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Salivary Proteomics in Early Diagnosis of Oral Cancer-A Review

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Abstract

Oral cancer is a predominant health issue in India affecting large number of population, with tobacco as one of the main preventable etiological factor for it. Early diagnosis is essential factor in lessening the burden of disease and molecular non-invasive (Salivary-based) diagnostics is considered as the up trending approach recently. Saliva is collected from individuals either from single or multiple glands and is generally stimulated or non-stimulated type. Saliva sample is prepared and fractionated to identify the proteome sequence using any of the advanced techniques.

Keywords: Oral cancer; salivary proteomics; early diagnosis

Introduction

Oral Squamous Cell Carcinoma (OSCC) in India has a high incidence, i.e. 20 per 1 Lakh population, 5-year survival rate (35%) in India, and therefore qualify as a major public health problem, not only in India but also globally [1,2]. OSCC is a tobacco-induced multi-step process resulting from an interdependent series of genetic, proteins and biochemical alterations rather than a single decisive event [3]. All these molecular responses are the signs that could be identified as biomarkers for various purposes. The proteomic era is also called as post-genomic, as they have interactive relationships as a result of biological responses to different reactions in the body [4]. The Saliva-based collection, in comparison with the blood-based collection, has a few clinical advantages like it is non-invasive, easy handling and preparation, easily transportable, cost-effective, safe and effective, requires no highly trained personnel and can be performed easily and readily and requires no expensive tools [5].

Salivary proteomics

The salivary proteomics signifies the comprehensive spectrum of proteins for both general and oral health [6]. These proteins are produced as a result of cellular changes which present as isoforms and post-translational forms either in the cells itself or in saliva as comparative low levels. The ascertained levels of proteins usually express completely or in part and are generally dependent on the network binding components [7]. The protein content of the whole saliva is generally derived from the three

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major paired salivary glands, which comprise the contralateral major (parotid, submandibular and sublingual) and minor salivary glands [8].

To further the proteomics, both quantitatively and qualitatively could reveal the morbidity or mortality monograms that are very potent early diagnostics or progression or monitoring paradigm in medicine practice [9]. The salivary proteins range from 0.5 mg/ml to 3.0 mg/ml, with at least 300 sequences of human origin [10].

Salivary proteomics mandates a carefully designed saliva sample collection/pretreatment protocol [11]. Commonly used profiling method includes 2D gel electrophoresis or liquid chromatography, followed by tryptic digestion, LC-MS/MS, and database search to identify the selected protein targets of interest [12]. Western blotting or any other proteomic approaches can be used to quantify protein levels for validation purpose [13].

Collection of saliva samples and storage kinetics for proteomic assessment could be categorized into the following factors

Saliva collection may include draining, spitting, suction or swab methods without any universal protocol per se [5]. The salivary proteome levels indicate both the physiological condition and also the pathological variations [8]. The oral fluid collector devices for the collection of unstimulated saliva includes Orasure HIV 1, Uplink, Salivette, Toothette plus, BBL culture swabs, transorb wicks, oral diffusion sink and ultra-filtrate saliva collector [14].

Physiological factors affecting saliva collection: This includes the flow rate, circadian rhythm, whether is whole saliva or gland specific, stimulated or unstimulated, type of stimulus used prior-in case of stimulated saliva, abstain from eating, drinking, smoking or using oral hygiene products [8], physiological conditions or status and the methods of collection involved [5]. These procedures hold good for health and diseased individuals. The saliva collection from oral cancer patients must be biopsy confirmed and adherent to the standard protocol [15].

Patient-related factors: All the patients must sign a mandatory informed consent, prior to the procedure [15]. Saliva is collected from healthy individuals up to one hour of abstinence from any kind of oral activities like food intake. Some studies also indicated brushing of teeth or chewing of paraffin wax, so as to facilitate salivary production. Although the saliva collection is indicated to be within 12 pm ideally 9 am to 10 am is recommended [8]. Saliva is collected onto the ice tray or small transportation case. A Lashley cup could be used for gland specific saliva collection [5]. The saliva collected must be centrifuged almost immediately and aliquoted and frozen [14].

