Salivary Biomarkers

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Abstract: Salivary biomarkers, a non-invasive alternative to serum and tissue-based biomarkers may be an effective modality for early diagnosis, prognosis and monitoring of post therapy status. At present, various technologies provide opportunity for high-throughput approaches to proteomics; which have been used to evaluate altered expressions of gene and protein targets in saliva of oral cancer patients. The emerging field of saliva based biomarkers has great potential to prove its clinical significance in combating disease. This review summarizes the importance of several salivary proteomic biomarkers for various diseases.

Keywords: Human Saliva, Biomarker, tobacco, oral cancer.

1. INTRODUCTION:

Worldwide, tobacco-use continues to be one of the leading causes of preventable death and has been estimated to kill more than five million people annually[1]. Lately, the epidemic of tobacco-use has shifted from developed to developing countries[2]. It is estimated that by 2030 almost 10 million people will die from tobacco-use per year, with 70% of these deaths occurring in developing countries. India accounts for one-sixth of the tobacco-related illnesses worldwide and is estimated to face an exponential increase in tobacco-related mortality from 1.4% of all deaths in 1990 to 13.3% in 2020[3]. In 2010, out of 52.8 million deaths that occurred worldwide out of which 34.5 million deaths were attributable to non communicable diseases; more than a quarter of these occur in low income and middle income countries[4-5]. Use of tobacco is one of the major risk factor for non-communicable disease which is slowly threatening human life[5].

Tobacco smoking in any form constitutes a major risk factor for coronary artery disease (CAD), hypertension (HTN), chronic obstructive pulmonary disease (COPD), oral, nasopharyngeal, bronchial and other visceral malignancies[6] . Smoking 1-4 cigarettes per day significantly increases the risk of cardiovascular disease[7]. Smoking also increases the risk of thrombosis[8] (8). Smokers are 3 times more likely to develop type 2 diabetes than the non smokers. Smokeless tobacco users have a higher incidence of diabetes and smokeless tobacco (SLT) has been associated with insulin resistance in people with diabetes. Smokers also have difficulty in controlling their blood glucose levels because insulin resistance is increased by smoking. Tobacco use is associated with various core components of metabolic syndrome that include a constellation of abdominal obesity, atherogenic dyslipidemia, raised blood pressure, insulin resistance (with and without glucose intolerance), pro-inflammatory state, and pro-thrombotic state[9]. According to the Inter heart study, tobacco use is one of the most important causes of acute myocardial infarction (AMI) especially in males. All forms of tobacco use, including different types of smoking and chewing tobacco and inhalation of second hand smokes are known to cause AMI[10].

The oral cavity is the first organ to be exposed to tobacco. Tobacco alters normal homeostasis of the oral cavity, including the saliva's antioxidant and other protective systems. This may lead to oral inflammatory diseases like type II diabetes mellitus, cardiovascular disorder and oral cancers[11-14]. Early

tumorigenic activities have been detected in normal oral mucosa of heavy smokers who have no overt precancerous or cancerous lesions[15]. The mucosal changes in smokers may also arise from the drying effects of the mucosa, high intraoral temperatures, intraoral pH changes, local alteration of membrane barriers and immune responses, or altered resistance to bacteria, fungal and viral infections. Tobacco-related cell damage may leave molecular footprints in the saliva, offering the potential for non-invasive early diagnosis of tobacco-related oral diseases.

Changes in saliva quality and quantity are indicative of the wellness of the patient [16]. Human saliva serves as the mirror of oral and systemic health and provides valuable information. Its especially proteins that can serve as a biomarker for the unique physiologic aspects of periodontal and systemic diseases. Compared to blood, saliva has been clinically shown to produce more accurate results and is relatively inexpensive and convenient. The diagnostic potential of saliva has been exploited in many laboratories due to its advantages over other biological fluids to identify potential biomarkers for many diseases. Unlike plasma, saliva can be readily analysed as it will not clot. Its non-invasive approach renders it an effective alternate to blood for monitoring patient's health condition[17].

