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Meijer, Jiska Marianne

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Shen Hu¹, Jianghua Wang¹, Jiska M Meijer⁸, Sonya leong¹,
Yongming Xie⁶, Tianwei Yu¹, Hui Zhou¹, Sharon Henry¹,
Arjan Vissink⁸, Justin Pijpe⁸, Cees GM Kallenberg⁹, David
Elashoff⁷, Joseph A Loo^{4,5,6}, David T Wong^{1, 2, 3, 4, 5}

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Chapter 4b

Salivary proteomic and genomic biomarkers for primary Sjögren's syndrome

¹School of Dentistry and Dental Research Institute, ²Division of Head & Neck Surgery/
Otolaryngology, David Geffen School of Medicine, ³Henry Samueli School of Engineering,
⁴Jonsson Comprehensive Cancer Center, ⁵Molecular Biology Institute, ⁶Department of
Chemistry and Biochemistry and ⁷School of Public Health, University of California Los
Angeles, Los Angeles, California, USA, ⁸Department of Oral and Maxillofacial Surgery and
⁹Clinical Immunology, University Medical Center Groningen, University of Groningen, The
Netherlands

Abstract

Objective To identify a panel of protein and messenger RNA (mRNA) biomarkers in human whole saliva (WS) that may be used in the detection of primary Sjögren's syndrome (pSS).

Methods Mass spectrometry and expression microarray profiling were used to identify candidate protein and mRNA biomarkers of pSS in WS samples. Validation of the discovered mRNA and protein biomarkers was also demonstrated using real-time quantitative polymerase chain reaction and immunoblotting techniques.

Results Sixteen WS proteins were found to be down-regulated and 25 WS proteins were found to be up-regulated in pSS patients compared with matched healthy control subjects. These proteins reflected the damage of glandular cells and inflammation of the oral cavity system in patients with pSS. In addition, 16 WS peptides (10 up-regulated and 6 downregulated in pSS) were found at significantly different levels ($p < 0.05$) in pSS patients and controls. Using stringent criteria (3-fold change; $p < 0.0005$), 27 mRNA in saliva samples were found to be significantly up-regulated in the pSS patients. Strikingly, 19 of 27 genes that were found to be overexpressed were interferon-inducible or were related to lymphocyte filtration and antigen presentation known to be involved in the pathogenesis of pSS.

Conclusion Our preliminary study has indicated that WS from patients with pSS contains molecular signatures that reflect damaged glandular cells and an activated immune response in this autoimmune disease. These candidate proteomic and genomic biomarkers may improve the clinical detection of pSS once they have been further validated. We also found that WS contains more informative proteins, peptides, and mRNA, as compared with gland-specific saliva, that can be used in generating candidate biomarkers for the detection of pSS.

Introduction

Sjögren's syndrome (SS), which was first described in 1933 by the Swedish physician Henrik Sjögren (1), is a chronic autoimmune disorder clinically characterized by a dry mouth (xerostomia) and dry eyes (keratoconjunctivitis sicca). The disease primarily affects women, with a ratio of 9:1 over the occurrence in men. While SS affects up to 4 million Americans, about half of the cases are primary SS (pSS). pSS occurs alone, whereas secondary SS presents in connection with another autoimmune disease, such as rheumatoid arthritis or systemic lupus erythematosus (SLE). Histologically, SS is characterized by infiltration of exocrine gland tissues by predominantly CD4 T lymphocytes. At the molecular level, glandular epithelial cells express high levels of HLA-DR, which has led to the speculation that these cells are presenting antigen (viral antigen or autoantigen) to the invading T cells. Cytokine production follows, with interferon (IFN) and interleukin-2 (IL-2) being especially important. There is also evidence of B cell activation with autoantibody production and an increase in B cell malignancy. SS patients exhibit a 40-fold increased risk of developing lymphoma.

SS is a complex disease that can go undiagnosed for several months to years. Although the underlying immune-mediated glandular destruction is thought to develop slowly over several years, a long delay from the start of symptoms to the final diagnosis has been frequently reported. SS presumably involves the interplay of genetic and environmental factors. To date, few of these factors are well understood. As a result, there is a lack of early diagnostic markers, and diagnosis usually lags symptom onset by years. A new international consensus for the diagnosis of SS requires objective signs and symptoms of dryness, including a characteristic appearance of a biopsy sample from a minor or major salivary gland and/or the presence of autoantibody such as anti-SSA.(2-4) However, establishing the diagnosis of pSS has been difficult in light of its nonspecific symptoms (dry eyes and mouth) and the lack of both sensitive and specific biomarkers, either body fluid- or tissue-based, for its detection. It is widely believed that developing molecular biomarkers for the early diagnosis of pSS will improve the application of systematic therapies and the setting of criteria with which to monitor therapies and assess prognosis (e.g., lymphoma development).

Saliva is the product of 3 pairs of major salivary glands (the parotid, submandibular, and sublingual glands) and multiple minor salivary glands that lie beneath the oral mucosa. Human saliva contains many informative proteins that can be used for the detection of diseases. Saliva is an attractive diagnostic fluid because testing of saliva provides several key advantages, including low cost, noninvasiveness, and easy sample collection and processing. This biologic fluid has been used for the survey of general health and for the diagnosis of diseases in humans, such as human immunodeficiency virus, periodontal diseases, and autoimmune diseases.(5-8) Our laboratory is active in the comprehensive analysis of the saliva proteome (for more information, see www.hspp.ucla.edu), thus providing the technologies and expertise to contrast proteomic constituents in pSS with those in control saliva.(9-11) Thus far, we have identified over 1,000 proteins in whole saliva (WS). In addition, we have recently identified and cataloged ~3,000 messenger RNAs (mRNA) in human WS.(12) These studies have provided a solid foundation for the discovery of biomarkers in the saliva of patients with pSS. We have previously demonstrated proteome- and genome-wide approaches to harnessing saliva protein and mRNA signatures for the detection of oral cancer in humans.(13,14)

There have been continuous efforts in the search for biomarkers in human serum or saliva for the diagnosis of pSS. Some gene products were found at elevated levels in SS

patient sera or saliva, including β_2 -microglobulin (β_2m), soluble IL-2 receptor, IL-6, anti-Ro/SSA, anti-La/SSB, and anti- α -fodrin autoantibodies.(15-20) However, none of them individually is sensitive or specific enough to use for the confirmative diagnosis of SS.(15) Therefore, it is crucial to use emerging proteome- and genome-wide approaches to discover a wide spectrum of informative and discriminatory biomarkers that can be combined to improve the sensitivity and specificity for the detection of pSS.

