Salivary Diagnostics in Oral Cancer

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1. Introduction

Saliva is a complex fluid produced by the major and minor salivary glands and is a mixture of several constituents of non-salivary origin such as gingival crevicular fluid, expectorated bronchial and nasal secretions, serum and blood derivatives from oral wounds, microorganisms, desquamated epithelial cells, other cellular components and food debris (Kaufmann & Lamster, 2002).

Saliva is considered a mirror of body health and is composed of variety of analytes from systemic sources that reach the oral cavity through various pathways. Because water is a major constituent, saliva plays a key role in the lubrication and repair of oral mucosa, formation and swallowing of food bolus, digestion of starch, facilitation of food tasting and control of oropharyngeal microbial population (Lawrence, 2002).

Salivary constituents comprises both organic and inorganic components in generally small quantities that vary with changes in flow, yet continually providing an array of functions. The components, especially proteins are multifunctional, redundant, and amphifunctional as research into the complex roles of proteins and mucins support this theory. The multifarious components within the saliva not only protect the integrity of oral tissues, but also provide clues to various local and systemic conditions and diseases. These salivary components are constantly being explored as markers of various diseases and to monitor general health. Despite not being one of the popular bodily fluids as it is lacks the drama of blood, sincerity of sweat and emotional appeal of tears; a growing number of researchers from various fields including oncology are finding saliva as a useful diagnostic tool (Mandel, 1990).

The role of saliva as a diagnostic tool has advanced exponentially over the past decade. The ability to measure a wide range of molecular components in saliva and compare them with serum coupled with the easy and non-invasive method of collection has made it feasible to study microbes, chemical and immunological markers. As a consequence these advances in technology have helped to move saliva beyond measuring oral health characteristics to where it may now be used to measure essential features of overall health (Streckfus & Bigler, 2003)

The scientific literature on using human saliva as a diagnostic tool began to emerge in 1960's. Early attempts at the usage of saliva as a diagnostic tool was confusing as the studies

were not precise and lacked uniformity. Over the years, identification of important constituents in the saliva, invention of more sensitive techniques for their detection aided with the description of various methods of saliva collection and the type of saliva to be collected, contributed in transforming saliva as a major diagnostic tool.

A large number of diagnostic analytes have been shown to be present in saliva including steroid hormones and HIV antibody. The past few years have seen the development of salivary diagnostic tools to monitor various oral diseases ranging from periodontal diseases, dental caries to infections and autoimmune diseases. The challenge of salivary diagnostics is to discover its potential and optimizing engineering technologies for the use of this biofluid. The challenge of making salivary diagnostics a clinical reality is in establishing the scientific foundation and clinical validations needed to position it as a highly accurate and feasible technology that can achieve definite point of care assessment of health and diseases states (Wong, 2006).

The various conditions where saliva has been used as a diagnostic tool includes autoimmune diseases like Sjogrens syndrome (Kalk et al., 2002; Tishler et al., 1997), head and neck cancer and cancer of other systemic sites, infectious diseases inclusive of viral, bacterial and fungal diseases, hereditary diseases like cystic fibrosis, drug and hormone monitoring and also for the diagnosis of systemic diseases like cardiovascular diseases, respiratory diseases, renal diseases and psychosomatic disorders. Of the lot, use of saliva as a diagnostic aid in oral cavity cancer is gaining immense popularity due to the close anatomic proximity of saliva to both pre-malignant and malignant neoplasms making it ideal for screening of these lesions.

Oral squamous cell carcinoma (OSCC) is one of the most common epithelial malignancies with significance morbidity and mortality. Inspite of diagnostic and therapeutic advances over the decades, the disease still remains a challenge for medical professionals with the five year survival rate being 30%-50% (Li et al., 2004). Recent observations indicate that the clinical and histological appearance of oral mucosa may not truly depict the damage occurring at the genetic level. This phenotypic and genotypic disparity may account in part for the failure to establish effective screening and surveillance protocols based on traditional clinical and microscopic examination (Li et al., 2004). Carcinogenesis is multistep process involving initiation, promotion and progression and evidence indicates that these are driven by accumulation of specific gene alterations (Sun, 1990). An understanding of the molecular mechanisms involved in OSCC is helpful in providing a more complete picture of the ways in which tumor arise and advance and a rationale for novel strategies of cancer detection. The oral cavity is particularly conducive to such strategies, given the ease with which saliva and exfoliated cells can be collected (Westra & Califano, 2004).

Tumor cells inhabit or produce biochemical substances referred to as tumor markers. These can be normal endogenous products that are produced at a greater rate in cancer cells or the products of newly switched on genes that remain quiescent in the normal cells (Malati, 2007). Tumor marker may be present as intracellular substances in tissues or as released substances in circulating body fluids such as serum, urine, CSF, and saliva. Until recently, analysis for tumor markers were carried out in fluids other than saliva such as CSF, blood and urine. With recent diagnostic technological advances however, the role of saliva as a tool for diagnosis has advanced exponentially.

The source of information is largely derived from the variety of DNA's, RNA's and proteins present in the saliva. Salivary DNA represents the genetic information of the hosting human body, the oral microbiota and the infecting DNA-viruses. Salivary RNA provides information on the transcription rates of the host genes and those of oral microbiota. Salivary proteins represent genetic information and help to understand the translational regulation of the host body and the oral microbiota (Fabian et al., 2008). In addition, saliva is also useful in detection of other markers such as cell cycle markers (p16, p53 etc), growth factors (Epidermal growth factor, transforming growth factor etc), cell surface markers, oxidant and antioxidants among others.

2. Properties of saliva

Saliva is a complex hypotonic fluid composed of secretions from the major and the minor salivary glands. The major glands are the parotid, submandibular and sublingual glands which are located outside the oral cavity and the secretions are transported to the mouth through the duct system. The minor salivary glands are numerous and are scattered throughout the oral cavity. Saliva is formed in two stages, the formation of the primary saliva by acinar cells and intercalated ducts which is isotonic plasma like containing most of the organic components and all of the water secreted by salivary glands. The second stage involves the modification of the primary saliva by the removal or addition of various ions in the salivary ducts to produce a hypotonic fluid that enters the mouth (Turner & Sugiya, 2002).

