MicroRNA-mediated Regulation of Mucin-type O-glycosylation Pathway: A Putative Mechanism of Salivary Gland Dysfunction in Sjögren Syndrome

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ABSTRACT. Objective. To investigate microRNA (miRNA) that is potentially implicated in primary Sjögren syndrome (pSS)-related salivary hypofunction in labial salivary glands and to study miRNA-mediated mechanisms underlying oral dryness and altered rheology, focusing on the mucin O-glycosylation pathway.

Methods. We performed miRNA expression profiling in minor salivary gland samples of patients with pSS presenting a different impairment in their unstimulated salivary flow rate. A computational in silico analysis was performed to identify genes and pathways that might be modulated by the deregulated miRNA that we had identified. To confirm in silico analysis, expression levels of genes encoding for glycosyltransferases and glycan-processing enzymes were investigated using Human Glycosylation-RT² Profiler PCR Array.

Results. Among 754 miRNA analyzed, we identified 126 miRNA that were significantly deregulated in pSS compared to controls, with a trend that was inversely proportional with the impairment of salivary flow rates. An in silico approach pinpointed that several upregulated miRNA in patients with pSS target important genes in the mucin O-glycosylation. We confirmed this prediction by quantitative real-time PCR, highlighting the downregulation of some glycosyltransferase and glycosidase genes in pSS samples compared to controls, such as GALNT1, responsible for mucin-7 glycosylation.

Conclusion. Collectively, our data suggest that the expression of different predicted miRNA-target genes in the mucin type O-glycan biosynthesis pathway is altered in pSS patients with low salivary flow and that the miRNA expression profile could influence the glycosidase expression levels and consequently the rheology in pSS. (First Release August 15 2019; J Rheumatol 2019;46:1485–94; doi:10.3899/jrheum.180549)

Key Indexing Terms:

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Primary Sjögren syndrome (pSS) is a complex, heterogeneous, and disabling disorder primarily affecting the exocrine glands but potentially involving any other organ or system. The hallmark of the disease is the lymphocytic infiltration and hypofunction of salivary and lacrimal glands that lead progressively to the typical oral and ocular dryness.

Pathogenetic mechanisms underlying salivary hypofunction in pSS have been only partially elucidated to date, especially because the correlation between salivary gland inflammation and dysfunction has appeared relatively scarcely.

Several studies have ultimately aimed at better clarifying
pSS salivary impairment with the general hypothesis that epigenetic mechanisms may influence salivary flow, protein expression, and saliva properties.

From this perspective, a number of differently expressed microRNA (miRNA) have been described in minor salivary gland biopsies of patients with pSS and associated with either salivary gland focus score or salivary flow decrease. In parallel, proteomic analysis of saliva has highlighted several changes in the protein composition of saliva in pSS, potentially associated to the alteration of salivary secretion\textsuperscript{10,11,12,13}. In particular, several quantitative differences have been described in the salivary proteome of patients with pSS compared to that of healthy volunteers, with a significant increase of proteins related to inflammation and immune response and a decrease of normal constituents of saliva. Moreover, qualitative differences in protein posttranslational modifications have been highlighted as well, especially in the mucin O-glycosylation processes\textsuperscript{14,15,16,17,18}. Mechanistic studies suggested that changes in the glycosylation of mucins rather than the proteins themselves may occur as the direct result of local inflammation induced by proinflammatory mediators, such as interleukin 1\textsuperscript{19,20}. Overall, the altered expression of the mucins in pSS saliva and tears has appeared to reduce the rheology of the salivary flow and the tears, ultimately contributing to oral and ocular dryness\textsuperscript{19,20}.

In this descriptive study, therefore, we aimed to investigate whether a miRNA-mediated mechanism could be involved in salivary dysfunction, influencing salivary proteome and mucin alterations. More specifically, we first identified miRNA differentially expressed in pSS patients with decreased salivary flow compared to pSS patients with normal salivary flow and to healthy controls. Subsequently, we examined the pathways targeted by the putative deregulated miRNA, including the mucin-type O-glycosylation pathway, to ultimately evaluate the effect of epigenetics on salivary rheology.