Human saliva is plasma ultra-filtrate and contains proteins either synthesized *in situ* in the salivary glands or derived from blood. Thus it contains biomarkers derived from serum, gingival crevicular fluid, and mucosal transudate. To date, researchers have identified 2,340 proteins in the salivary proteome, of which 20– 30% are also present in blood[18], an encouraging indicator of clinical utility of saliva as a diagnostic fluid. In contrast to the plasma proteome, in which 99% of total protein content is contributed by 22 highly abundant proteins, the 20 most abundant proteins in whole saliva constitute only 40% of total protein content[19]. This composition suggests that detecting biomolecules of clinical importance with sensitivity and specificity would be more practicable and easier in saliva than in blood. How molecules of blood transport in saliva may also be important for successful use of saliva as diagnostic fluid. Lipophilic molecules such as steroid hormones passively diffuse into saliva, while water and electrolytes pass through the pores of acinar cells. Various peptides in blood move through protein channels, and large proteins are transported via pinocytosis[20].

2. ISSUES AND CHALLENGES IN SALIVARY BIOMARKER DISCOVERY:

Saliva obtained by passive drool, a widely used procedure of sample collection, may present high viscosity which makes its handling in laboratory difficult. To overcome this drawback, saliva collection devices such as Salivettes[21-22]became very popular, as these are easy to handle and after centrifugation, the resulting saliva presents a lower contribution of mucins, being consequently less viscous and allowing a better sample processing. Topkas et al[23] evaluated the effect of different saliva collection devices on the composition of this fluid by immunoassay of C-reactive protein, myoglobin and IgE and detected significant differences in analyte levels based on the collection method and device's material type. In addition, significant differences in the salivary flow rates were also observed depending on the saliva collection method. Although the most appropriate saliva sample collection method, according to our experience, is passive drool, in special cases such in case of xerostemia, special collection devices are needed for saliva collection for proteome analysis[24].

Besides issues concerning saliva collections, variables such as gender, age[25-28], diet[27-30], circadian rhythm[31], inter-individual variability[32-33] and sample stability[34-37]might influence the result of proteomic analysis. Several reports have appeared in the last decade that have addressed the standardsation of protocols for saliva collection and processing. Since saliva contains microorganisms and proteases which may impact sample stability/protein degradation, careful control of temperature during saliva collection and sample storage is crucial.

It is recommended that saliva collection should be performed on ice with the addition of protease inhibitor cocktail[36]. Xiao and Wong[33] also proposed the addition of ethanol to stabilize the salivary proteome without significant degradation at room temperature, for a maximum period of 2 weeks. Nevertheless, ir should be noted that higher levels of salivary peptides-derived fragments can be produced with increased sample freezing rate independently of donor nutritional status as observed by De Jong et al.[38] Furthermore, nutritional status as well as circadian rhythm influences protein expression as observed by Quintana et al.[32] Thus, it is recommended that saliva samples should always be collected at the same time of the day to reduce the effect of circadian rhythm, and at least 2 h after eating, with a previous mouth wash.