A normal healthy adult produces 1-1.5liters/day of saliva composing of mixture of serous and mucinous material at a rate 0.5ml/min. Resting saliva is mainly composed of submandibular secretion while stimulated saliva is made of mainly parotid saliva. Each individual type of salivary gland secretes a characteristic type of saliva such as the secretion of serous saliva from parotid gland.

Salivary output and composition depends on the activity of the autonomic nervous system through the sympathetic and parasympathetic systems. The parasympathetic supply is through the branch of facial nerve to sublingual and submandibular gland and a branch of IXn to the parotid gland. The sympathetic supply is through the fibers arising from first and the fourth thoracic segment and relaying in the superior sympathetic ganglion (Jenkins 1978). Parasympathetic stimulation results in a high flow of saliva containing low levels of organic and inorganic compounds. Sympathetic stimulation produces a low volume of protein rich and potassium rich saliva (Chiappin et al, 2007).

Saliva is composed of 99.5% water and 0.5% solid material which is inclusive of organic and inorganic constituents. The inorganic constituents are made of sodium, potassium, chlorine, bicarbonate, magnesium, calcium, phosphate, Thiocyanate, fluoride, lead, cadmium, copper, nitrite and nitrate. Sodium, potassium and chlorine contribute to the osmolarity of saliva and their concentration give diagnostic information related to the efficiency of ductal transport system. Bicarbonates are the most important buffer present in saliva resisting changes in salivary pH. Calcium, phosphate and fluoride have anti-caries activity. Thiocyanate acts as a anti-bacterial agent (Fergusson, 1994). Nitrate estimation in saliva provides a means of monitoring nitrate uptake and may also predict the future development of carcinoma.

The organic constituents are made up of proteins which include mucins and proline rich proteins which have lubricating properties, amylase and lipase with digestive properties, proteins such as sialoperoxidase, lysozyme (Thorn et al., 1989), lactoferrins, chitinase, cystatins, histatins, defensins, salivary leukocyte proteinase inhibitors, calprotectin, peroxidase, acid phosphatase, chromogranin A, sialin, agglutinin which have anti-microbial properties (Lamkin & Oppenheim, 1993). Other proteins include statherin, mucoproteins and glycoproteins. The rest of the organic constituents is made of blood group substances, enzymes such as aldolase, β-glucoronidase, succinic dehydrogenase, kallikrein, hormones, cacrbohydrates, lipids, nitrogen containing compounds, vitamins, oral microorganisms, various gases like oxygen, nitrogen and carbon dioxide, growth factors like epidermal growth factor and cells of desquamated epithelium (Jenkins, 1978).

The components present in the saliva could either be the inherent component of the saliva itself or the metabolites transferred from the plasma. The passage of plasma components into saliva involves several processes like, ultrafilteration through gap junctions between cells of secretory units, transudation of plasma compounds into oral cavity, from crevicular fluid or directly from oral mucosa and selective transport through cellular membranes by passive diffusion of lipophilic molecules or by active transport through protein channels (Chiappin et al., 2007).

2.1 Collection and storage of saliva

Saliva can be collected under both resting and stimulated conditions. The duration of the collecting period is important because flow rates vary with time. It is therefore important to standardize the collection procedure during the whole sampling period to keep the secretion rate as constant as possible. The methods of saliva collection comprise of collection of whole saliva and collection of glandular saliva. Among these, whole saliva collection is easiest and most feasible method. Specific glandular saliva can be collected with less easy methods.

The common methods of resting-saliva collection include draining method, spitting method, suction method and swab method. Methods of stimulated saliva collection include masticatory and gustatory method. For collection of specific saliva, following methods can be used; a) for parotid saliva, saliva can be collected in a two-chambered type of suction and collection cup according to Lashley (Hu et al., 2004) and b) for submandibular saliva, saliva is collected by placing the tip of collection device at the orifice of the Wharton's duct after occluding the parotid and sublingual ducts (Chiappin et al., 2007).

Various oral fluid collector devices are currently available which aid in collection of unstimulated saliva. These include Orasure HIV 1, Uplink, Salivette, Toothette plus, BBL culture swabs, transorb wicks, oral diffusion sink and ultrafilterate saliva collector (Hansen et al., 2004).

Saliva specimens after collection should preferably be kept on ice, aliquoted and frozen as soon as possible to maintain the sample integrity. The refrigeration prevents the degradation of some molecules in saliva and when necessary, bacterial growth may also be prevented (Chiappin et al., 2007). Storage procedure and time from the collection mainly affect the analysis of the biochemical variables characterized by temperature instability and bacterial growth. Some salivary compounds can have a very short half-life so that the sample to be analyzed needs a narrow range of time after collection, other substances can

remain stable for a longer time and may be detected and quantified after sometime (Nurkka et al., 2003).

Certain approaches to store saliva in order to prevent degradation of salivary compounds include (Chiappan et al., 2007)

- Immediate storage without any processing; if analysis is to be done within 30-90min, saliva can be stored at room temperature; for analysis after 3 to 6hrs from collection, storage is to be done at $+4^0C$ and if analysis is to done after days to months after collection, storage is to be done at -20^0C or still better at -80^0C.
- Snap freezing of saliva in liquid nitrogen
- Inhibition of enzyme activity in saliva by mixing with certain enzyme inhibitors
- Addition of sodium azide to retard bacterial growth
- Addition of trifluor acetate to denature salivary enzymes that could degrade salivary compounds such as proteins and steroid hormones.

For salivary DNA analysis, since the main source is from desquamated oral mucosal cells, pre-clearing of saliva before analysis is not recommended. In appropriate buffers of DNA extraction kits, saliva can be stored at room temperature for atleast one year. However before adding such buffer, saliva should be stored on ice to prevent microbial growth and to decrease salivary DNAase activity. Saliva can also be frozen and stored at -20^0C or -80^0C before extraction or after extraction before use (Fabian et al., 2008).

For RNA analysis, saliva sample is usually centrifuged because the majority of RNAs in the centrifuged whole saliva are genuine human RNA's (Park et al., 2007). For RNA studies focusing on oral microbiota, any kind of pre-clearing of saliva should be avoided. In order to avoid destruction of salivary RNA, the use of RNAase inhibitors are recommended. With the use of inhibitors, mRNA can be stabilized for longer run even in room temperature although cooling of saliva in ice is useful. RNA can be preserved in saliva by freezing at -80^0C also (Fabian et al., 2008).