**MATERIALS AND METHODS**

**Patients and sample collection.** We selected for this study minor salivary gland biopsies (MSGB) from patients newly diagnosed with pSS [American-European Consensus Group (AECG) criteria 2002]\textsuperscript{21}. All the patients underwent a complete examination for the diagnosis of the disease, including sialometry. We defined patients as having a low salivary flow (LF) if their unstimulated salivary flow rate (USFR) was ≤ 1.5 ml/15 min and as having a preserved salivary flow (i.e., high flow, HF) if their USFR was >1.5 ml/15 min. For the purposes of this study, to reduce the heterogeneity derived from different degrees of inflammation in the minor salivary gland samples, we excluded pSS patients with a low or moderate infiltrate, and we retrieved only biopsies with a focus score ≥ 322. Subsequently, we subdivided our samples into 2 groups: (a) biopsies from patients having an HF, and (b) biopsies from patients having an LF. Controls were represented by patients undergoing an MSGB under the suspicion of pSS, who at the end of the examination were not diagnosed with pSS and that at the MSGB histology presented a mild aspecific inflammation. All patients gave written informed consent for all procedures, which were carried out with local ethics committee approval (Comitato per la Sperimentazione Clinica dei Mediciinali, University of Pisa, prot N° 3062/2010).

**Minor salivary gland biopsy.** Minor salivary gland samples, formalin-fixed, paraffin embedded, sectioned and H&E-stained, were evaluated by the same pathologist. If a diagnosis of focal lymphocytic sialadenitis was made, the focus score (FS) was then determined according to the method of Greenspan, et al\textsuperscript{23}. A focus was defined as an aggregate of ≥ 50 mononuclear cells (mostly lymphocytes). The FS was reported as the number of foci per 4 mm\textsuperscript{2} of tissue, up to a maximum of 12 foci. Only samples with an FS ≥ 3 were selected for the study, to limit the samples to those homogeneous in their inflammatory infiltrate composition and to minimize the effect of inflammation on salivary gland epithelium function.

**Sialometry.** According to the AECG 2002 criteria\textsuperscript{21}, patients were asked to sit quietly, without talking or chewing, and spit any saliva that accumulates in the floor of their mouth into a pre-weighed tube for a total of 15 min.

**RNA extraction.** Total RNA was purified with the miRNeasy mini kit (Qiagen), following the manufacturer’s recommendations. RNA concentration was evaluated using a NanoDrop spectrophotometer (Thermo Fisher Scientific).

**TaqMan low-density arrays (TLDA) for miRNA profiling.** MicroRNA profiling of samples was done with TaqMan Array Human MicroRNA panels A and B (Life Technologies, Thermo Fisher Scientific) to analyze 754 human miRNA. Reverse transcription and pre-amplification were done following the manufacturer’s instructions (Life Technologies, Thermo Fisher Scientific). Quantitative real-time PCR (qRT-PCR) was performed with the Applied Biosystems 7900 HT Real-Time PCR system. For each miRNA, the expression level was determined by the equation 2-ΔΔCT\textsuperscript{24}. U6 snRNA (001973) was used as a control to normalize data. Gene Expression Suite software (v1.0.4) and Data Assist software (v 3.01; Life Technologies, Thermo Fisher Scientific) were further used to process the array data.

In silico analysis. A computational analysis was performed to identify genes and pathways that might be modulated by the deregulated miRNA that we identified. For the in silico analysis, we used DIANA-miPath v.3\textsuperscript{25} to identify potential miRNA target genes and pathways (as settings, we used microT-CDS for target prediction with a threshold of 0.85). Results were visualized with a p value threshold of 0.05 using the option “genes union,” according to the Kyoto Encyclopedia of Genes and Genomes (KEGG). In addition, we used Cytoscape\textsuperscript{26} to visualize selected miRNA target genes’ interaction network.

**Analysis of glycosylation enzyme genes.** The cDNA for each RNA sample was obtained using RT\textsuperscript{1} First Strand kit (SABioscience Corporation, Qiagen), according to the manufacturer’s instructions. The expression levels of glycosyltransferases and glycosidases were analyzed using RT\textsuperscript{2} Profiler PCR Array — Human Glycosylation (PAHS-046ZC; SABiosciences Corp., Qiagen), following the manufacturer’s instructions. An Applied Biosystems StepOne Plus PCR System (Life Technologies, Thermo Fisher Scientific) was used for measurements. The PCR array data were analyzed by RT\textsuperscript{2} Profiler PCR Data Analysis software (SABiosciences Corp.). Relative quantification for each gene was assessed by 2-ΔΔCT calculation for each miRNA. All test samples were run in duplicate and template-negative reactions served as controls. Expression of each gene was classified as high or low, based on the level of expression after grouping patients.

**Statistical analysis.** qRT-PCR data processing and analysis were conducted using tools from RQ-manager (v1.2, Life Technologies, Thermo Fisher Scientific), Expression Suite software (v1.0.4, Life Technologies, Thermo Fisher Scientific), Microsoft Excel and Prism GraphPad V5.0d software (GraphPad Software). In addition, real-time PCR array data were analyzed using the RT\textsuperscript{2} Profiler PCR Array Data Analysis program (SABioscience, Qiagen).

To summarize the most relevant features of patients with pSS, descriptive statistics were used. In particular, for categorical variables we reported the distribution of absolute frequencies, percentages, and cumulative, while for continuous variables the mean and standard error of the mean.