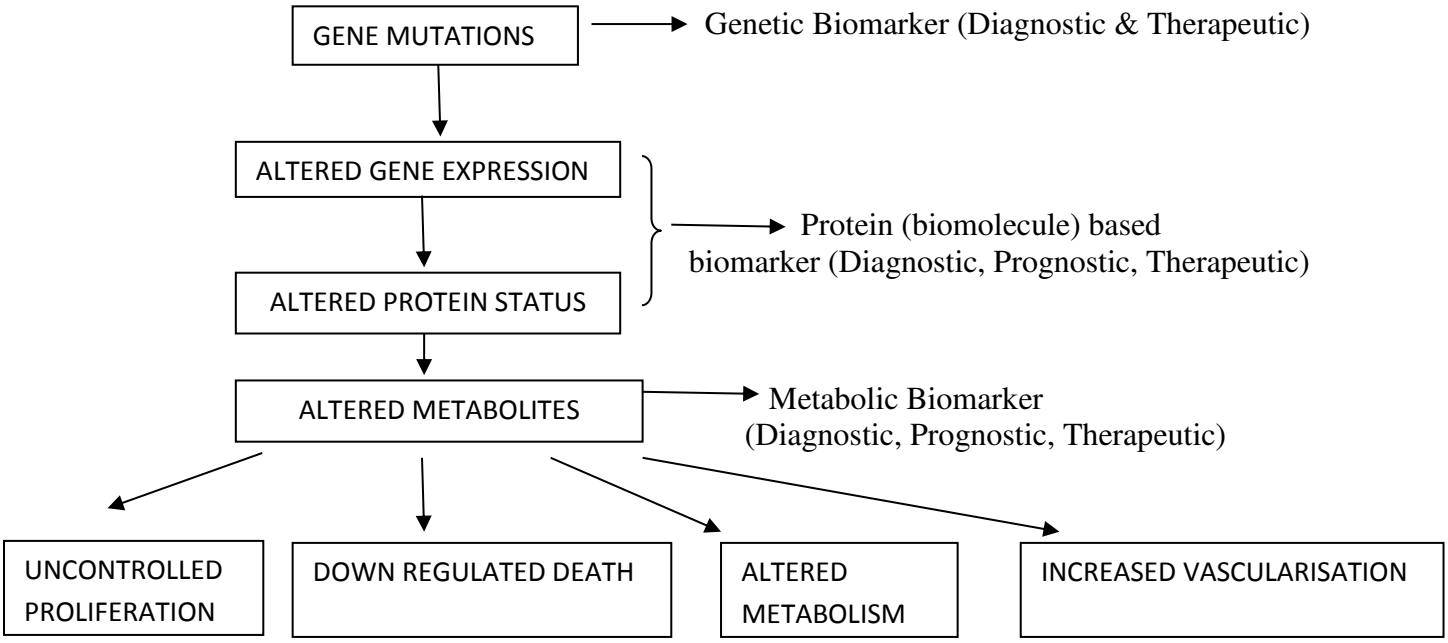

Fig: Opportunities for Biomarker (Bhatt AN, Mathur R, Farooque A, Verma A, Dwarakanath BS. Cancer biomarkers - current perspectives. Indian J Med Res 2010;132:129-49.)

3. POTENTIAL SALIVARY BIOMARKERS:

A number of markers have been identified in saliva that can be of clinical significance. Some of the known compound with strong potential for use as biomarker are listed in Table I,II and III:

I. Potential salivary biomarkers for oral cancer detection, reported as of 2016. [101]			
Category	**Potential OSCC salivary biomarkers**	**Techniques employed in studying**	**Authors/year**
Non-organic compound	Na, Ca, F, and Mg	Flame Photometry, atomic absorption, spectrophotometry	Shpitzer et al./2007[39]
Peptide	Defensin-1	High performance liquid chromatography	Mizukawa et al./1998[40]
Proteins	P53 autoantibody	Enzyme linked immunosorbent assay	Warnakulasuriya et al./2000[41]

	α-amylase	Matrix assisted laser desorption/ionization time o flight mass spectrometry (MALDI-TOF MS)	Chen et al./2002[42]
	IL-8	Enzyme linked immunosorbent assay	St. John et al./2004[43]
		-do-	Rhodus et al./2005 [44]
		-do-	Arellano-Garcia et al./2008[45]
		-do-	Brinkmann et al./2011[46]
		-do-	Elashoff et al./2012[47]
	TNF-α	Enzyme linked immunosorbent assay	Rhodus et al./2005 [48]
	IL-1	-do-	
	IL-6	-do-	Katakura et al./2007[49]
		-do-	Saheb-Jamee et al./2008[50]
		-do-	Sato et al./2010[51]
		-do-	
		Enzyme linked immunosorbent assay	Cheng et al./2013[52]
	Basic fibroblast growth factor	Enzyme linked immunosorbent assay	Vucicevic et al./2005[53]
		-do-	
	Statherin	Enzyme linked immunosorbent assay	Gorugantula et al./2012[54]
		High performance liquid chromatography	Contucci et al./2005[55]
	Cyfra 21.1	Radio Immuno assay	Nagler et al./2006[56]
		-do-	
		-do-	
	Tissue polypeptide antigen (TPA)	-do-	
	Cancer antigen 125 (CA125)	Radio immunoassay	Nagler et al./2006 [56]
		-do-	
		-do-	Balan et al./2012[57]
	Endothelin-1	Enzyme linked immunosorbent assay	Pickering et al./2007 [58]
		-do-	Cheng et al./2011[59]
		-do-	
	IL-1β	-do-	Katakura et al./2007[60]
		-do-	
		-do-	Brinkmann et al./2011[46]
		-do-	Elashoff et al./2012 [47]
	CD44	Enzyme linked immunosorbent assay	Franzmann et al./2007[61]
	Total salivary protein	-do-	Shpitzer et al./2007 [39]
		-do-	
	Insulin growth factor 1 (IGF-1)	-do-	
		-do-	
		-do-	
	MMP-2	-do-	
		-do-	
	MMP-9	Enzyme linked immunosorbent assay	
		-do-	
		-do-	