For saliva protein analysis, centrifugation and/or microfiltration can be done. Pre-cleared saliva can be stored on ice without significant protein degradation for only few hours. Addition of protease inhibitors is advantageous especially for time consuming analysis procedures. Samples can be stored for few days at -20^0C. However prolonged storage at any temperature may lead to significant protein precipitation (Fabian et al., 2008).

3. Technologies for saliva based diagnosis

The complex nature of saliva consisting of mixture of various components makes it difficult to identify specific constituents. Recently, significant inroads have been made in discovery of a range of technologies which have high specificity and sensitivity in detection of salivary components. For assessment of the diagnostic technologies, a hierarchial model exist which consist of five basic levels of analysis at which the effectiveness of any diagnostic test should be evaluated. These include, analytic (precision and accuracy), diagnostic (sensitivity and specificity), patient outcome efficacy (medical decision making), operational (predictive value and efficiency) and cost / benefit (societal efficacy). This section discusses the various technologies available for saliva based diagnosis followed by the application aspect in oral cancer.

3.1 Proteomics

The proteome represents the complete set of proteins encoded by genome and proteomics is the study of the proteome that investigates the cellular levels of all the isoforms and post-translational modifications of proteins that are encoded by the genome of the cell under a given set of circumstances. Whilst a genome is more or less static, the protein levels in a cell can change dramatically as genes get turned on and off during the cells response to its environment. The proteomic analysis can ascertain function by either looking for changes in the expression of either all or subset of proteins, or by identifying binding partners for particular proteins and seeing how their interaction is affected by biological perturbation (Crocker & Murray, 2003).

The proteomics in bodily fluids are valuable tool for diagnosis due to their high clinical potential as sources of disease markers. In principle a global analysis of the human salivary proteomes can provide a comprehensive spectrum of oral and general health. Furthermore, analysis of salivary proteomes over the course of complications may unveil morbidity signatures in the early stage and monitor disease progression (Lee & Wong, 2009). The total amount of protein in whole saliva ranges between 0.5-3mg/ml. This proteome consist of roughly 1000 protein sequences from which around 300 sequences are of human origin (Fabian et al., 2008).

Proteomic analysis involves the following stages (Crocker & Murray, 2003):

i.   Separation of proteins: Before analyzing protein expression and abundance levels, proteins have to be isolated into a purified state. This is generally done by using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). To resolve the complex composition of saliva, 2D- PAGE, allows separation not only of different molecules of similar molecular weights, but also of different modification patterns or isoforms of the same protein (Lee & Wong, 2009).

ii. Analysis of comparative expression: Once separated, it is necessary to carry out some form of analysis to assess the relative abundance of the protein present.

iii. Identification of protein species: Once a set of proteins showing differences in abundance between two or more states has been identified, mass spectrometric analysis is to be used to determine their identities. Proteins that are primarily identified by mass spectrometry (MS) can be further characterized by ionization methods such as electrospray ionization (ESI) and matrix assisted laser desorption ionization (MALDI) (Lee & Wong, 2003).

iv. Confirmatory experiments.

Two main methods are used to resolve the protein mixture and then to visualize the individual components in such a way that their relative abundance can be quantified. The two methods are i) 2D gel electrophoresis followed by a variety of in-gel staining methods and ii) liquid chromatographic separation with subsequent UV &/or mass spectrometric detection.

In 2D-PAGE, the proteins are initially separated by isoelectric focusing in the first dimension according to charge and then by SDS-PAGE in the second dimension according to size. This type of separation has the capacity to resolve complex protein mixtures, thus permitting analysis of hundreds of proteins at a time. For visualization, staining methods like

Coomassie staining, silver staining or Sypro post- electrophoretic fluorescent stains can be used.

The non-gel based methods are used to overcome the problems associated with 2D-PAGE such as the difficulty in detecting low abundance proteins particularly integral membrane proteins. Two dimensional high performance liquid chromatography (2D-HPLC) is recently employed which uses a capillary HPLC column with a strong cation exchange matrix at the front end of the column which is followed by a reverse phase packing at the back end. Their approach involves the tryptic digestion of soluble and insoluble protein fractions of the entire yeast proteome, followed by the application of total tryptic peptides from the two fractions onto the strong cationic exchange matrix at the top of the column. A salt step gradient is then used to displace a fraction of the peptides onto the reverse phase packing. Displaced peptides are then eluted into the mass spectrometer using a solvent gradient which generates fragmented data that are used for automated searches against protein databases and identification. This procedure is than repeated in steps, each time using an increasing amount of salt to release further peptides from cation exchange to the reverse phase packing.

Mass spectrometry is a powerful technique used for the identification of proteins from two dimensional cells. The ionization methods such as MALDI and ESI were developed by Karas & Hillenkomp (1988) and Fenn et al (1989) respectively. The combination of either of the above mass spectrometric techniques with the separation of proteins by 2D-PAGE is an established method of proteome analysis. In both the cases identification takes place at the peptide level and is therefore necessary to convert proteins in the excised gel pieces into peptides which can be extracted for analysis. Peptides can also be analyzed by MALDI to produce peptide mass fingerprints which are then matched against protein databases in order to identify corresponding proteins. It is also useful to identify which peptides in a tryptic digest have undergone post-translational modification like phosphorylation and glycosylation that are mediated by kinases and glycosyl transferases. An alternative mass spectrometric based method for protein identification is nanoelectrospray. This utilizes very fine glass capillaries with gold coated tips in order to produce a very fine spray which subsequently enables extremely low levels of protein digest mixtures to be analyzed at flow rated of approximately 20-50nl/min.

3.2 Transcriptomics

Salivary transcriptome diagnostics constitutes a novel clinical approach where a large panel of human RNAs can be readily detected in saliva. The large panel of human mRNA is determined by the use of microarray technology and after profiling, validation of transcriptome biomarkers will be done by quantitative real time PCR. Sometimes multiplex reverse transcriptase PCR are used to overcome problems with quantitative real time PCR (Lee & Wong, 2009).

Microarray technologies have the advantage of simultaneously detecting and quantifying the expression of large number of genes health and disease. The technology involves the use of robotic automated miniaturized microscopic spots of aliquots of cDNAs or oligonucleotides from specific genes in a standardized high density gridded arrangement on glass (Muelker & Young, 2004).