Patients and methods

Patient cohort

Because sample quality is critical for clinical proteomics studies, a standardized procedure, in strict accordance with the American-European Consensus Group Criteria for SS (2), was used for the identification and recruitment of pSS patients for this study. A diagnostic evaluation of SS was performed in all patients and included assessments of subjective complaints of oral and ocular dryness, sialometry (unstimulated WS), sialography, histopathology of salivary gland tissue, serology (SSA and SSB antibodies), eye tests (rose bengal staining and Schirmer's test) according to the American-European classification criteria for SS (2), and screening for extraglandular manifestations. Three of the pSS patients were being treated with hydroxychloroquine, and 1 patient was being treated with prednisolone. Eight patients had a focus score of >1 on examination of parotid gland biopsy tissue.

The enrolled pSS patients and healthy control subjects were well matched for age, sex, and ethnicity. The mean \pm SD age was 37.2 ± 9.8 years in the pSS patients ($n=10$) and 37.0 ± 10.6 years in the healthy control subjects ($n=10$). All subjects enrolled in this study were Caucasian women, since pSS mainly affects women. All of the enrolled control subjects were negative for serum anti-SSA/SSB antibodies, and none of them reported any sicca symptoms, including oral and ocular dryness.

Saliva sample collection

Samples of WS and saliva from the parotid and submandibular/sublingual glands were collected from each pSS patient and control subject for comparative analysis. Saliva sample collection was performed at the University Medical Center Groningen, using our standardized saliva collection protocols. Subjects were asked to refrain from eating, drinking, smoking, or performing oral hygiene procedures for at least 1 hour prior to the collection. Samples were collected in the morning, at least 2 hours after eating and rinsing the mouth with water, according to established protocols.(21,22) WS was stimulated by chewing paraffin and was collected over a period of 15 minutes. Glandular saliva specimens from individual parotid glands and, simultaneously, from the submandibular/sublingual glands were collected into Lashley cups (placed over the orifices of the Stenson's duct) and by syringe aspiration (from the orifices of the Warton's duct, located anteriorly in the floor of the mouth), respectively.

After collection, the saliva samples were immediately mixed with protease inhibitors (Sigma, St. Louis, MO) to ensure preservation of the integrity of the proteins and then centrifuged at $2,600g$ for 15 minutes at $4^\circ C$. The supernatant was removed from the pellet, immediately aliquoted, and stored at $-80^\circ C$. All samples were kept on ice during the process. Two patients who had very low submandibular/sublingual gland salivary flow rates (0.03 ml/minute) did not produce enough submandibular/sublingual gland saliva for this study.

Sample preparation for proteomic analysis

The saliva samples were precipitated overnight at -20°C with cold ethanol. Following centrifugation at 14,000g for 20 minutes, the supernatants were collected and dried with a speed vacuum for use in the peptide biomarker study. The pellet was then washed once with cold ethanol and collected for assay of total protein using a 2-D Quant kit (Amersham, Piscataway, NJ). We pooled saliva samples according to the total protein content from all patients with pSS and those from all control subjects. However, both the patients and controls were analyzed individually for the peptide profiling experiment.

Matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF-MS)

Profiling of saliva peptides in 10 pSS patients and 10 matched control subjects was performed using a MALDI-TOF-MS system (Applied Biosystems, Foster City, CA). The peptide fraction from each patient ($n=10$) and control ($n=10$) sample was dissolved in 10 μl of 50% acetonitrile (ACN)/0.1% trifluoroacetic acid (TFA). The sample was mixed with α -cyano-4-hydroxycinnamic acid (10 mg/ml in 50% ACN/0.1% TFA) at a ratio of 1:2, and 1 μl of the mixture was spotted on the MALDI plate for measurement. Three measurements were performed for each sample, and the signals were averaged for subsequent data analysis.

In order to achieve an accurate comparison of the MALDI-TOF-MS data between the patient and control groups, baseline correction and Gaussian smoothing were initially performed to eliminate broad artifacts and noise spikes. Afterward, peak alignment was undertaken to ensure accurate alignment of the mass/charge (m/z) values across the set of spectra, and peak normalization was performed against the total peak intensity. These steps ensured comparability of the MALDI-TOF-MS spectra among all subjects. Subsequent statistical analysis (t-test) was used to reveal peptides that were present at significantly different levels in the pSS patients as compared with the control subjects.

Two-dimensional gel electrophoresis

Saliva samples from the 10 pSS patients and from the 10 control subjects were equally pooled according to the total protein content and then precipitated using the same procedures described above. The pellet was washed once with cold ethanol and then resuspended in rehydration buffer. A total of 100 μg of proteins was loaded onto each gel for the 2-D gel separation procedure. Isoelectric focusing was performed using immobilized pH gradient strips (11 cm long, with an isoelectric point [pI] of 3-10 nonlinear) on a Protean isoelectric focusing cell (Bio-Rad, Hercules, CA), and sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed in 8-16% precast Criterion gels on a Criterion Dodeca Cell (Bio-Rad). Fluorescent SYPRO Ruby stain (Invitrogen, Carlsbad, CA) was used to visualize the protein spots.

The gel images were acquired and analyzed using PDQuest software (Bio-Rad). The images were initially processed through transformation, filtering, automated spot detection, normalization, and matching. The 2-D gel image was transformed to adjust the intensity of the protein spot and filtered to remove small noise features without affecting the protein spot. The images were then normalized based on the total density of the gel image. The 2-D gel images of the pSS patients (master gel) and the control subjects were used as a “match set” for automated detection of the protein spots on the gel. Within the match set, the detected spots were reviewed manually, and the relative protein levels in the patient sample compared with the control sample were summarized.