	CD59	-do-	Hu et al./2008[62]
		-do-	
	Catalase	-do-	
		-do-	
	Profilin	-do-	
		-do-	
	S100A9/MRP14	-do-	
		-do-	
	M2BP	-do-	Hu et al./2008[62]
		-do-	
		-do-	Brinkmann et al./2011[46]
		-do-	Elashoff et al./2012[47]
	Carcinoembryonic antigen (CEA)	Enzyme linked immunosorbent assay	He et al./2009[63]
		-do-	
	Carcinoma associated antigen CA-50	-do-	
		-do-	
	Salivary carbonyls	-do-	Shipitzer et al./2009[39]
		-do-	
	Cyclin D1	-do-	
		-do-	
	Maspin	-do-	
		-do-	
	8-oxoguanine DNA glycosylase (OGG1)	-do-	
	Phosphorylated-Src	-do-	
		-do-	
		-do-	
	Ki-67	-do-	
	Lactate dehydrogenase	Colorimetric (mostly commercially available) assays	Shipitzer et al./2009[39]
		-do-	
		-do-	Shetty et al./2012[64]
		-do-	
	Transferrin	Colorimetric (mostly commercially available) assays	Jou et al./2010[45]
	Zinc finger protein 501 peptide	Matrix assisted laser desorption/ionization time o flight mass spectrometry (MALDI-TOF MS)	Jou et al./2011[65]
	Hemopexin	Two dimensional gel electrophoresis followed by LC tandem MS	Jessie et al./2013[66]
		-do-	
	Haptoglobin	-do-	
		-do-	
	Complement C3	-do-	
		-do-	
	Transthyretin	-do-	
		-do-	
	α1-antitrypsin	-do-	
		-do-	
		-do-	
	Thredoxin	-do-	
		-do-	Hu et al./2008[62]

DNAs	p53 gene codon 63	Polymerase chain reaction	Liao et al./2000[63]
	Loss of heterozygosity in the combination of markers D3S1234, D9S156, and D17S799	Polymerase chain reaction	El-Naggar et al./2001[68]
	Mitochondrial DNAs (cytochrome c oxidase I and cytochrome c oxidase II)	Polymerase chain reaction	Jiang et al./2005[69]
	Hypermethylation of promoters in tumor suppressor genes: DAPK, DCC, MINT-31, TIMP-31, TIMP-3, p16, MGMT, CCNA1	-do-	Carvalho et al./2011[70]
mRNAs	IL-8	Microarray followed byqPCR	Li et al./2004[71]
		-do-	Brinkmann et al./2011[46]
		-do-	Elashoff et al./2012 [47]
		-do-	
		-do-	
	IL-1β	Microarray followed byqPCR	Li et al./2004[71]
		-do-	Elashoff et al./2012[47]
	DUSP1 (dual specificity phosphatase 1)	Microarray followed byqPCR	Li et al./2004[71]
		-do-	Elashoff et al./2012[47]
		-do-	Cheng et al./2013[72]
	H3F3A (H3 histone family 3A)	Microarray followed byqPCR	Li et al./2004[71]
		-do-	Elashoff et al./2012[47]
	OAZ1 (ornithin decarboxylase antizyme 1)	Microarray followed byqPCR	Li et al./2004[71]
		-do-	Elashoff et al./2012[47]
		-do-	Cheng et al./2013[59]
	S100P (S100 calcium binding protein P)	Microarray followed byqPCR	Li et al./2004[71]
		-do-	Brinkmann et al./2011[46]
		-do-	Elashoff et al./2012[47]
		-do-	Cheng et al./2013[59]
		-do-	
	SAT (spermidine/spermine N1-acetyltransferase EST)	qPCR	Li et al./2004[71]
		-do-	Brinkmann et al./2011[46]
		-do-	Elashoff et al./2012[47]
MicroRNAs	miR-125a	qPCR	Park et al./2009[73]
	miR-200a	-do-	
	miR-31	-do-	Liu et al./2012[74]
Long non-coding RNAs	HOTAIR	qPCR	Tang et al./2013[75]
		-do-	
		-do-	
Oxidative stress-related molecules	Reactive nitrogen species (RNS) such as nitric oxide (NO), nitrites (NO2) and nitrates (NO3)	Colorimetric (mostly commercially available) assays	Bahar et al./2007[76]