The steps involved in microarray analysis consist of RNA extraction, sample labeling, hybridization and processing, image capture analysis and data analysis. RNA is first extracted from tissues or cells of interest. The quality of RNA extracted is paramount to the overall success of the microarray experiment as impurities in the sample can affect both the probe labeling efficiency and also stability of the fluorescent label. Further extraction of mRNA from the total RNA can be done resulting in a purer starting material (Crocker & Murray, 2003).

The mRNA is transcribed in vitro with the concomitant inclusion of labeled nucleotides. The labels may be fluorescent or radioactive. After labeling, the cDNA is purified (to remove unincorporated nucleotides), mixed with a hybridization buffer and then applied to a cDNA microarray slide. The sample and the slide are heated prior to hybridization in order to separate double stranded DNA. A coverslip is applied or preferably a hybridization chamber is used to avoid evaporation and enable even hybridization. The hybridization and subsequent wash steps are carried out at a buffer stringency and temperature that enables hybridization of complementary strands of DNA but reduces non-specific binding. After hybridization, the microarray slides are scanned using either a laser or a phosphorimager. The images are analyzed using software that measures the intensity of the signal from the hybridized spotted genes which provide a measurement of the amount of cDNA bound. Thus, the initial concentration of mRNA is inferred. After the profiling of RNA by microarray, quantitative polymerase chain reaction is used to validate a subset of differently expressed transcripts that are identified by microarray analysis (Crocker & Murray, 2003).

3.3 Polymerase chain reaction (PCR)

PCR is a simple in-vitro method for amplification of specific short segments of DNA or cDNA reverse transcribed from RNA. The technique greatly simplifies genetic analysis and permits the study of all types of clinical samples. Oligonucleotide primers binding to the flanking regions of target sequence are used to initiate specific copying of DNA strands by DNA polymerase. The requirement for the reactions are template DNA to be studied, short single strand DNA primers, complementary to opposite strands of the flanking regions of the fragment of interest, the four nucleotide triphosphatases, a thermostable DNA polymerase and an appropriate buffer solution (Crocker & Murray, 2003).

PCR involves three major steps which are denaturation at 94-96^{0}C for 30 seconds, annealing at 50-60^{0}C for 30 seconds and extension at 70-72^{0}C for one minute. The procedure begins with isolation of DNA from the sample. Heating separates complementary double stranded DNA into single strand forms intended to act as a template dictating nucleotide sequence in vitro. Two short oligonucleotides primers are designed to anneal to the template and flank the region of interest. A thermostable DNA polymerase known as Taq polymerase catalyzes the sequential addition of the four nucleotides to the primers. Cooling the solution permits the primers to bind to template DNA and then Taq polymerase catalyzes the addition of dNTPs to the template between the primers. Salt and buffers permit the Taq polymerase to catalyze the reaction. The reaction is repeated for around 35 to 40 cycles (Jordan et al., 2001).

Reverse transcriptase-PCR (RT-PCR) is a modification of PCR which is important in transcriptomics as it permits analysis of mRNA and thus the study of gene transcription and easier analysis of multiple exons. RNA is extracted and mRNA reverse transcribed using

reverse transcriptase. The standard PCR is then carried out after generation of cDNA to amplify the desired region. Quantitative PCR (qPCR) is used for accurate quantification. The generated PCR products can be monitored during the reaction process using fluorescent labeled probes (Crocker & Murray, 2003).

The visualization of amplification is done by agarose gel electrophoresis, southern blot, dot blot and reverse blot assays. The simplest method is agarose gel or polyacrylamide gel electrophoresis followed by ethidium bromide staining and viewing under UV illumination. The choice depends in the product size and the amount of resolution required (Crocker & Murray, 2003).

3.4 Genomics

Genomic analysis is one of the recent advances in the diagnosis of oral cancer and considerable research is being performed in this field. With the availability of high thoroughput technologies to harness genetic information from various sources like blood, saliva, etc., their usage has advanced exponentially. Stable cell free circulating DNA in plasma was first observed almost 60yrs ago. Plasma DNA were shown to exhibit tumor specific characteristics such as somatic mutations in tumor suppressor genes or oncogenes, microsatellite alterations, abnormal protein methylation, mitochondrial DNA mutations and presence of tumor related viral RNA (Zimmermann et al., 2007). These DNA related changes were also found in saliva, the identification of which helps a great deal in diagnosis of oral cancer. The techniques that are available for detection of genetic changes are discussed below.

3.4.1 Hybridization methods

Hybridization refers to the pairing of complementary DNA or RNA strands to produce double stranded nucleic acids. This method uses a radio-labeled or fluorescence labeled DNA or RNA probe that binds to the target molecule of interest, permitting visualization. The target nucleic acids can either be immobilized in a membrane (blotting) or examined in tissue sections (in situ) (Jordan et al., 2001).

Blotting technique involves the isolation of cell free mixture containing the biomolecules of interest, resolving the mixture into its component parts, transfer of the component parts onto a suitable membrane and detection of the biomolecules of interest. The blots are named according to the type of molecule that is blotted on the membrane i.e. DNA, RNA or protein as Southern, Northern and Western blotting respectively (Crocker & Murray, 2003).

The southern blot technique is used to detect specific sequences within the mixture of DNA; this was first described by Southern in 1975. The DNA is size fractioned by gel electrophoresis and then transferred by capillary action to a membrane. The technique involves the transfer or blotting of DNA fragments onto a membrane. DNA is first enzymatically cleaved into smaller pieces by restricted endonucleases, then size separated by gel electrophoresis. After fragment separation, the DNA is transferred from the gel to nylon or nitrocellulose membrane through capillary action of a buffer as it is absorbed by blotting paper. Then the DNA is bound to the membrane by baking the membrane in a vacuum oven or by ultra-violet light cross linking. Finally specific DNA fragments can be

identified by hybridizing the membrane with labeled complementary DNA probes followed by detection of label by autoradiography or chemiluminescence (Jordan et al., 2001).

The northern blot analysis is used for RNA detection wherein RNA as a target molecule are size separated by agar gel electrophoresis, transferred to nylon or nitrocellulose membrane and hybridized with specific probes (Jordan et al., 2001). However, precautions should be taken to prevent RNA degradation that can be caused by the presence of ribonucleases (Crocker & Murray, 2003).