Table 1 Salivary proteins differentially expressed between pSS patients and healthy control subjects, as identified by LC-MS/MS and Mascot database searching*

Spot No.	Accession	Protein name	Mascot score	Peptide matched	PI	Mt	Ratio (pSS/ctrl)
1	IPI00295105	Carbonic anhydrase VI	163	4	6.65	35343	0.22
2	IPI00295105	Carbonic anhydrase VI	114	5	6.65	35343	0.35
3	IPI00295105	Carbonic anhydrase VI	78	2	6.65	35343	0.29
4	IPI00004573	Polymeric-immunoglobulin receptor	235	5	5.58	83262	0.48
5	IPI00004573	Polymeric-immunoglobulin receptor	293	7	5.58	83262	0.39
6	IPI00004573	Polymeric-immunoglobulin receptor	182	4	5.58	83262	0.56
7	IPI00019038	Lysozyme C	103	2	9.38	16526	0.21
8	IPI00022974	Prolactin-inducible protein	147	3	8.26	16562	0.52
9	IPI00009650	Von Ebner's gland protein	239	4	5.39	19238	0.32
10	IPI00032293	Cystatin C	153	3	9.0	15789	0.43
11	IPI00013382	Cystatin SN	152	3	6.82	16361	0.46
12	IPI00013382	Cystatin SN	130	3	6.82	16361	0.61
13	IPI00002851	Cystatin D	50	1	6.70	16070	0.56
14	IPI00032294 IPI00013382	Cystatin S Cystatin SA	166 208	3 4	4.95 4.85	16 214 16 445	0.65
15	IPI00007047	Calgranulin A	104	2	6.51	10828	0.53
16	IPI00007047	Calgranulin A	79	2	6.51	10828	Absent in pSS
17	IPI00027462	Calgranulin B	126	4	5.71	13234	1.05
18	IPI00219806	Psoriasin	133	4	6.28	11464	1.44
19	IPI00410714	Hemoglobin alpha-I globin chain	157	5	7.96	15292	Absent in control
20	IPI00218816	Hemoglobin beta chain	48	1	6.75	15988	2.73
21	IPI00218816	Hemoglobin beta chain	51	1	6.75	15988	7.58
22	IPI00007797	Fatty acid-binding protein, epidermal	211	6	6.60	15155	3.21
23	IPI00472762 IPI00472610 IPI00430840	IGHG1 protein Hypothetical protein Ig gamma-I chain C region	333 363 333	14 14 14	8.33 7.50 7.48	50822 52633 54866	22.64
24	IPI00472610 IPI00550718	IGHM protein Ig gamma-I chain C region	260 257	11 11	7.50 8.46	53270 53331	Absent in control
25	IPI00465248	Alpha-enolase	409	12	6.99	47139	4.37
26	IPI00300786	Salivary alpha-amylase, fragment	241	5	5.73	57731	3.41
27	IPI00300786	Salivary alpha-amylase, fragment	230	4	5.73	57731	2.19
28	IPI00300786	Salivary alpha-amylase, fragment	375	7	5.73	57731	31.53
29	IPI00300786	Salivary alpha-amylase, fragment	260	5	5.73	57731	2.57
30	IPI00300786	Salivary alpha-amylase, fragment	171	5	5.73	57731	2.50

Table I continued

Spot No.	Accession	Protein name	Mascot score	Peptide matched	PI	Mt	Ratio (pSS/ctrl)
31	IPI00300786	Salivary alpha-amylase, fragment	194	4	5.73	57731	11.92
32	IPI00300786	Salivary alpha-amylase, fragment	149	4	5.73	57731	1.57
33	IPI00300786	Salivary alpha-amylase, fragment	148	4	5.73	57731	4.03
34	IPI00549682	Fructose-bisphosphate aldolase A	218	4	8.75	52306	2.59
35	IPI00332161	Ig gamma-1 chain C region	138	5	8.46	36083	2.54
36	IPI00215983	Carbonic anhydrase I	119	4	6.59	28852	7.4
37	IPI00218414	Carbonic anhydrase II	98	2	8.67	31337	2.11
38	IPI00013885	Caspase-14	172	5	5.44	27662	3.32
39	IPI00419424	Ig kappa chain C region	263	7	5.82	27313	1.79
40	IPI00004656	Beta-2-microglobulin	62	2	6.06	13706	2.21
41	IPI00021439	Actin	461	11	5.29	41710	3.18
42	IPI00022434	Serum albumin, fragment	492	10	5.41	69321	Absent in control

* Liquid chromatography mass spectrometry/mass spectrometry (LC-MS/MS) analysis and Mascot database searching were performed to identify the proteins. Shown are the theoretical isoelectric point and molecular mass of the protein precursors, as well as the ratio of protein levels in patients with primary Sjögren's syndrome (SS) and matched control subjects, as detected by 2-dimensional gel electrophoresis.

Liquid chromatography tandem mass spectrometry (LC-MS/MS) and database searching

Protein spots showing differential protein levels were excised by a spot-excision robot (Proteome Works; Bio-Rad) and deposited into 96-well plates. Proteins in each gel spot were reduced with dithiothreitol, alkylated with iodoacetamide, and then digested overnight at 37°C with 10 ng of trypsin. After digestion, the peptides were extracted and stored at -80°C prior to LC-MS/MS analysis.

LC-MS/MS analysis of peptides was performed using an LC Packings Nano-LC system (Dionex, Sunnyvale, CA) with a nanoelectrospray interface (Protana, Odense, Denmark) and a quadrupole time-of-flight (Q-TOF) mass spectrometer (QSTAR XL; Applied Biosystems). A New Objective PicoTip tip (internal diameter 8 µm; New Objective, Woburn, MA) was used for spraying, with the voltage set at 1,850V for online MS and MS/MS analyses. The samples were first loaded onto an LC Packings PepMap C18 precolumn (300 µm x 1 mm; particle size 5 µm) and then injected onto an LC Packings PepMap C18 column (75 µm x 150 mm; particle size 5 µm) (both from Dionex) for nano-LC separation at a flow rate of 250 nl/minute. The eluents used for LC were 1) 0.1% formic acid and 2) 95% ACN/0.1% formic acid, and a 1%/minute gradient was used for the separation.

The acquired MS/MS data were searched against the International Protein Index (IPI) human protein database (available at <http://www.ebi.ac.uk/IPI/IPIhelp.html>) using the Mascot (Matrix Science, Boston, MA) database search engine. Positive protein identification was based on standard Mascot criteria for statistical analysis of LC-MS/MS data.

Immunoblotting

Western blot analysis of α -enolase was performed on the same set of saliva samples (10 pSS and 10 control samples). Proteins were separated on 12% NuPAGE gels (Invitrogen) at 150V and then transferred to a polyvinylidene difluoride membrane (Bio-Rad) using an Invitrogen blot transfer cell. After saturating with 5% milk in Tris buffered saline-Tween buffer (overnight at 4°C), the blots were sequentially incubated for 2 hours at room temperature with polyclonal goat α -enolase primary antibody and horseradish peroxidase-conjugated anti-goat IgG secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The bands were detected by enhanced chemiluminescence (Amersham) and quantified using Quantity One software (Bio-Rad).