Clinician/researcher related factors: Salivary proteomic analysis is based on predominant three principles:

A. Protein separation
B. Analysis of comparative expression of proteins/peptides
C. Identification of proteins/peptides

It is very essential that the patients be made aware of the research perspectives, pre-collection instructions, and etiquettes during the collection of saliva. The biochemical instability of the salivary proteins mandates immediate assaying due to its perishable properties.

Storage criteria are to be followed [14]:

- If the analysis is done within an hour, the saliva can be stored at room temperature
- If the analysis is scheduled for up to 6 hours, the saliva can be stored at 4°C
- if the analysis is postponed from a few days to months, it is recommended that the saliva be stored at either -20°C or still better at -80°C

The sample has to be centrifuged to remove the macromolecules at 14,000 to 15,000 rpm after thawing to room temperature [16] **(TABLE 1).**

TABLE 1. **Different saliva collection methods.**

Different saliva collection methods	
Type of saliva collected	**Method and type of collection device**
Whole Saliva (WS)	Patients should refrain from eating, drinking, and any of oral hygiene/ Dental treatment procedures for at least 1 h before saliva collection (Optimum collection time is 8 am to 10 am), Before collection, perform a 1 min oral rinse with distilled water and then after 5 min collects 5 ml of saliva, The collected sample must be processed in the laboratory within 1 hour
Unstimulated Whole Saliva (USWS [7]	**Passive drooling**: In this method restrict oral movement and drain saliva from the lower lip into a plastic vial.
	Spitting method:
	A thorough clinical examination is conducted
	Subjects are asked to rinse their mouth with distilled water prior to sample collection
	Volunteers are then asked to generate saliva and spit into a wide-mouthed sterile sample collection bottle for the duration of 5 min to 10 min and generally, 2 ml to 5 ml is collected from each subject
	The collection containers should be placed into ice buckets immediately. Samples are then to be transferred to Eppendorf tubes and centrifuged twice at 5000 rpm for the duration of 10 min to remove any macromolecules and to remove exfoliated cells from the salivary sample.
	The supernatant of the saliva is distributed into 3 to 5 aliquots, for further storage at -80°C for short-term storage
	This method has the disadvantage of containing 14 times more bacterial load, compared to the drooling technique and hence is less recommended [17]
Stimulated Whole Saliva (SWS) [16]	For the stimulation of glands, chewing different things like natural gum, a piece of paraffin wax, citric acid, and powdered drink crystals have been used. Stimulated saliva is frequently obtained after paraffin mastication, or after sour taste stimulation.
Parotid Gland	a) For parotid saliva, saliva can be collected in a two-chambered type of suction and collection cup according to Lashley [18] and The method introduced by Carlson and Crittenden. In this method, a double-chambered metallic cup with two outlet tubes is used. One end holds the cup in place using vacuum suction. The second half acts as a collection vehicle for saliva. Specimen collection can be enhanced by smearing citric acid (10%; 1 mL) on the dorsum of the tongue every 30 s. Discard the first 1.5 ml of saliva prior to sample collection.
Submandibular gland [16]	For submandibular saliva, saliva is collected by placing the tip of the collection device at the orifice of the Wharton's duct after occluding the parotid and sublingual ducts
Minor glands	Kutscher used capillary tubes for collecting saliva from Minor Glands Salimetrics Oral Swab (SOS) [20]. Minor glands located at the everted surface of the lower lips.

[16]	

Materials and Methods

Methods for protein extraction and purification

The samples are thawed to 40°C and then centrifuged to remove macromolecules including cell debris. Proteins would be precipitated overnight at -20°C by addition of TCA in acetone to the supernatant [16].