To evaluate differences between pSS samples, ANOVA was used. Bartlett’s test was also applied to assess the homoscedasticity assumption. P values < 0.05 were considered statistically significant. All statistical
analyses were performed using STATA 13.1 and R 3.3. To visualize principal component analysis (PCA), the R functions “prcomp” and “plot” were used.27,28.

RESULTS

Cellular miRNA expression profile (miRNome) significantly changes in patients with pSS. To study the role of miRNome in salivary glands of pSS, we analyzed miRNA expression by qRT-PCR arrays in human MSGB from non-SS controls (n = 5), pSS patients with HF (n = 5), and those with LF (n = 5). Controls (i.e., non-SS controls) were represented by 5 women aged 54.9 ± 17 years with sicca symptoms, negative anti-Ro/SSA, and a mild aspecific sialoadenitis in the MSGB. Regarding patients with pSS, we chose to investigate the miRNome in pSS with an FS ≥ 3, but different salivary flow phenotypes, to reduce the variability of B cell infiltrates that might cause misleading results in the salivary flow mechanism comprehension. No statistically significant difference in the FS was observed between HF and LF pSS patients. Supplementary Table 1 (available with the online version of this article) summarizes the clinical and serological features of the patients enrolled in the study. As shown in Figure 1A, we found that the expression levels of several miRNA have a very specific pattern and the samples strongly cluster following the salivary flow stratification and with clinical characteristics.

To evaluate whether the TLDA results could separate controls, HF samples, and LF samples, an unsupervised PCA using the 126 significant differentially expressed miRNA in pSS samples was performed (Figure 1B).

The PCA showed that LF and control samples clustered into 2 separate populations, indicating that the 2 groups have a very different miRNA expression profile, while miRNA expression levels in HF are intermediate between controls and LF samples, corroborating the heat map diagram results.

Among 754 human miRNA on the arrays (Figure 1A and 1B), our analysis displayed a unique pattern of miRNA expression, highlighted by a marked overexpression of members of let-7, miR-30, miR-17/92, and miR-200 microRNA families, in pSS samples relative to controls. Interestingly, the overexpression in pSS shows a trend that is inversely proportional with the impairment of salivary flow rates (Supplementary Figure 1, available with the online version of this article).

We selected 126 microRNA that were significantly deregulated in pSS compared to control (Supplementary Table 2, available with the online version of this article). Among these 126 microRNA, 100 showed a very interesting expression pattern, with an overexpression inversely proportional to the salivary flow rate, suggesting a global expression change in the miRNome of impaired salivary glands. In Figure 2, we show 6 representative graphs that represent the expression level of 4 upregulated miRNA (hsa-miR-18b, hsa-miR-20a, hsa-miR-106a, hsa-miR-146b) with an inversely proportional pattern to the salivary flow rate, and 2 downregulated (hsa-miR-635 and hsa-miR-372) with a directly proportional pattern to the salivary flow rate.

MiRNA target prediction and pathway enrichment analysis. To identify potential pathways that could be targeted by the most 100 deregulated miRNA in the pSS samples (fold change ≥ 2; p < 0.05), we performed a computational analysis using DIANA-miRPath software.

KEGG pathways that theoretically might be affected by the upregulated miRNA are reported in Figure 3A, and among which the most significant (p = 2.98e-13) was the “Mucin type O-Glycan biosynthesis” pathway.

We reported the list of the deregulated miRNA used for the bioinformatics analysis and of the predicted target genes of the pathway “Mucin type O-Glycan biosynthesis” in Supplementary Table 2 (available with the online version of this article).

Because glycosylated mucins are important for saliva rheological properties19,29 and to confirm in silico analysis, we decided to investigate glycosyltransferase and glycosidase expression level changes in patients with pSS and to evaluate the possible correlation with salivary flow.

Human glycosylation profiling analysis in patients with pSS. A total of 84 genes encoding enzymes that add glycans to proteins or remove glycans from glycoproteins were analyzed using Human Glycosylation RT² Profiler PCR Array in 4 healthy volunteers, 4 pSS patients with HF, and 4 patients with LF. The samples used in miRNA and gene expression arrays were the same. The array results for the 3 groups are reported in Supplementary Table 3 (available with the online version of this article).

Hierarchical clustering showed systematic variations in the gene expression among the different groups, confirming that pSS samples (HF and LF) had similar gene expression profiles, and both were different from controls (Figure 3B). As shown in volcano plots (Figure 3C and 3D), using a threshold value of 2-fold expression change and p < 0.05, we found a gene expression deregulation of some glycosyltransferases and glycosidases.