Profiling of salivary mRNA by high-density oligonucleotide microarray analysis

Samples of stimulated parotid gland saliva or WS from 10 pSS patients and 8 matched controls were preserved in RNAlater reagent (Qiagen, Valencia, CA) at a 1:1 ratio and then frozen at -80°C . Total salivary RNA was isolated from 560 μl of RNAlater-preserved saliva (280 μl of parotid gland saliva/WS and 280 μl of RNAlater) using a viral RNA mini kit (Qiagen) as described previously (12). Isolated total RNA was treated with 2 rounds of recombinant DNase I (Ambion, Austin, TX) digestion, and the RNA concentration was measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). The salivary RNA quality was examined by real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis for expression of the salivary internal reference gene transcripts S100 calcium-binding protein A8 and annexin A2 (data not shown).

For microarray study, total salivary RNA was subjected to 2 rounds of T7-based RNA linear amplification (10). One microliter (200 ng/ μl) of poly(dI-dC) (Amersham) was added to 11 μl of the salivary RNA sample, and 2 rounds of first-strand and second-strand complementary DNA (cDNA) synthesis were performed with a RiboAmp HS RNA amplification kit (Arcturus, Mountain View, CA) according to the manufacturer's instructions. After purification, the cDNA were in vitro transcribed to RNA and then biotinylated with GeneChip Expression 3'-Amplification Reagents for in vitro transcription labeling (Affymetrix, Santa Clara, CA). The labeled RNA was purified with the reagents provided with the RiboAmp HS RNA Amplification kit. The quality and quantity of amplified RNA were determined by spectrophotometry, with optical densities at 260/280 nm > 1.9 for all samples.

Biotinylated RNA samples (15 μg each) were subsequently fragmented, and the quality of the fragmented RNA was assessed using an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA). The Affymetrix human genome UI33 Plus 2.0 array, which contains $>54,000$ probe sets representing $>47,000$ transcripts and variants, including $\sim 38,500$ well-characterized human genes, was applied to salivary mRNA profiling. Fragmented RNA were hybridized overnight to the microarrays. After a high-stringency wash to remove the unbound probes, the hybridized chips were stained and scanned according to the manufacturer's standard expression protocol. The scanned images were read with the Affymetrix microarray Robust Multiarray Average (RMA) software.(23) We deposited the microarray data we obtained into a Minimum Information About a Microarray Experiment (MIAME)-compliant database (available at <http://.mged.org/workgroups/MIAME/miame.html>); the accession number is GSE7451.

Statistical analysis for the mRNA study

The expression microarrays were scanned, and the fluorescence intensity was measured using Microarray Suite 5.0 software (Affymetrix). The arrays were then imported into the statistical software R (24). After quantile normalization and RMA background correction, the RMA expression index was computed in R using the Bioconductor routine.(25) Since most human RNAs are not present in saliva (12), we used the present/absent call generated by the Affymetrix Microarray Suite 5.0 software to exclude probe sets that were assigned an “absent” call in most (>75%) of the samples. Principal components analysis was performed to assess the information contained in the data to separate pSS and control cases. Student’s 2-tailed t-test was used for comparison of the average gene expression signal intensity between samples from the SS patients (n=10) and controls (n=8). P values were adjusted with the Benjamini and Hochberg false discovery rate (FDR) criterion.(26) Fold ratios between SS and control samples were calculated for the transcripts. For the further validation study using real-time quantitative PCR, we applied stringent criteria: an alpha level of 0.001 for the t-test, which corresponded to a 5% FDR based on the data, and a fold ratio of 3. For functional analysis using MAPPFinder (27), we applied an alpha level of 0.01, which corresponded to an 8% FDR, and a fold ratio of 2, to obtain a larger list of genes.

Real-time quantitative RT-PCR

The biomarker candidates generated by microarray profiling were validated by real-time quantitative RT-PCR on the same set of samples used for the microarray analysis. All primers used for quantitative PCR were designed with the Primer3 program and synthesized by Sigma. Total RNA was reverse-transcribed using reverse transcriptase and gene-specific primers. One microliter of total RNA was used in a 20- μ l volume of cDNA synthesis reaction and then subjected to the following thermal cycling conditions: 25°C for 10 minutes, 42°C for 45 minutes, and 95°C for 5 minutes. Three microliters of cDNA was used as template for each 20- μ l PCR, which contained forward primer (200 nM), reverse primer (200 nM), and 10 μ l of 2 x SYBR Green PCR Master Mix (Applied Biosystems). PCRs were performed in a 96-well plate on the Bio-Rad iCycler or IQ5 instrument (95°C for 3 minutes followed by 50 cycles of 95°C for 30 seconds, 62°C for 30 seconds, and 72°C for 30 seconds). All PCRs were performed in duplicate for all candidate mRNA.

The specificity of the PCR was confirmed according to the melting curve of each gene, and the average threshold cycle (C_t) was examined. The relative expression of the candidate genes was calculated according to the $2^{(-\Delta C_t)}$ method, where $\Delta C_t = C_t$ in pSS patients – C_t in controls. The expression ratio ([pSS patients/controls] = $2^{(-\Delta C_t)}$) is shown as the fold change.(28)

Pathway analysis

PathwayArchitect software, version 1.1.0 (Stratagene, La Jolla, CA) was used to investigate the functional pathways presented by the differentially expressed genes.

Results

Salivary flow rate and total salivary protein and mRNA contents in pSS patients

Patients with pSS who had been carefully diagnosed and monitored were enrolled in this study. All 10 patients were positive for anti-SSA/Ro antibodies, and 9 of them were also positive for anti-SSB/La antibodies. Their mean \pm SD IgG level was 23.4 ± 7.4 gm/liter, and

their mean \pm SD IgM rheumatoid factor level was 136.3 ± 99.6 kIU/liter. These patients exhibited significantly lower ($\sim 50\%$) salivary flow rates than did the age-, sex-, and ethnicity-matched healthy control subjects. The mean \pm SD stimulated salivary flow rates in the 10 pSS patients were 0.13 ± 0.12 ml/minute for the parotid glands (per gland), 0.32 ± 0.38 ml/minute for the submandibular/sublingual glands, and 0.61 ± 0.23 ml/minute for WS. These rates in the 10 control subjects were 0.21 ± 0.07 ml/minute for the parotid glands (per gland), 0.78 ± 0.36 ml/minute for the submandibular/sublingual glands, and 1.03 ± 0.31 ml/minute for WS. Due to the low volume of saliva obtained from the pSS patients, the salivary proteins were equally pooled for the 10 pSS patients and separately for the 10 control subjects for the 2-DE analyses.