Protein extraction methods [19]:

1. Electrophoresis
2. High-performance liquid chromatography

According to Lamy et al. [19], Electrophoresis is frequently used for salivary protein separation:

1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)
2. PAGE in non-denaturing conditions
3. Isoelectric focusing
4. Two-dimensional electrophoresis (2-DE)
5. Capillary electrophoresis (CE) and
6. Free flow electrophoresis has all been used in saliva studies, with different purposes

According to Doustjalali et al. [20], Sample fractionation is done with the first-dimension Isoelectric Focusing (IEF) by using the PROTEAN IEF system (Bio-Rad Laboratories, USA). The second dimension separation could be carried out at 16°C on 12.5% SDS slab gels using 2-DE system (Bio-Rad Laboratories, USA), with the IPG strips sealed on the top of the gels with 0.5% agarose. SDS-PAGE run for 40 mA/gel at 50V for the first 30 minutes.

The Methodology Proteomic techniques such as:

1. Two-dimensional electrophoresis (2-DE)
2. 2D-liquid chromatography/mass spectrometry (2D-LC/MS)
3. Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF/MS) [16] and
4. Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF/MS), have been used in saliva studies [8]

The commonly identified proteins could be enlisted as follows **(TABLES 2 and 3):**

TABLE 2. **A brief summary of different salivary proteomics estimated in different studies.**

SL. No.	Salivary protein	Description	Method	Number	Concentration	p-value
1	Total sialic acid	Protein-bound monosaccharide-glycoprotein	Skoza and Mohos, ninhydrin method	Cases (OSCC)-40	102.12 ± 15.38	0
				Controls-20	40.941 ± 5.772	
2	Total proteins	Defensin-1 and statherin	Biuret method, lowry's method	Cases-40	1.68 ± 0.19	0.322

				Controls-20	1.63 ± 0.15	
3	Total sugars	Glycoconjugates	Phenol- sulphuric acid	Cases-40	2.497 ± 0.55	0.311
				Control-20	2.63 ± 0.3339	
4	MMP1	Matrix metalloprotease	LC-Multiple reaction monitoring technique	Control- 76	0.9 ng/ml	<0.001
				Cases-113	76.7 ng/ml	
5	MMP3	Matrix metalloprotease	LC-Multiple reaction monitoring technique	Control-170	3.6 ng/ml	<0.001
				Cases-125	15.9 ng/ml	
6	MMP 9	Metalloprotease that participates in cancer pathogenesis as they degrade type IV collagen.	LC-Multiple reaction monitoring technique	Control-180	28.9 ng/ml	<0.001
				Cases-138	93.8 ng/ml	
7	KNG1	(Kininogen 1)	LC-Multiple reaction monitoring technique	Controls-197	107.3 ng/ml	<0.001
				Cases-131	586.3 ng/ml	
8	ANXA2	(Annexin) A2	LC-Multiple reaction monitoring technique	Control-187	12.4 ng/ml	<0.001
				Cases-131	63.7 ng/ml	
9	Soluble CD44	Adhesion molecule released in soluble form by protease	Sandwich type ELISA	Controls-84	9.31 (14.85)	<0.001
				Cases-102	24.44 (32.01)	

TABLE 3. **The salivary proteomics-significant approach used for its detection.**

SL No.	**Salivary protein**	**Description**	**Method of detection**
1.	M2BP (Mac 2 binding protein)	Tumor antigen was found significantly unregulated in nasopharyngeal carcinoma.	Subtractive proteomics refers to direct profiling of proteins expressed in samples from two cellular or pathologic states using multidimensional LC separation and data-dependent MS/MS analysis.
2.	MRP14 myeloid-related protein 14[2]	Tissue cells of oral tongue cancer	
3.	CD 59	Compliment restriction factors that are overexpressed on tumor cells and they enable tumor cells to escape from compliment-dependent and antibody-mediated killing.	
4.	Profiling	Regulator of the microfilament system and is involved in various signaling pathways via interactions with cytoplasmic and nuclear ligands	
5.	Catalase	Protects the cell against oxidative stress and altered levels of catalase are evident in many human tumors and are fundamentally involved in carcinogenesis and tumor progression.	

Conclusion

Salivary proteomics is a valuable tool for early diagnosis of Oral cancer due to their high clinical potential. The identification of these biomarkers, however, requires further validation in a larger population. These biomarkers as a result of biological variations not only emphasize the physiological conditions but also the pathological conditions at a very early stage so that it could be prevented. This indeed is very cost-effective.

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