Published in final edited form as:

Rheum Dis Clin North Am. 2016 August ; 42(3): 449–456. doi:10.1016/j.rdc.2016.03.004.

The proteomics of saliva in Sjögren's syndrome

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Synopsis

One of the main characteristics of primary Sjögren's syndrome (pSS) is chronic dysfunction and destruction of the salivary and lacrimal glands; hence, their secretory biofluids should reflect the glandular biological status. Saliva is a heterogeneous biofluid comprised of bio-molecules and omics constituents are altered in response to various diseases including pSS. In recent years, scientific effort has been undertaken for the purpose of evaluating saliva proteome to diagnose, monitor or prognosticate pSS. This review article overviews the recent advances in the field of salivary proteomics in the context of pSS, highlighting the most significant and promising findings. Determining saliva as a credible means of early disease detection could lead to translational advantages and significant clinical opportunities for pSS.

Keywords

Sjögren's syndrome; saliva; proteomics; biomarkers; saliva diagnostics

I. Introduction

Primary Sjögren's syndrome (pSS) is characterized by chronic dysfunction and destruction of exocrine glands, particularly the salivary and lacrimal glands, leading to persistent dryness of the mouth and eyes⁽¹⁾. Due to the direct involvement of those glands in the pathophysiology of the disease, saliva and tears are thought to reflect the glandular dysfunction and destruction. As a matter of fact, saliva and tears drain the main targets of autoimmune response, which in the case of pSS are the salivary and lacrimal glands.

Saliva presents obvious advantages in terms of accessibility and unstimulated secretion, hence, ease of collection. While primarily considered an indispensable element of early digestion, saliva is a heterogeneous biofluid comprised of bio-molecules and omics constituents, which may become altered in response to various diseases including pSS. The

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Disclosure David Wong is co-founder of RNameTRIX Inc., a molecular diagnostic company. He holds equity in RNameTRIX, and serves as a company Director and Scientific Advisor. The University of California also holds equity in RNameTRIX. Intellectual property that David Wong invented and which was patented by the University of California has been licensed to RNameTRIX.

advancements of high throughput technologies and analytical techniques have made saliva proteomics an ideal tool to study the underlying pSS glandular autoimmune exocrinopathy. In recent years, scientific effort has been undertaken for the purpose of evaluating saliva proteome to diagnose, monitor or prognosticate pSS. The results from these studies are encouraging, thus, saliva has been emerged as a novel and promising biofluid for the discovery of definitive disease-specific biomarkers for pSS with potential clinical and translational impact^(2; 3).

We begin our discussion by exploring the production, secretion and composition of saliva. Next, we delve into the proteomics of saliva in the context of pSS by describing a number of studies conducted from different groups including ours. Last, we address potential pitfalls and highlight promising topics for the near future of saliva-proteome research in pSS.

II. Saliva: production and secretion

Saliva is produced by several salivary glands located around the oral cavity including the parotid, submandibular, sublingual, minor salivary glands, and posterior deep lingual glands (von Ebner's glands). Salivary glands are comprised of clustered acinar cells called acini, which concertedly produce about 500 to 1500 ml of saliva daily⁽⁴⁾.

Two categories of acinar cells are found in the salivary glands: (1) serous cells (most commonly found in the parotid gland), which secrete a non-viscous watery product, and (2) mucous cells (predominant in the sublingual gland), which secrete a mucous-like product of high viscosity. The solution produced by these cells contains electrolytes, mucins and enzymes, which subsequently flow into collecting tubes, where their composition can be further altered by the reabsorption of specific molecules before release into the mouth as saliva.