Western blotting detects antigenic determinants on protein molecules using polyclonal or monoclonal antibodies and often is described as immunoblotting. The first step involves solubilization of protein samples using sodium dodecyl sulphate and reducing agents like dithiothreitol or 2-mercaptoethanol. Individual proteins are then resolved by SDS polyacrylamide gel electrophoresis prior to electrophoretic transfer. Following blotting, conjugation with antibodies is performed and markers are visualized by autoradiography or chemiluminescence. The molecular weight of proteins is determined by comparison with a set of molecular weight markers which are co-electrophoresed (Crocker & Murray, 2003).

3.4.2 Flow cytometry

Flow cytometry is an important method used to analyze cell kinetics and protein expression in normal and tumor cells (Jordan et al., 2001). It is routinely used to measure DNA content of cells using dyes whose fluorescence is enhanced by binding to nucleic acids and accurately reflecting DNA content (Crocker & Murray, 2003). The dye most commonly used is propidium iodide. It can be excited by the blue line (488nm) of an argon ion laser, fitted into flow cytometers. When bound to double stranded nucleic acids, the dye fluoresces red. Other dyes that can be used are ethidium bromide, 7-aminoactinomycin, DRAQ5, Acridine orange etc. The labeled cells are then directed in a single file along a charged column through a laser beam which excites the fluorescent dye bound to the cell. The fluorescent emission from the excited cells are then collected by a fluorescence detector and analyzed. Cell size can also be detected by using data from the forward scatter of the excitation laser passing through the stream of single cells (Jordan et al., 2001).
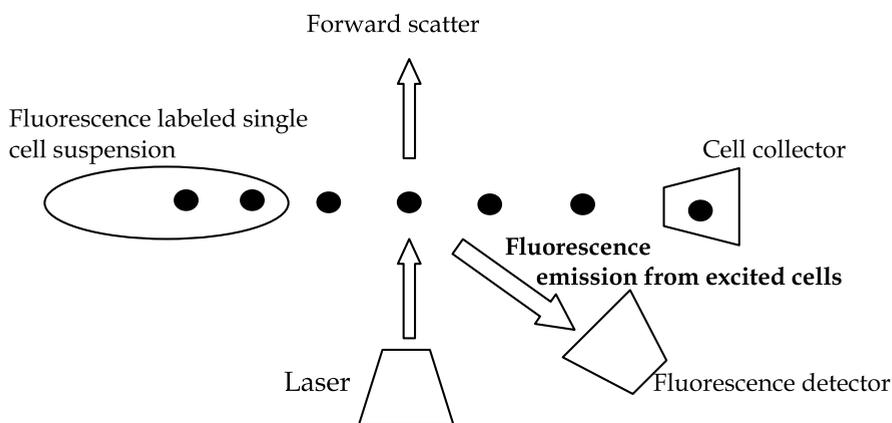
Diagrammatic representation of flow cytometric analysis

3.5 Point of care diagnostics

In recent years, the concept and practice of Point of Care Testing (POCT) is gathering growing interests and remains an important discovery of the present century. POCT is advantageous over standard laboratory procedures by providing timely information to medical teams, facilitating rational, time critical decisions and has been demonstrated to improve patient outcomes in critical care settings. POCT facilitates personalized medicine allowing the caregiver to customize the therapy according to the patient needs. It reduces turnaround time for results which avoids the patients not following up with their caregiver after a test. POCT avoids some of the cost associated with sample handling, packaging, tracking and shipping to centralized labs and reduces the likelihood of samples being contaminated, mixed up, lost &/or degraded. POCT assays must be highly automated to minimize error, sample contamination and sample degradation. Early POCT were based on lateral flow (LF) assays. Recently microfluidics has been known to offer greater functionality and sophisticated flow control than lateral flow assays. The field is often referred to as micro-total analysis or lab on a chip (LOC). POCT microfludic devices are classified as instrumented or un-instrumented devices. Instrumented devices consist of a disposable cassette accompanied by a portable, durable analyzer. Un-instrumented devices are a reincarnation of the LF strips with added capabilities and sophistication. POCT can be used for detection of proteins and nucleic acids. For immunoassay test for small molecules and proteins, enzyme linked immunosorbent assay (ELISA) is the gold standard which shows high specificity and sensitivity. They utilize the high specificity of antibody binding to their target antigen ligand. The most ubiquitous point of care immunoassay is the LF strip also known as immunochromatographic strip. Another assay that is employed at point of care is based on microbeads. Microbeads with various functionalization are ubiquitous solid supports for capturing target molecules in both bench top and microfluidic system (Hart et al., 2011).

In 1999, The NIDCR initiated a research workshop aimed at applications of microfluidics and micro/nanoelectromechanical system (MEMS/NEMS) to saliva based diagnostics. MEMS/NEMS is an integrated system that consists of a central unit for processing data and several other components that connect with the outside interface like microsensors. In continuation of development of saliva diagnostic technologies, in 2002, the NIDCR funded seven projects that explored different point of care systems to detect salivary analytes and provide an overall profile that correlated with a particular disease states. Electrochemical sensing, on chip PCR/RTPCR, microsphere based nano-biochip, microsphere based optical fiber assay, high thoroughput DNA microarray, surface plasmon resonance optical system and microchip electrophoretic immunoassay (Lee & Wong, 2009).

Point of care microfluidic systems are being developed for diagnosis of oral and breast cancers. They permit concurrent detection of multiple salivary analytes including protein and nucleic acid. Oral fluid nanosensor test (OFNASET) is a handheld, automated, easy to use integrated system that will enable simultaneous and rapid detection of multiple salivary proteins and nucleic acids (Wong, 2006).

4. Salivary tumor markers in oral cancer

4.1 Protein markers

Protein biomarkers in saliva are being analyzed both individually and as a panel of markers to aid in early detection of oral cancer and in implementing appropriate therapeutic regime.

Hu S et al (2008) tried to discover and validate differentially expressed proteins in saliva of oral squamous cell carcinoma (OSCC) patients which could serve as potential markers in its detection. The study was performed using subtractive proteomics approach to profile salivary proteins from oral cancer and matched healthy subjects followed by validation by immunoassay to identify a co-panel of candidate protein biomarkers for OSCC detection. 461 and 438 non-redundant proteins were identified in OSCC and control groups respectively. Of these, 409 proteins were found in both the groups while the rest were specific for individual groups. Among the large number of proteins, many of them were differentially expressed as reflected by the differential number of mass spectrometric analysis. Proteins like MRP14 which is a calcium binding protein were significantly overexpressed in OSCC group. Polymeric immunoglobulin receptor (PIGR) was significantly downregulated in saliva of OSCC patients.