On average, the mean \pm SD total protein concentrations in the controls were determined to be 1.26 ± 0.40 mg/ml in submandibular/sublingual gland saliva ($n=8$ subjects), 0.93 ± 0.38 mg/ml in parotid gland saliva ($n=10$ subjects), and 0.95 ± 0.52 mg/ml in WS ($n=10$ subjects). The total protein concentrations in the pSS patients were 1.45 ± 0.49 mg/ml in submandibular/sublingual gland saliva ($n=8$ patients), 1.40 ± 0.56 mg/ml in parotid gland saliva ($n=10$ patients), and 1.38 ± 0.37 mg/ml in WS ($n=10$ patients). There were consistently higher concentrations of proteins in the SS patients (WS, submandibular/sublingual gland saliva, and parotid gland saliva) than in the matched healthy control subjects. In addition, saliva from the pSS patients appeared to contain a higher concentration of total RNA than did that from the matched controls. In parotid gland saliva, the mean \pm SD RNA concentration was determined to be 5.8 ± 3.1 μ g/ml in the pSS patients and $3. \pm 5$ μ g/ml in the controls ($p=0.05$). In WS, the average RNA concentration was 10.9 ± 5.4 μ g/ml for pSS patients and 6.6 ± 3.6 μ g/ml for matched controls ($p=0.057$).

Discovery of candidate peptide markers for pSS

The expression of 16 WS peptides was found to be significantly different ($p=0.0046$ – 0.0441) in pSS patients ($n=10$) and controls ($n=10$). Ten of the 16 peptides were overexpressed (m/z 1.107, 1.224, 1.333, 1.380, 1.451, 1.471, 1.680, 1.767, 1.818, and 2.039) and 6 were underexpressed (m/z 2.534, 2.915, 2.953, 3.311, 3.930, and 4.187) in the pSS patients. The peptide with an m/z of 1.451 exhibited the highest up-regulation (25.9-fold) in pSS patients (results not shown). We also compared the native peptide patterns in saliva from the parotid and submandibular/sublingual glands between pSS patients and control subjects (results not shown). WS was found to contain more informative peptides than did gland-specific (parotid or submandibular/sublingual) saliva. On average, 53 MALDI peaks were observed in WS from the 10 pSS patients, with only 24 peaks and 26 peaks detectable in saliva from their parotid and submandibular/sublingual glands, respectively.

Findings of 2-DE of WS proteins from pSS patients and matched control subjects

Figure 1 presents the 2-DE patterns of the proteins in pooled WS samples from 10 pSS patients and 10 control subjects. A number of proteins were found to be differentially expressed between the patient and control groups. By performing the PDQuest analysis and normalizing the protein spot signals, the relative levels of these proteins were quantified. The differentially expressed proteins (figure 1, spots 1-42) were excised and subsequently identified using in-gel tryptic digestion and LC-Q-TOF-MS. Pooled parotid and submandibular/sublingual gland saliva from pSS patients and control subjects was also analyzed by 2-DE (results not shown). WS was again found to be more informative than parotid or submandibular/sublingual gland saliva for generating candidate protein biomarkers

for the detection of pSS. A total of 325 protein spots were detected by 2-DE analysis of WS, whereas 232 and 267 spots were detected by 2-DE analysis of parotid and submandibular/sublingual gland saliva, respectively.

LC-Q-TOF-MS identification of proteins at altered expression levels

The differentially expressed WS proteins identified by LC-Q-TOF-MS and Mascot database searching, as well as their theoretical isoelectric point (pI), relative molecular mass (Mr), IPI accession number, the number of peptides matched, and ratios of expression levels between the pSS patient and matched control groups are shown in table 1.

Figure 2A depicts the tandem MS spectrum of a double-charged tryptic peptide (m/z 450.3). The precursor ion was well fragmented to yield sufficient structural information for confident identification of the peptide sequence TIAPALVSK, which originated from α -enolase. Mascot database searching indicated that 12 peptides were matched to this protein, resulting in a sequence coverage of 31%. Validation of α -enolase was also performed by Western blotting of the same set of samples used for the 2-DE study. (figure 2B) An equal amount of total proteins from each sample was used for immunoblotting of α -enolase and actin. Both α -enolase and actin were found to be up-regulated in WS from pSS patients, which is consistent with the 2-DE results. (table 1) P values were calculated to be 0.006 for α -enolase without actin normalization and 0.037 with actin normalization for comparisons between the pSS patient and healthy control groups.

Figure 1

Comparative analysis of proteins in whole saliva (WS) samples from patients with primary Sjögren's syndrome (pSS) and age-, sex-, and ethnicity-matched control subjects, as determined by 2-dimensional gel electrophoresis (2-DE) and liquid chromatography-quadrupole time-of-flight mass spectrometry (LC-Q-TOF-MS). Shown are the 2-DE patterns of proteins in pooled WS from 10 control subjects and 10 pSS patients. A total of 100 μ g of total proteins from each pooled sample was used for the 2-D gel separation. The differentially expressed proteins (spots 1-42; see Table 1 for the complete list) were identified using in-gel tryptic digestion and LC-Q-TOF-MS.