III. Composition

Saliva is a clear, slightly acidic, hypotonic fluid, which is continuously secreted and is predominantly composed of water (99.5%). The remaining 0.5% is comprised of inorganic ions, including sodium, chloride, potassium and calcium along with organic components, such as proteins, amino acids, antibodies, hormones, enzymes, lipids and cytokines, among many others⁽⁵⁾. In addition, recent studies have shown that saliva actually contains a variety of genomic, transcriptomic, proteomic, microbiologic and immunologic analytes^(6; 7; 8; 9) that may be capable of identifying both local and systemic disorders in afflicted individuals. Therefore, saliva is now the focal point of multiple investigations aimed at establishing oral fluids as the preferred diagnostic medium.

In 2004 the National Institute of Dental and Craniofacial Research (NIDCR) provided funding to three research groups comprising the Saliva Proteome Consortium, in an effort to identify and catalogue the human saliva proteome including saliva proteins as well as their structurally modified forms (e.g., glycosylated and phosphorylated). These studies revealed the salivary proteome as a sizeable collection of up to 1166 protein molecules - 914 in parotid and 917 in submandibular/sublingual saliva⁽¹⁰⁾. The majority of these proteins are synthesized and secreted into the oral cavity by the acinar cells of the salivary glands. In

consideration, a high proportion of proteins that are found in plasma and/or tears are also present in saliva along with unique components^(8; 10; 11; 12). The proteins identified are involved in numerous molecular processes ranging from structural functions to enzymatic and catalytic activities, with the majority of them been mapped to the extracellular and secretory compartments.

IV. Function

Saliva plays a key role in maintaining the oral health and homeostasis, by lubricating and moistening the oral tissues to aid in swallowing, chewing, speech, and taste⁽¹³⁾. Saliva also has a critical role in initiating and facilitating digestion. In addition, maintenance of oral health largely depends upon saliva's cleansing actions and intrinsic antipathogenic characteristics⁽¹⁴⁾.

V. Saliva for biomarker discovery in pSS

A. An attractive biofluid

The revelation that saliva is comprised of analytes capable of reflecting health status presents a significant translation potential. In considering the simplicity of saliva collection and its potential as a diagnostic medium, oral fluids have rapidly become the focus of investigation for several disease biomarkers. In that sense, saliva is nowadays widely recognized as an attractive biofluid for study of pSS, featuring several undisputable advantages over blood, the most important being that it can be obtained using noninvasive techniques. As clinical tool, saliva can be easily collected, stored and shipped compared to serum. More importantly, for patients, the non-invasive collection techniques significantly reduce anxiety and discomfort and make procurement of repeated collections a cost-effective approach for longitudinal monitoring^(8; 15; 16).

B. Challenges

While saliva exerts several compelling advantages over serum and other invasively collected biofluids, there are challenges that need to be overcome⁽¹⁷⁾. Unlike blood, saliva proteome appears very sensitive to degradation and research has been carried out to minimize those processes. Hence, a major challenge is to collect and store saliva under conditions that prevent proteolysis, degradation or dephosphorylation. We have shown that collection of saliva into ice-cold tubes, addition of protease inhibitors and storage immediately at -80°C , results in minimal proteolysis⁽¹⁸⁾. Another critical step for proper saliva proteomic analysis is the removal of the mucins. These proteins are responsible for the “sticky” appearance of saliva and might interact with standard immunological assays. Mucins removal can be achieved either by centrifugation. or using special filter-containing collection devices.

C. Immunological proteins in saliva

One of the current criteria being used for diagnosis of pSS is serum positivity for anti-Ro/SSA and/or anti-La/SSB⁽¹⁹⁾. Studies from different groups have demonstrated the presence of these two autoantibodies in whole and parotid saliva collected from pSS patients^(20; 21; 22; 23). This finding is of fundamental importance for pSS because it provides

evidence that oral fluids are capable of reflecting the autoantibody load, thus, presenting an alternative, non-invasive procedure for the diagnosis of the disease. Other autoantibodies that have been described in saliva are anti-mAChR, anti-spectrin, and rheumatoid factor^(24; 25; 26; 27). In an attempt not only to discover and validate potential autoantibody biomarkers in saliva, our laboratory has identified 24 potential autoantibodies that can discriminate patients with SS from both patients with SLE and healthy individuals. Four of these saliva autoantibodies, namely anti-transglutaminase, anti-histone, anti-Ro/SSA, and anti-La/SSB, were further successfully validated in independent SS, SLE, and healthy control subjects⁽²²⁾. Hence, saliva autoantibodies appear as promising biomarkers to be used in a clinical setting.