The study showed up-regulation of 12 markers in OSCC group. Among these, 5 showed significant difference which included M2BP, MRP14, CD59, catalase and prolifin. Some proteins which were downregulated include clusterin (protein involved in apoptosis) that was absent in OSCC and present in control group. MRP14 expression which is also a calcium binding protein was found to be increased in OSCC group. MRP14 was show to have increased expression in another study performed in tongue cancer by He QY et al (2004). CD 59 (protectin) which was overexpressed in OSCC group enables the tumor cells to escape from complement dependent and antibody mediated killing (Ravindranath & Shuler, 2007). Profilin 1, another upregulated protein is a regulator of microfilament system and is involved in various signaling pathways via interaction with cytoplasmic and nuclear ligands. It may be secreted into tumor microenvironments during the early progressive stage of tumor formation. Catalase protects the cell against oxidative stress and altered levels of catalase are involved in carcinogenesis and tumor progression (Hu et al., 2008).

Protein	Functions
M2BP	Tumor antigen
MRP14	Calcium binding protein
CD 59	Enables the tumor cells to escape from complement dependent and antibody mediated killing
Prifilin 1	Regulator of microfilament system and is involved in various signaling pathways
Catalase	Protects against oxidative stress

Table 1. Overexpression of proteins in OSCC and their functional role.

Analysis of salivary epithelial markers was performed in a study which included CA125, CA19-9, tissue polypeptide antigen (TPA), carcino-embryonic antigen (CEA), SCC and Cyfra 21-1. The study found that the salivary concentration of all these markers was increased in cancer patients than in normal controls. However concentration of SCC, CA19-9 and CEA showed statistically significant differences while the others did not (Nagler et al., 2006).

Contucci et al., (2005) analyzed statherin levels in oral pre-cancer and cancer and found decreased statherin levels compared to healthy controls. Statherin possess high affinity for calcium and phosphorus minerals and provides protective effect which can delay and decrease the level of proliferation induced by carcinogen and reduction of this protective

effect is associated with an increased penetration of environmental carcinogens through the mucosal surface.

Cyfra 21-1 is a soluble fragment of cytokeratin 19, which is a component of cytoskeletal protein with a molecular weight of 40kDa. Cleavage of CK19 through caspases 3 activity releases cyfra21-1 into the extracellular space. A study evaluated salivary cyfra21-1 levels in OSCC using ELISA kits and compared with normal and found significantly increased levels in OSCC group. The study also found that the pre-operative saliva Cyfra 21-1 levels were significantly higher in patients suffering from tumor recurrence than in patients without recurrence. The study highlights the usefulness of Cyfra 21-1 for tumor detection and predicting recurrence. It has been postulated that increased Cyfra 21-1 could be due to increased CK19 expression in tissues (Ping et al., 2007).

Salivary soluble CD44 levels were evaluated in head and neck squamous cell carcinoma compared with controls (Franzmann et al., 2005). CD44 are adhesion molecules expressed on normal T-lymphocytes and is used by these cells to migrate to selective sites in the lymphoid tissue. Such migration is accompanied by binding of CD44 to hyaluronate on high endothelial venules and overexpression of this molecule may favor metastatic spread (Kumar et al., 2005). These isoforms arise from alterative splicing of a variable exon present in CD44 mRNA. Franzmann et al., (2005) performed the study using ELISA and western blotting and demonstrated increased value in head and neck squamous cell carcinoma than in controls, suggesting their usage in early detection of cancer.

P53 gene is lovated on chromosome 17p and functions as a critical gatekeeper against development of cancer. They also function as critical modulator of the cellular response to exogenous and endogenous stress. The main functional activity of p53 protein is cell cycle arrest and initiation of apoptosis in response to DNA damage. In a study by Liao et al., (2000), mutation of p53 was observed in DNA extracted from saliva of OSCC patients suggesting a potential use as biomarker for oral cancer detection. The study concentrated on p53 exon 4 codon 63 mutations which was significantly higher.

Hyaluronic acid (HA) is a non-sulfated glycosaminoglycan made of repeating disaccharide units, D-glucoronic acid and N-acetyl- D- glucosamine responsible for regulation of cell adhesion, migration and proliferation. They support metastasis by promoting tumor cell migration offering protection against immune surveillance and causing a partial loss of contact mediated inhibition of cell growth and migration. Hyaluronidase is an endoglycosidase that degrades HA into small angiogenic HA fragments. HAase is shown to alter the expression of CD44 isoforms which may be involved in tumor progression (Franznmann et al., 2003). Franznmann et al., (2003) in their study showed statistically significant rise in HA and HAase in OSCC patients than normal controls suggesting their role as potential marker for diagnosis and prognosis of head and neck SCC.

Balicki et al., (2005) estimated EGF levels in saliva of oral cancer and compared with controls and found significantly decreased EGF levels in OSCC patients. It was suggested that the impaired ability to heal oral mucosal damage in neoplastic diseases may be related to low EGF concentration in saliva. The weaker mitogenic effect due to decreased EGF concentration reduces the reconstructive potential of oral mucus membrane epithelium in patients suffering from oral cavity cancer.

Analysis of interleukin 6 & 8 in OSCC patients revealed significantly higher values than in normal controls. Increased interleukin 6 has been shown to promote immune unresponsiveness and induction of wasting, cachexia and hypercalcemia. Interleukin 8 plays a role in stimulation of angiogenesis, proliferation and chemotaxis of granulocytes and mecrophages which are prominent constituents in the stroma of OSCC (St. John et al., 2004).

Estimation of salivary sialic acid as a tumor marker was considered as aberrant glycosylation are universal features of cancer. A study performed to evaluate salivary sialic acid, total protein and total sugar in OSCC patients found significantly increased levels in OSCC patients than in healthy controls (Sanjay et al., 2008, Debensteen et al., 1991). Another protein evaluated was statherin and results have shown increased levels in OSCC group than in normal controls (Contucci et al., 2005).