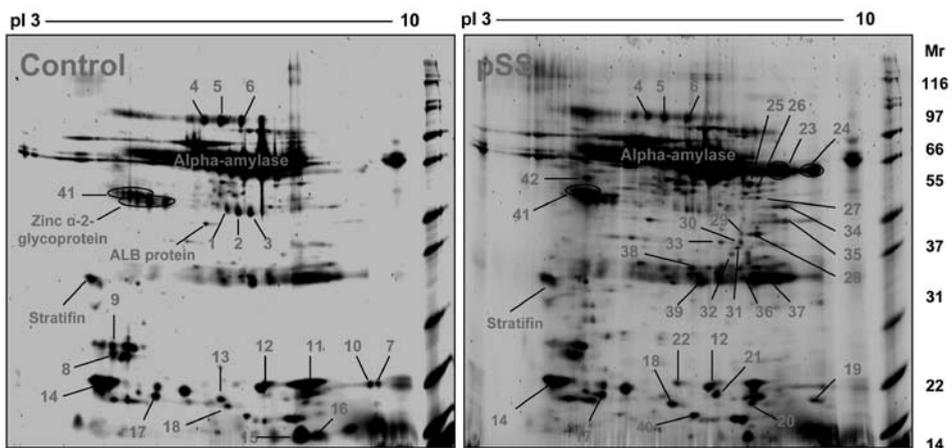


Table 2 Real-time quantitative RT-PCR validation of 13 genes selected from the top 27 genes found to be differentially expressed in pSS patients and healthy control subjects*

Gene	Average Ct Control	Average Ct pSS	Δ Ct (Control/pSS)	quantitative RT-PCR, fold change $2^{-(\Delta Ct)}$	P value (t-test)	Microarray fold change
GIP2	44.5±1.9	35.5±2.1	9.0	495.5	<0.001	15.76
B2M	45.0±2.1	38.8±3.4	6.2	72.1	<0.001	8.67
IFIT2	41.1±2.0	35.9±2.6	5.1	35.5	<0.001	12.19
BTG2	38.5±5.3	33.5±2.0	5.0	32.4	0.01	3.22
IFIT3	43.8±0.5	39.1±2.4	4.7	25.3	<0.001	122.82
MNDA	37.3±1.2	33.7±2.1	3.7	12.7	<0.001	8.67
FCGR3B	40.6±1.5	36.9±2.2	3.6	12.5	<0.001	25.32
TXNIP	39.2±2.1	35.6±3.2	3.6	11.7	0.01	3.42
IL18	45.3±2.1	41.8±2.5	3.5	11.5	0.01	6.12
HLAB	36.4±2.7	32.9±2.0	3.5	11.2	0.01	4.34
EGRI	37.4±2.4	33.9±2.0	3.4	10.3	0.01	7.20
COPI	40.5±1.5	38.7±3.3	1.8	3.4	0.18	7.62
TNSF	39.6±0.4	38.9±2.9	0.7	1.6	0.95	8.03

* All real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) analyses were performed in duplicate. See Patients and Methods for calculations of the fold change (primary Sjögren's syndrome (SS) patients/healthy controls) and threshold cycle (C_t) data.

Identification of candidate genomic markers of pSS in saliva samples

For all the arrays, the mean \pm SD percentage of genes present was $13.2 \pm 2.9\%$. This is similar to the finding in our previous study (12) and indicates consistency of the techniques used for sample preparation. Microarray profiling indicated that WS contains >10 times more informative mRNA than does parotid gland saliva. A total of 328 mRNA had a >2 -fold change in WS from pSS patients, while only 21 mRNA had a >2 -fold change in parotid gland saliva from these patients. Therefore, we focused on the discovery and validation of WS candidate mRNA biomarkers using microarray and real-time quantitative RT-PCR strategies.

Gene expression profiles of individual WS samples from 10 pSS patients and 8 controls were compared. After filtering the transcripts by the criteria of being "present" in $>25\%$ of the samples, a total of 6,413 transcripts were retained for further analysis. This number is consistent with our previous results, showing that only a small number of RNAs are present in saliva (12). Principal components analysis indicated that the information contained in the data could well segregate control subjects and pSS patients. (figure 3) We then performed statistical testing and fold change analysis to identify differentially expressed genes. Only a few mRNA were found at significantly lower levels in pSS patients as compared with the controls when using a threshold of >2 -fold change and a significance level of $P < 0.01$ (FDR 0.08). Yet, by the same criteria, 162 genes showed significant up-regulation in samples from patients with pSS.

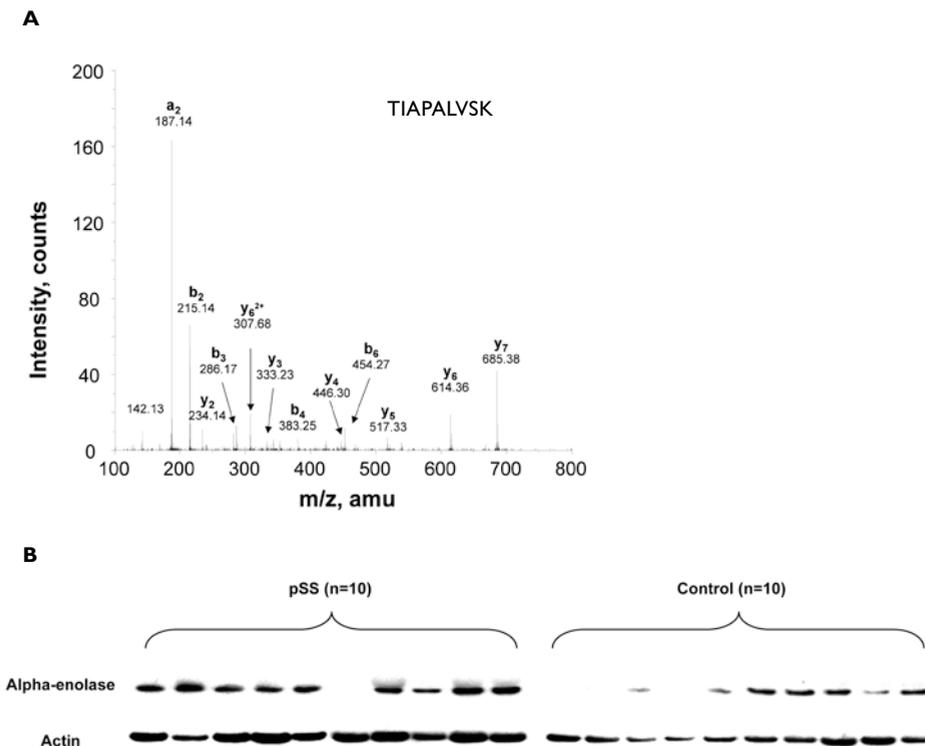
Pathway analysis indicated that 37 genes were involved in the IFN- α pathway, and most

of them have been reported to be IFN- α or IFN- β inducible.(29;30) These results suggest that activation of IFN pathways is involved in the pathogenesis of pSS and that the related information is reflected in the saliva. To facilitate biomarker discovery, we narrowed the number of candidate biomarkers by using more stringent threshold criteria of $P < 0.001$ (FDR 0.05) and 3-fold change. Based on these criteria, we found 27 genes that were highly overexpressed in samples from pSS patients. These genes are sufficiently informative for segregating the pSS patients from the control subjects. (figure 4)

Among the top 27 genes, 13 were validated by real-time quantitative RT-PCR. Eleven of the 13 genes were found to be significantly up-regulated in pSS patients (>10-fold change), including the IFN-inducible protein GIP2, which showed an ~500-fold change in pSS patients. Table 2 shows the average Ct values of these genes in pSS patients and control subjects, as well as the quantitative PCR fold change in comparison with that of microarray profiling.