A few studies have reported saliva cytokines in the context of pSS. Data from these reports congruently show significantly higher levels of Th1, Th2 and Th17 cytokines in saliva of pSS patients^(28; 29; 30; 31). However the lack of appropriate controls in the majority of these studies, in fact non-SS sicca patients, does not allow a definitive conclusion regarding the specificity of these salivary cytokines to discriminate pSS from non-SS sicca subjects. Kang et al. found that salivary Th-1/Th-2 ratios, represented by INF- γ /IL-4 and TNF- α /IL-4 ratios, were features that most differentiated SS and non-SS sicca, and were correlated with the clinical parameters of SS⁽²⁸⁾.

To conclude, the available data indicate that immunological proteins including the major pSS-related autoantibodies and cytokines can be detected in saliva and their levels are indeed significantly increased in patients suffering from pSS. These findings, although not independently validated in large clinical cohorts, confirm that saliva is capable of reflecting the autoimmune exocrinopathy in pSS.

D. Salivary proteomics for pSS diagnosis

Since saliva is the product of salivary glands, the primary targets of the autoimmune response in pSS, it is believed that this secreted fluid can directly mirror the glands' pathophysiology. To analyze the proteomic content of saliva, scientists often employ traditional techniques including liquid chromatography, gel and capillary electrophoresis, nuclear magnetic resonance, mass spectrography and immunoassays⁽³²⁾. However, more contemporary methods including immune-response protoarrays and 2DE coupled with mass spectrography are also utilized and have allowed investigators to analyze several salivary analytes^(22; 33; 34). Nowadays, the development of emerging high throughput proteomic approaches allows the investigation of the whole and gland-specific protein composition.

The main goal of these studies has been the discovery, verification and validation of a panel of protein biomarkers so that they can be utilized in early detection of pSS. Such approaches have highlighted distinct protein patterns characteristic of pSS^(2; 3; 33; 34; 35; 36; 37; 38; 39). These protein signatures mostly comprise secretory proteins, enzymes, calcium-binding proteins, and abundantly expressed immune-related molecules such as β -2-microglobulin^(33; 35; 37; 39). Other protein molecules that have received particular attention include cathepsin-D, α -enolase, cystatins, defensins and Ig γ -light chain. Collectively, these studies have presented evidence that inflammatory phase proteins are elevated in saliva from pSS patients and this finding correlates with the chronic autoimmune inflammation of the

salivary glands in pSS. Similarly, the increased expression of salivary $\beta 2$ -microglobulin, Ig κ -light chain and Ig γ -light chain was attributed to B-cell activation in the periphery. In an effort to catalogue salivary biomarkers according to their biological pathways, a recent study showed that SS-associated salivary proteome appears profoundly altered with respect to several aspects of immunity, immune cell differentiation, and tissue homeostasis⁽⁴⁰⁾. The congruency between the proteomic signatures identified in this study and the hallmarks of salivary gland pathology in pSS, greatly supports that salivary proteome reflects the biologic state of the glands. Collectively, these data indicate that saliva has the capability of revealing changes in the biologic state of the salivary glands and proteomic approaches appear as a promising tool for improving early diagnosis of pSS.