	Peroxidase	Colorimetric (mostly commercially available) assays	
	Glutathione S-transferase (GST)	Colorimetric (mostly commercially available) assays	
	Superoxide dismutase (SOD)	-do-	Agha-Hosseini et al./2012[77]
	8-hydroxy-2-deoxyguanosine (8-OHdG)	-do-	
	Glutathione	HPLC	Almadori et al./2007[78]
	Malondialdehyde (MDA)	Colorimetric (mostly commercially available) assays	Agha-Hosseini et al./2012[77]
Glucocorticoid	Cortisol	Colorimetric (mostly commercially available) assays	Bernabé et al./2012[79]
Metabolomics	Cadaverine, alpha-aminobutyric acid, alanine, C5H14N5, piperidine, taurine piperideine, pipercolic acid, C4H9N, C8H9N, pyrroline hydroxycarboxylic acid, betaine, C6H6N2O2, leucine+isoleucine, tyrosine, histidine, tryptophan, beta-alanine, glutamic acid, threonine, serine, glutamine, choline, carnitine, C4H5N2O11P	HPLC	Sugimoto et al./2010[80]
	Phenylalanine	-do-	Wei et al./2011[81]
		-do-	Sugimoto et al./2010[80]
	Valine	Capillary electrophoresis TOF MS	Wei et al./2011[81]
		-do-	Sugimoto et al./2010[80]
	Lactic acid	HPLC with quadrupole/TOF MS	Wei et al./2011[81]
Glycosylation-related molecules	Sialic acid	Colorimetric (mostly commercially available) assays	Vajaria et al./2013 [82]
	α-L-fucosidase	-do-	
Other	Telomerase activity	Colorimetric (mostly commercially available) assays	Zhong et al./2005[83]

II.Potential salivary biomarkers for Cardiovascular Disease, reported as of 2016			
Category	Potential salivary biomarker	Techniques used in studying	Author
Proteins/ Inflamatory markers and enymes	C-reactive protein (CRP), myoglobin (MYO), creatinine kinase myocardial band (CK-MB), cardiac troponins (cTn), and myeloperoxidase,	Spectrophotometry, enzymatic assays	Floriano et al/2009[84].
	CRP, CK-MB, sCD40 ligand	ELISA, Flow Cytometry	Miller CS et al/2014[85]
	Irisin, increased Troponin-I, CK, CK-MB	Kit Based	Aydin S et al/2014[86]
		-do-	
	Ischemia-modified albumin	Colorimetric	Toker A etal/2013[87]
	cTnI*	Kit Based	Mirzaii-Dizgah et al 2013[88]

	CK-MB	-do-	
	CK	-do-	
	polymorphonuclear leukocyte MMP-8	HPLC, ELISA, RIA, 2 D Electrophoresis,LC-MS, MALDI-TOF MS.	Buduneli E etal/2011[89]
	CRP, MMP-9, IL-1β, sICAM-1, MPO, adiponectin, monocyte chemoattractant protein 1, GRO-α, decreased TNF-α, sCD40 ligand, IL-6		Floriano PN,2009[90]
	NT pro BNP, GDF-15, Cys C	Spectrophotometry	Rathnayake etal/2014[91]

