic profile of saliva from OSCC patients differs from the profile for OSCC-free controls. In 2008, a panel of candidate protein biomarkers for the detection of OSCC was identified by immunoassay validation. The combination of five candidate protein markers, myeloid related protein 14 (MRP14), profiling, CD59, catalase, and Mac-2-binding protein (M2BP), had a sensitivity of 90 % and specificity of 83 % for OSCC detection. Mass

spectrometry-based proteomics was used to discover differences in salivary protein abundance between subjects with pre-malignant and malignant oral lesions. Biochemical validation showed that myosin and actin are promising salivary biomarkers capable of discriminating malignant oral lesions. Actin and myosin are key cytoskeletal proteins that facilitate cell motility and invasion, behavior central to epithelial tumorigenesis.

In another study, salivary biomarkers for early-stage OSCC were identified by two-dimensional gel electrophoresis and mass spectrometry, and then validated by Western blot analysis and ELISA. Transferrin levels were elevated in the saliva from a mostly male sample of 41 OSCC patients compared with 30 OSCC-free controls. The increase in salivary transferrin correlated with increasing tumor size. Transferrin is needed for the growth of rapidly growing cells and is involved in DNA synthesis, electron transport, mitogenic signaling pathways, proliferation, and cell survival.

Fibroblast growth factors (FGFs) are heparin-binding proteins involved in angiogenesis, wound healing, embryonic development, and various endocrine signalling pathways. FGFs are key players in the proliferation and differentiation of a wide variety of cells and tissues. Basic FGF (bFGF) is a solid mitogen that stimulates the proliferation of cells of mesodermal and neuroectodermal origin and is reported to be involved in wound healing, hematopoiesis, angiogenesis, and tumor progression.(Maciag, 1989) Salivary bFGF levels were found to be significantly elevated in patients newly diagnosed with OSCC compared with healthy controls. The findings suggest that bFGF could be a potential biomarker for the detection of OSCC in patients with no oral mucosal disease, such as oral lichen planus, geographic tongue, aphthous ulcer, or candidiasis. Salivary bFGF could also be used to detect recurrence, as the levels have been reported to be higher in patients with newly diagnosed OSCC than in those who had completed treatment and not exhibited any recurrence for at least 2 years(Gorugantula et al., 2012).

Speaking about the advantages and disadvantages of these analytical and immunological techniques, one has to consider that a successful approach or method should demonstrate much higher throughput capability, significantly lower amounts of the sample needed, higher sensitivity, higher resolution at low mass ranges, lower costs and easy to use, compared with the usual method in order to be considered for clinical purposes. The highly sensitive determination of volatile compounds is essentially performed by various GC techniques. Although, the head-space sample handling seems to be cumbersome as the determination of alcohol or acetone in one's breath is inconvenient because these volatile compounds are easily lost during saliva sample pre-treatment before the GC analysis. Nowadays, electrochemical immunosensors with different sensing receptors and transducers are considered promising tools in screening methods. Specifically, over conventional ELISA, the use of electrochemical biosensors presents some advantages such as increased sensitivity, lower limits of detection, low cost, simple design, ease of manipulation, low consumption of expensive and/or toxic reagent. Another advantage of using electrochemical biosensors for saliva analysis is the possibility of sample processing just after saliva sampling, without separation steps, the response being obtained within minutes or even seconds. Furthermore, the biocomponent that is immobilized at the electrode leads to the increase of the selectivity and even the specificity for the target analyte, which make the sensor suitable for measurements in complex matrix as saliva. Test duration is also very important when dealing with real samples. Thus, assays

based on chromatographic separation coupled with mass spectrometry separation (HPLC-MS) proved to be very useful in salivary biomarkers testing since it allowed the non-invasive, simple, and reliable analysis after a total run time of few minutes. Moreover, electrochemical sensors based on Nano composite materials meant to increase the sensitivity and selectivity for the target, perform the analysis within seconds(Kim et al., 2014)(Lee and Compton, 2015)

Conclusion

The desirable goals of healthcare research is to assess the health or illness states, to monitor the progression of disease and post-treatment therapeutic outcomes through non-invasive methodologies. Further, in the field of toxicological, biochemical and immunological diagnostics, Saliva represents a very remarkable biofluid with high potential to monitor health. In contrast to blood sample, which is prone to clotting, saliva is much easier to handle and its sampling is non-invasive, requiring less pre-analysis manipulation.

Salivary diagnostic serves as a portion of the greater field of molecular diagnostics and it has developed into a more sophisticated discipline. Further, it is recognized as a central competitor in basic, biomedical and clinical research. Modern advances, mainly through standardization of specimen collection have made it easier for secure, effortless and non-invasive collection of samples. Further, to make salivary diagnostics a certainty for different pathologies, extensive research is required. Particularly, it can be envisioned that saliva chemistry mapping could be a striking possibility as a routine chair-side test included in a comprehensive diagnostic and risk assessment tool.

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