Free radical generation result in production of reactive oxygen species which at high levels are known to cause DNA based alteration, strand breaks, damaged tumor suppressor genes and enhanced expression of proto-oncogenes. Numerous anti-oxidants exist in the body that protects from the damaging effects of reactive oxygen species and reactive nitrogen species. Any imbalance in oxidant-antioxidant balance contributes to cancer development and hence determination of salivary oxidant and antioxidant levels may be helpful in early detection, treatment planning and prevention of tumor recurrence.

Salivary nitrites are produced by tobacco and fungal organisms which are of special importance in carcinogenesis as they form nitrosamines. Studies have shown that salivary DNA and proteins to be profoundly oxidized with increased salivary reactive nitrogen species while all salivary antioxidants were significantly reduced (Bahar et al., 2007). Studies by Rai et al., (2008) showed significantly elevated levels of malondialdehyde in pre-cancer and cancer.

In addition to the use of saliva in oral cancer detection, evidence support the usage of saliva in diagnosis of cancer from other sites such owing to the fact that many markers which are identified in other cancers can be isolated from saliva. Markers such as C-erbB2 and CA15-3 for breast cancer, CA125 for ovarian cancer, kallikrein and epidermal growth factor for breast cancer have been evaluated and found to be very useful in the detection of distant tumors (Streckfus & Bigler, 2005)

4.2 Genomic markers

Tumor specific genomic markers consisting of DNA and RNA markers can be identified in saliva for detection of oral cancer considering that the initiation and progression of malignant tumors is driven by the accumulation of specific genetic alterations. DNA shows tumor specific characteristics such as somatic mutations in tumor suppressor genes and p53, microsatellite alteration, abnormal promoter methylation, mitochondrial DNA mutations and presence of tumor related viral DNA. In addition transcript levels of mRNA, microRNA levels are also considered as diagnostic markers for oral cancer.

Microsatellite DNA consist of tandem repeats of one to six nucleotides scattered throughout the genome. DNA microsatellites are highly polymorphic and vary between every individual. These microsatellite sequences are fixed for an individual and same in every tissue. Any error in mismatch repair, creates microsatellite instability which has been

successfully used as molecular markers for analysis of tumorigenesis in head and neck cancer (Kumar et al, 2004). Loss of heterozygosity (LOH) is a term used to describe a condition where the cell becomes homozygous to a mutant allele resulting in cancer development. LOH can occur through mechanisms such as loss of a chromosome through mitotic non-disjunction, deletion on the chromosome carrying the corresponding allele and cross over between two homologous genes leading to homozygosity for the mutant allele (Muelker & Young, 2004).

Studies using microsatellite markers from different chromosomal arms in head and neck SCC have shown alterations at certain regions on chromosome 3p, 9p, 17p & 18q to be associated with development of tumors (El- Naggar et al, 2001). El- Naggar et al., (2001) evaluated salivary samples to analyze DNA and showed highest incidence of microsatellite LOH of chromosome 9p, 3p &17p.

Methylation is the main epigenetic modification in humans and changes in methylation pattern plays a main role in tumorigenesis. Epigenesis is the alteration of gene activity without change in their structure (Kumar et al., 2004). Certain tumor suppressor genes are inactivated by hypermethylation of promoter sequences without a change in DNA base sequence resulting in loss of expression.

p16INK4A also known as CDKN2A is a cyclin dependent kinase inhibitor which is critical in cyclin D-Rb pathway for maintaining Rb protein in its active, non-phosphorylated state. It blocks CDK4 binding to cyclin thus helping in cell cycle regulation. Any mutation of p16INK4A occurring in tumors result in inability to block cyclin D-CDK4 activity and preventing Rb phosphorylation during cell cycle. O-methylguanine-DNA-methyltransferase (MGMT) is DNA repair gene for guanosine methyl adduct; death associated protein kinase (DAP-K) is a novel serine/ threonine kinase whose expression is required for interferon induced apoptosis which functions as a potential metastasis inhibitor (Rosas et al., 2001).The study by Rosas et al., (2001) determined the promoter hypermethylation of the above three markers in saliva of patients with head and neck SCC and normal controls and found that 56% of the cases exhibited promoter hypermethylation in atleast one of the genes. Among the cases, 47% exhibited hypermethylation at p16, 33% at DAP-K and 23% at MGMT. The authors suggested that these alterations can be used for early detection of cancer and also for detection of natural progression from neoplasia to cancer in at risk population.

Righini et al., (2007) tried to define a gene methylation profile in head and neck SCC tumors and saliva for diagnosis and follow up by analyzing eleven genes. They found that six methylated genes were most frequently found which included tissue inhibitors of metalloproteinase (TIMP 40%), ECAD (36%), MGMT (29%), p16(29%), DAPK (27%) & RASSF1A (20%). It was suggested that aberrant methylation of these 6 genes in exfoliated malignant cells of the saliva reflects tumor status. TIMP3 encodes for metalloproteinase inhibitor that suppresses tumor growth, angiogenesis, invasion and metastasis.

Detection of telomerase activity in saliva of OSCC was performed by Zhong et al., (2005) and they detected telomerase positivity in 75% of cases suggesting that telomerase detection could be used as assistant marker in OSCC. Telomerase is a ribonucleoprotein aid to elongate repeat sequence at the end of the chromosomes. Reactivation of telomerase is considered to be a pre-requisite for development of malignant tumor cells from somatic cells.

Adenosine deaminase (ADA) and 5′- nucleotidase (5′-NT) are important enzymes participating in purine and DNA metabolisms. Defect in ADA results in intracellular accumulation of adenosine and deoxyadenosine which are toxic to living cells. Deoxyadenosine causes dATP accumulation which is strong inhibitor of ribonucleotide reductase and cause some aberrations in DNA synthesis. They also interfere with critical methylation dependent process such as synthesis, maturation or function of DNA. Saracoglu et al., (2005) found decreased ADA in oral cancer patients and suggested that it may be a compensatory mechanism against rapid purine and DNA metabolism in cancer cells. Decreased ADA activities cause higher dATP and AMP concentration in the cell, thereby lowering dATP and dAMP ratio and leading to decreased energy production. This may be a limiting factor against rapid proliferation of cancer cells.