III. Potential salivary biomarkers for Type 2 Diabetes Mellitus reported as of 2016			
Category	Potential salivary biomarker	Techniques used in studying	Author
Inflamatory marker	TNF-α, IL6, Acylated ghrelin, Deacylated ghrelin, Resistin ,Visfatin	Flow cytometry, ELISA, Kit based technique.	Mythili et al,2015[93]
	TNF-alpha, INF gama, IL 2, IL6,1L8,CIP,MIP1 alpha, MIP1 beta, MMP1,MMP2,MMP 9, TIMP2,Pro CT.	ELISA	Nikolaos etal/2014[94]
Proteins	Chromogranin A		Martini etal/2010[95]
	anhydrase (↑384), Glycogen-phosphorylase	Multidimensional liquid chromatography , LCMS	Rao et al 2009[92]
	A1AT, CysC, A2MG,TTR,RBP,FABP,Complement C6, Carbonic Anhydrase, Glycogenphosphorylase		
	Alpha 2 macroglobulin	ELISA	Juan Pablo etal/2015[97]

III. Potential salivary biomarkers for lung cancer reported as of 2016			
Category	Potential salivary biomarker	Techniques used in studying	Author
Genetic	EGFR, BRAF, CCNI, FGF19, FRS2, GREB1, LZTS1	PCR, Microarray	Zhang L, Xiao H, Zhou H, et al.[98]

IV. Potential salivary biomarkers for pancreatic cancer reported as of 2016			
Category	Potential salivary biomarker	Techniques used in studying	Author
Genetic	KRAS,	PCR, Microarray	Zhang L, Farrell JJ et al.[99]
	MBD3L2,		
	ACRV1,		
	DPM1		

V. Potential salivary biomarkers for Breast cancer reported as of 2016			
Category	Potential salivary biomarker	Techniques used in studying	Author
Proteins	c-erbB-2 ,	ELISA, CLIA	
	CA 15.3		Bigler LR, Streckfus CF et al.[100]

V. Potential salivary biomarkers for Tobacco user reported as of 2016			
Category	Potential salivary biomarker	Techniques used in studying	Author
Metabolomic	Thiocyanate	Colorimetric (mostly commercially available) assays	Fawaz Pullishery et al 2015[102]
	Uric acid		
	Cotinine	ELISA	C. Nuca et al/2012 [96]
	Cortisol	-do-	Nao Suzuk/2016 et al [103]
Inflammatory / Protein	SIgA	-do-	"
	(IL)-1β	-do-	"
	IL-6	-do-	"
	TNF-α	-do-	"

4. CONCLUSION:

The saliva research field is a rapidly evolving and advancing field due to the use of novel approaches including metabolomics, genomics, proteomics and bioinformatics. Implication of saliva as a diagnostic tool for various diseases has proved that saliva contains more clinically useful information than serum, apart from its functional importance. Due to its proximity to oral cavity and non-invasive collection procedure, salivary screening may probably be the best choice as primary screening test for oral cancer. The systematic analysis of salivary genomics and proteomic biomarkers facilitates the identification of sensitive and specific parameters for oral cancer that may aid in effective screening to identify patients with high risk. It may also help in designing better treatment modalities thus improving the survival of oral cancer patients. Collectively, the promising field of salivary genomic and proteomic biomarker analysis may strengthen and transform the field of oral cancer diagnosis. This would enable clinicians to monitor patients' saliva for diagnosis and prognostication of oral cancer. It will thus advance the clinical efforts to overcome the severity of the disease. However, there may be certain cultural and behavioral perceptions against using saliva; these barriers are needed to be overcome with time. Further, enormous efforts from researchers and clinicians are essential to turn salivary diagnostics into clinical and commercial reality to combat oral cancer.

Overall, the identified biomarkers and their expression demonstrate the potential use of a combination of significant biomarkers to structure a more complete diagnostic tool. The potential exists for combinations of identified biomarker expression, or the correlation of biomarker expression and clinical assessments, to be utilized to achieve effective disease diagnosis. The proteomic profiling of specific disorders viz oral cancer, type 2 diabetes mellitus and cardiovascular disorder in smokers and smokeless tobacco users has not been ventured in detail. As the salivary proteome significantly changes much in these diseases, the differentially expressed proteins may be used as early biomarkers to indicate risks of tobacco-related diseases.

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