Figure 2

Analysis of α -enolase by electrospray ionization tandem mass spectrometry (ESI-MS/MS) and immunoblotting. A, ESI-MS/MS spectrum of the tryptic peptide TIAPALVSK (mass/charge [m/z] 450.3 atomic mass units [amu]) from α -enolase. This protein was found to be overexpressed in whole saliva from patients with primary Sjögren's syndrome (pSS), as determined by 2-dimensional gel electrophoresis. B, Immunoblotting of whole saliva from 10 patients with pSS and 10 age-, sex-, and ethnicity-matched control subjects for α -enolase and actin. An equal amount of proteins from each sample was used for the immunoblots.



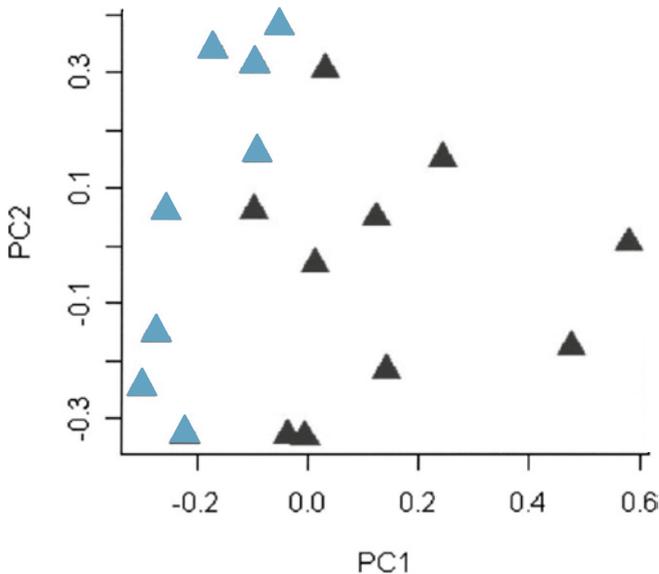
Discussion

Although saliva has been extensively explored as a source of information that can be used in the diagnosis of pSS, most of the previously published studies mainly examined individual components of the saliva. High-throughput profiling techniques, such as proteomics and expression microarray analysis, enable us to explore salivary proteins and mRNA in a global manner and may therefore provide new and deeper insights that may lead to the discovery of salivary biomarkers for pSS. Recently, surface-enhanced laser desorption ionization time-of-flight mass spectrometry and differential gel electrophoresis have been used to identify very promising candidate biomarkers of SS in tears and in parotid gland saliva.(31;32) It was found that the proteomic profile of parotid gland saliva from SS patients is a mixture of increased inflammatory proteins and decreased acinar proteins as compared with the profile in non-SS controls.(32)

In order to determine which oral fluid compartment is more informative for the discovery of biomarkers that can be used to detect pSS, we used both proteomic and microarray approaches to profile peptides, proteins, and mRNA in WS, parotid gland saliva, and submandibular/sublingual gland saliva from each study subject. WS as a fluid includes secretions from 3 major salivary glands, numerous minor salivary glands, and gingival fluid, as well as cell debris. There has therefore been concern about the complex background in WS for discovery of disease biomarkers, whereas parotid gland saliva, if collected carefully, may contain more specific biomarkers for pSS. Yet, there are no published reports of any advantage of using gland-specific saliva versus WS in terms of the diagnostic potential for

Figure 3

Principal components analysis of the gene expression data in patients with primary Sjögren's syndrome (SS) and in age-, sex-, and ethnicity-matched control subjects. Results of the principal components (PC1 and PC2) analysis suggest that the gene expression data we obtained segregated the 8 control subjects (green symbols) from the 10 pSS patients (black symbols).



pSS. The findings of our study allow us to conclude that WS is more informative than glandular saliva for generating biomarkers to be used for the detection of pSS.

Microarray profiling indicated that WS from pSS patients contained 328 mRNA with 2-fold change in expression, whereas the parotid gland saliva from pSS patients contained only 21 mRNA with a >2-fold change in expression. Similarly, findings of the MALDI-TOF-MS and 2-DE analyses suggested that WS from pSS patients has more informative proteomic components than does parotid or submandibular/sublingual gland saliva. Since the salivary flow rate varies from person to person, the peptide or protein composition among different individuals could be affected by the very low salivary flow rate of the parotid and submandibular/sublingual glands. With regard to the low flow rate of glandular saliva, as well as the additional skill set and clinical time necessary to collect gland-specific saliva, WS may be a more appropriate clinical diagnostic fluid for the discovery and detection of biomarkers of pSS.

The panel of candidate peptide/protein markers for pSS is completely distinct from the panel we obtained for oral cancer.(13) This suggests that the panels of discriminatory salivary proteomic components are likely to be different for different diseases. The majority of underexpressed proteins found in WS from pSS patients are secretory proteins, including 3 glycoforms of carbonic anhydrase VI (figure 1, spots 1-3), cystatins, lysozyme C, polymeric immunoglobulin receptor (pIgR), calgranulin A, prolactin-inducible protein, and von Ebner gland protein. This suggests that the level of secretory proteins in WS from pSS patients may be directly affected by injury to salivary glandular cells. Several of these down-regulated proteins in the WS of pSS patients, including pIgR, lysozyme C, and cystatin C, were found up-regulated in the parotid gland saliva of pSS patients in a previously published study.(32) This may be factual, as evidenced by our comparative analysis of parotid gland salivary proteins in pSS patients and control subjects (results not shown). For example, in our 2-DE study, pIgR was also found to be up-regulated in the pooled parotid gland saliva of pSS patients as compared with the matched control subjects (results not shown). A future study of salivary proteins from the parotid gland versus WS in the same pSS patients would be of interest to the pSS research community.