E. Prognostic biomarkers of lymphoma in pSS

Considering the lack of well-validated prediction biomarkers^(41; 42), saliva might be a significant pool of candidate molecules for early identification of pSS patients at higher risk for developing MALT lymphoma. Our group has performed a proteomic and transcriptomic analysis of human parotid glands from patients with pSS and patients with pSS and MALT lymphoma⁽⁴³⁾. This study revealed that 70 proteins were up-regulated in SS/MALT lymphoma samples as compared with both non-SS control and pSS samples. Intriguingly, 45% of the up-regulated proteins had an mRNA transcript (gene-expression level) that was concordantly differentially expressed. Most of the proteins with up-regulated levels in primary SS/MALT lymphoma were related to signal transduction, gene regulation, apoptosis, and the immune response. Amongst these targets, a few have previously been linked to lymphoma, and, particularly, 2 cancer-related proteins, Rho-GDP dissociation inhibitor (Rho-GDI) and cyclophilin A (CypA), are of biologic significance.

In another proteomic approach to analyze whole saliva from pSS and pSS/MALT lymphoma patients, Baldini, et al. showed several qualitative and quantitative modifications in the expression of putative albumin, immunoglobulin J chain, Ig kappa chain C region, alpha-1-antitrypsin, haptoglobin and Ig alpha-1 chain C region.⁽⁴⁴⁾ These studies suggest that clinical and functional changes of the salivary glands driven by lymphoproliferative processes might be reflected in patients' whole saliva, providing further insights into the molecular mechanisms of pSS and pSS/MALT lymphoma. Reasonably, once validated and confirmed, the identified protein candidates could be translated into early prognostic biomarkers for non-Hodgkin's lymphoma susceptible pSS patients.

VI. Summary

In the last few years saliva proteomics has emerged as a promising source for the discovery of pSS biomarkers, for use in diagnosis, classification and/or predicting the prognosis of patients with pSS. Saliva is the obvious pool of these biomarkers, since it is being directly and constantly secreted by the primarily affected exocrine glands. With this in mind, the translation of basic research findings into clinical practice is of great importance. In this regard, effort is been undertaken by multiple research groups, for the definitive validation of salivary biomarkers in large, independent multi-center cohorts. These ongoing studies will validate the previously identified and verified salivary proteins and will strengthen their

potential as biomarkers for pSS. Furthermore, the correlation of salivary biomarker profiles with salivary function and clinical diseases would help in the stratification of pSS patients, which is required for their proper treatment. The dependency on lip biopsy hampers efficient early assessment of an individual with suspicious pSS, thus, the development of such biomarkers has the potential to resolve several challenges that hinder effective on-time evaluation and diagnosis. In conclusion, saliva proteomics have potential for the development of biomarkers and for the identification of pathogenic pathways underlying the different subsets of pSS, leading to the development of new treatment strategies (Table 1).

Acknowledgments

Supported by research grants to David Wong: NIDCR U01DE017593, and to Stergios Katsiogiannis: Hirshberg Foundation Seed grant 2015.

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Key Points

- SS saliva proteome analysis reveals a unique expression profile.
- Major classification biomarkers of SS are present in saliva including anti-Ro/SSA and anti-La/SSB.
- Pre-validation studies suggest salivary autoantibodies as strong biomarker candidates in SS.
- Further clinical validation of the verified and pre-validated proteomic signatures is needed in independent multicenter validation cohorts.

Table I

Salivary autoantibodies in pSS

Salivary autoantibodies	Saliva sample	Year	Authors
IgA-, IgM-rheumatoid factor	Whole saliva	1979	Dunne JV (24)
Anti-Ro/SSA, anti-La/SSB	Whole saliva	1993	Ben-Chetrit E (23)
IgA- rheumatoid factor	Whole saliva	1993	Markusse HM (21)
Anti-spectrin	Parotid	2001	Moody M (26)
Anti-Ro52, anti-Ro60	Whole saliva	2011	Ching KH (20)
Anti-Ro/SSA, anti-La/SSB, Anti-Histone, anti-transglutaminase	Whole saliva	2011	Hu S (22)
Anti-M ₃ mAChR IgA	Whole saliva	2002	Berra A (25)
Anti- M ₃ mAChR-220	Whole saliva	2012	He J (27)