In a study conducted by Yang et al., (2004), microarray analysis of salivary transcriptome showed that there are 1679 genes exhibiting different expression levels between cancer patients and controls. Of these, seventeen cancer related biomarkers showed significant elevation (3.5 fold) and quantitative PCR was performed to validate these findings. Nine markers were then selected based on their reported cancer association (DUSP 1, GADD45B, H3F3A, IL1B, IL8, OAZ1, RGS2, S100P & SAT). The results confirmed that seven of these nine markers showed significant elevation in saliva of OSCC patients. The validated seven genes could be classified in three ranks by the magnitude of increase into highly upregulated mRNA (IL-8), moderately upregulated mRNA (H3F3A, IL1B, S100P) and low upregulated mRNA (DUSP1, OAZ1 &SAT).

Gene symbol	Gene name	Functions
DUSP-1	Dual specificity phosphatase 1	Protein modification, signal transduction, oxidative stress
H3F3A	H3 histone, family 3A	DNA binding activity
IL1 β	Interleukin-1β	Signal transduction, proliferation, inflammation, apoptosis
IL 8	Interleukin-8	Angiogenesis, replication, Ca++ mediated signaling pathway, cell adhesion, chemotaxis, cell cycle arrest, immune response
OAZ-1	Ornithine decarboxylase antizyme-1	Polyamine synthesis
S-100P	S-100 Ca++ binding protein P	Protein binding, Ca++ ion binding
SAT	Spermidine / Spermine N1-acetyl transferase	Enzyme, transferase activity.

Table 2. Salivary mRNA showing significant changes in oral cancer with their functions.

MicroRNAs (miRNA) are a group of small RNAs, 19-25 nucleotides in length, involved in the regulation of development and cell differentiation, proliferation and survival. They exert their effects by two mechanisms; mRNA degradation and inhibition of translation. A single miRNA is capable of regulating the translation of a multitude of genes by targeting specific

regions in their mRNA transcript. As a single miRNA can regulate hundreds of genes and may act as a master regulator of processes; select subsets of miRNAs can be used as biomarkers of physiological and pathological states (Micheal et al., 2010).

Analysis of miRNA from saliva of OSCC patients was performed by Park et al., (2009). Of the total number of miRNA analyzed, four potential miRNA were identified as being present in statistically significant levels between the two groups. These included miR-200a, miR-125a, miR-142-3p & miR-93. Further analysis of these miRNA in a separate set of OSCC and normal patients suggested that miR-200a and miR-125a were present in significantly lower levels in OSCC than in healthy controls. These findings suggested that the detection of miRNA in saliva can be used as a non-invasive and rapid diagnostic tool for oral cancer detection. These were considered as the third diagnostic alphabet in saliva (Park et al., 2009).

4.3 Salivary microbiota

A multitude of microorganisms thrive in the oral cavity interacting with each other and at times causing clinical diseases. Oral cavity plays host to a wide array of microorganisms inclusive of variety of bacteria, viruses and fungi. This natural microflora is essential for the normal development of host physiology and contributes to host defenses by excluding exogenous microorganisms. Certain alterations in diet, medications, habits and host immune status may lead to overgrowth of minor components of oral microflora which can predispose the site to disease. Organisms such as candida act as opportunistic pathogens contributing to various oral diseases. The role of bacteria in oral cancer is currently being investigated to determine if the role is a causal or it is a co-incidental finding.

Kang et al., (2009) evaluated the salivary levels of group of organisms (cariogenic, periodontopathic and fungal) and demonstrated significant increase in the levels of *P. Gingivalis, T. Forsythia* & *C. albicans* in cancer group than in normal controls. The study also found that the prevalence of *S. sobrinus* in healthy group was significantly lower than in healthy head and neck tumors.

Mager et al., (2005) determined salivary counts of forty common oral bacteria in OSCC and normal controls using DNA hybridization techniques. They found significantly elevated levels of *P. gingivalis, P. melaninogenica* & *S.mitis* in saliva of OSCC patients thereby suggesting the role of salivary microbiota as a diagnostic indicator in OSCC.

Candida species are normal commensals of the oral cavity and evidence suggest their involvement in oral cancer development primarily through nitrosation of nitrosobenzene compounds (Krogh, 1990). Studies have found increased candidal carriage in salivary samples of OSCC group than in normal controls (Kang et al., 2009). This indicates that the salivary analysis of candida species might be useful as diagnostic and prognostic indicator of oral pre-cancer and cancer.

5. Conclusion

Saliva has been considered as the mirror image of blood for a long time and its various components acts as a mirror of body's health. Since the ancient times, saliva has been used for detection of various diseases ranging from autoimmune diseases to infections and cancer among others. The identification of many new components along with the introduction of

newer technologies has led researchers to believe that saliva could be used as an attractive tool. Salivary analysis is advantageous due to the easy and non-invasive method of collection, safety and the possibility of repeated collection without discomfort to the patient. However, saliva analysis was not considered to be effective owing to the inability to isolate minor components as a result of technological barriers. Emerging technologies with high sensitivity and specificity developed in the recent past have overcome these problems giving a new dimension to the use of saliva as a diagnostic tool.

Saliva is normally composed of many organic and inorganic constituents and identification of these proteins in disease states were considered useful from a diagnostic perspective. Salivary proteins, nucleic acids and microbiota have been studied in oral cancer with an aim to identify specific components that could serve as a useful marker for cancer detection as well as treatment planning. Advances like proteomics which is used to detect a panel of proteins, transcriptomics for DNA and RNA and genomics for determination of genetic damage are helpful for characterization of disease states. With point of care diagnostics being the requirement of the day salivary analysis could become a routine procedure for cancer detection.

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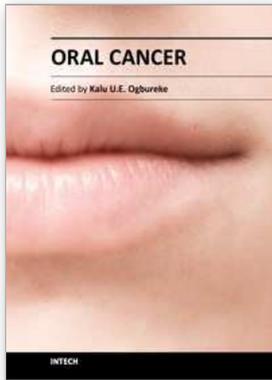
Oral Cancer

Edited by Dr. Kalu U. E. Ogbureke

Oral cancer is a significant public health challenge globally. Although the oral cavity is easily accessible, early diagnosis remains slow compared to the enhanced detection of cancers of the breast, colon, prostate, and melanoma. As a result, the mortality rate from oral cancer for the past four decades has remained high at over 50% in spite of advances in treatment modalities. This contrasts with considerable decrease in mortality rates for cancers of the breast, colon, prostate, and melanoma during the same period. This book attempts to provide a reference-friendly update on the etiologic/risk factors, current clinical diagnostic tools, management philosophies, molecular biomarkers, and progression indicators of oral cancer.

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