Two glycolysis enzymes, fructose-bisphosphate aldolase A and α -enolase, were found at elevated levels in the WS of pSS patients. Fructose-bisphosphate aldolase A plays a central role in glucose metabolism, catalyzing either net cleavage or synthesis during glycolysis or gluconeogenesis. Alpha-enolase is a multifunctional glycolysis enzyme involved in various processes, such as growth control, hypoxia tolerance, and allergic responses. Previously, α -enolase was identified as an autoantigen in Hashimoto encephalopathy, which is an autoimmune disease associated with Hashimoto thyroiditis.(33) Alpha-enolase was also found as an autoantigen in lymphocytic hypophysitis, and serum autoantibodies directed against α -enolase were detected in patients with lymphocytic hypophysitis as well as in patients with other autoimmune diseases. Excessive production of autoantibodies, which are generated as a consequence of uptake of enolase by antigen-presenting cells and subsequent B cell activation, can potentially initiate tissue injury as a result of immune complex deposition.(34;35) Overexpressed proteins in WS from patients with pSS also included psoriasin, fatty acid binding protein, carbonic anhydrases I and II, salivary amylase fragments, caspase 14, β_2 m, hemoglobin (β and α 1 global chains), and immunoglobulins. The elevated level of caspase 14 protein and caspases 1 and 4 RNA in pSS patients also suggested an interesting role of apoptosis in the pathogenesis of pSS.

Our study clearly demonstrates that pSS-related gene expression signatures are present

The mechanism of IFN pathway activation in the pathogenesis of pSS may be more complicated. Activation of IFN pathways (both type I and type II) in pSS suggests the involvement of viral infection in its pathogenesis. Immune complexes consisting of auto-antibodies and DNA- or RNA-containing autoantigens derived from apoptotic or necrotic cells are also able to induce the production of type I IFN. However, IFN itself is not among the genes we found to be overexpressed in the saliva of the pSS patients. On the other hand, low-dose IFN- α has been reported to be effective in the treatment of some patients with pSS. A single-blind controlled trial showed that IFN- α therapy significantly improved salivary gland dysfunction in SS patients.(40) Serial labial salivary gland biopsy in 9 patients responding to IFN- α therapy showed a significant decrease ($p < 0.02$) in lymphocytic infiltration and a significant increase ($p = 0.004$) in the proportion of intact salivary gland tissue after IFN- α treatment.(41)

Type I IFN pathway dysregulation, however, has been reported in such distinct diseases as SLE, dermatomyositis, psoriasis, and SS (36), indicating that the consequences of activation of this pathway are likely to be tissue type-dependent and, from a therapeutic point of view, that local immune modulation (e.g., direct infusion into salivary glands) may be more efficient than systemic interference. An initial viral infection-induced type I IFN production in salivary glands, with prolonged activation triggered by autoantibodies from nucleic acid-containing immune complexes, has been proposed as the mechanism of pSS.(42) More importantly, activation of this IFN pathway may provide potential therapeutic targets for pSS, and saliva may be used to monitor the response to the IFN-related target modulation.

One of the up-regulated genes seen in the saliva of patients with pSS is β_2m , which is also regulated by IFN. Significantly elevated levels of β_2m have previously been detected in saliva from patients with pSS.(43) The concentration of salivary (but not serum) β_2m was highly related to the salivary gland biopsy focus score.(43) The value of salivary β_2m protein as a biomarker for pSS has been evaluated, and it has been suggested that determination of β_2m levels in the saliva could be used as a noninvasive measurement for confirmation of the diagnosis of SS.(44) Interestingly, but not surprisingly, we found that both the mRNA and protein levels of β_2m are concordantly overexpressed in the saliva of patients with pSS.

From the top 27 mRNA found to be overexpressed in WS from pSS patients, as revealed by microarray profiling, we were able to validate 11 of the genes; expression of the other 16 genes was too low for quantitative PCR assessment. The most overexpressed mRNA was found to be GIP2, which has a function in cell signaling and has been reported to be up-regulated at the mRNA level in minor salivary glands from patients with pSS.(37) There were discrepancies with regard to the fold change as determined by the quantitative PCR and the microarray studies.

There are many factors that may contribute to the observed discrepancies, including the procedures unique to the microarray analysis, such as nonspecific and/or cross-hybridization of labeled targets to array probes, as well as those unique to real-time quantitative RT-PCR, such as amplification biases.(45) Also, the increased distance between the location of the PCR primers and the microarray probes on a given gene was found to decrease the correlation between the 2 methods.(46) In our study, the amplified RNA used for microarray assay and the unamplified RNA used for the real-time quantitative RT-PCR validation studies can introduce variances in the fold change between the 2 methods. Furthermore, we do not expect there to be perfect correlation between the fold change as determined by quantitative PCR and by microarray analyses, since there is considerable variability in the fold change statistic, especially in the case of genes that are near the limit

of detection by quantitative PCR. For genes with expression levels that are too low for the quantitative PCR techniques in current use, it is still possible that they may be validated when the technology improves. Nevertheless, these 11 highly expressed genes, once they are further validated in a new and independent patient cohort, may be used in the clinical detection of pSS.

There was little correlation between the protein and mRNA markers identified. This has been observed for biologic systems when efforts were made to correlate the gene expression at both the protein and mRNA levels.(47;48) In a previous correlation analysis of the human saliva proteome and transcriptome, we demonstrated that complementary validation (e.g., Western blotting for protein or RT-PCR for mRNA) is required in the conduct of RNA-protein correlation studies of individual genes after initial mass spectrometry and expression microarray profiling.(49) If mutual validation is performed, there may be higher correlations between the protein and mRNA candidate markers in saliva identified in the present study. Nevertheless, the discrepancy we found may suggest that the combination of both mRNA and protein markers is important for improving the detection of pSS.

As a result of this preliminary study, a number of promising salivary protein and mRNA candidates that are characteristic of pSS have been identified. Many of these candidate biomarkers have not previously been associated with SS and, in combination, they may eventually be validated as specific biomarkers of pSS, thus improving the clinical diagnosis of pSS. Ideally, the biomarkers would be very specific for pSS and would discriminate pSS from other autoimmune diseases of a similar immunopathologic background. Future studies will include new pSS patients as well as patients with other autoimmune diseases as control groups, aiming to validate the candidate genes either through the use of real-time quantitative RT-PCR for mRNA or immunoassays for proteins. Absolute quantification will provide a cutoff value for each biomarker selected, and combination of the mRNA and protein markers will allow the eventual construction of a multimarker prediction model that can be used as an adjunct to the current diagnostic criteria for the clinical diagnosis of pSS.

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