In particular, compared to controls, 1 gene was significantly overexpressed (ST8SIA4) and 4 were downregulated (B4GALT2, B4GALT3, GALNT1, GALNT16) in both pSS groups (HF and LF; Figure 3C and 3D). Other genes were significantly downregulated in HF or LF compared to controls, as reported in Supplementary Table 3 (available with the online version of this article). More specifically, we highlighted a trend for the expression of other genes, such as EDEM2, GALNT4, GLB1, which were highly downregulated in LF (p < 0.05) and less in HF, compared to controls.

Collectively, these data suggest that different predicted miRNA-target genes in the mucin type O-glycan biosynthesis pathway were altered and that miRNome could influence the glycosidase expression levels and consequently the glycosylation of mucins (Figure 4).
Figure 1. A. Unsupervised hierarchical clustering and analysis of miRNA expression profiles in controls and pSS samples. Expression data of 784 miRNA were obtained with Custom TaqMan Array MicroRNA Cards (Life Technologies, Thermo Fisher Scientific), analyzed using RQ-manager (v1.2) and DataAssist (v3.01). The 126 deregulated miRNA in pSS samples compared to controls are shown in the heat map. The distance measured is Pearson’s distance, and the clustering method is average linkage. Each column corresponds to the sample expression profile, and each row corresponds to an miRNA. The miRNA clustering tree is shown on the left. Branch lengths represent the degree of similarity between individual miRNA. Red and black colors indicate relatively high and low expression, respectively. B. Principal component analysis was performed on all samples (n = 5 for each group) and on the top 126 miRNA with p ≤ 0.05. Normalized values (Log_{10} 2^{-dCT}) were used for the analysis. Red = control group; green = HF group; blue = LF group. There is clear separation of LF and control groups, demonstrating a clearly different miRNA expression profile. miRNA: microRNA; pSS: primary Sjögren syndrome; HF: high salivary flow; LF: low salivary flow; CNT: controls; PC: principal component; PCA: PC analysis.
Figure 2. Expression levels of 6 deregulated miRNA in controls, HF, and LF patients with pSS. Values were normalized to U6 levels and expressed as the fold increase over controls. Data are shown as the mean ± SD. pSS: primary Sjögren syndrome; HF: high salivary flow; LF: low salivary flow; miRNA: microRNA.
Figure 3.
DISCUSSION
Pathogenetic mechanisms responsible for salivary hypofunction in pSS have been the object of various studies, especially because the correlation between salivary gland inflammation and dysfunction has still not been completely clarified.

In our present exploratory study we investigated miRNA that might be implicated in pSS salivary hypofunction and
altered rheology in pSS by array analysis of MSGB from pSS patients with HF or LF, as well as non-SS sicca controls. We identified 126 deregulated microRNA that were inversely associated with the impairment of salivary flow rates. Either overexpressed or downregulated, these microRNA followed perfectly the salivary flow rates in a proportional way. The plus/minus microRNA presence following the plus/minus salivary production strongly suggested a direct role of the global miRNome of impaired salivary glands.

In addition, among 754 human miRNA investigated, we found a marked overexpression of miR-30, miR-17/92, miR-200, and miR-let-7 family members, in pSS compared to controls. These miRNA families have been described as involved in several physiological and pathological processes including oncogenesis, heart development, lung development, repression of cancer stem cell differentiation, modulation of cell division and apoptosis, and cell differentiation and proliferation. Gourzi, et al. found that the levels of miR200b-3p were upregulated in salivary gland epithelial cells (SGEC) of patients with SS compared to sicca-complaining controls. The MSG levels of let7b were correlated positively with Ro52/TRIM21-mRNA. MiR200b-3p was correlated negatively with Ro52/TRIM21 and Ro60/TROVE2 mRNA in SGEC, whereas let7b and miR200b-5p were associated with La/SSB-mRNA. In peripheral blood mononuclear cells, let-7b were correlated with Ro52/TRIM21, whereas let-7b were also associated with La/SSB-mRNA expression. Significantly lower miR200b-5p levels were expressed in SS patients with mucosa-associated lymphoid tissue lymphoma compared to those without. In our study, for the first time to our knowledge, these miRNA families were associated with the impairment of salivary flow rates.

To assess the global effect of differentially expressed miRNA in pSS samples, an in silico approach was used to identify the major signaling pathways that could most likely be affected by the co-expressed miRNA.

We found that several upregulated miRNA in patients with pSS predicted the targeting of genes implicated in mucin type-O glycosylation. Indeed, qRT-PCR analysis confirmed the downregulation of some glycosyltransferase and glycosidase genes in pSS samples compared to controls, suggesting that the altered salivary function and glycosylation in pSS may be regulated by miRNA.

In particular we found that GALNT1, the predicted target of many upregulated miRNA (miR-24-2-5p, miR-23a, miR-590-3p, miR-30b, miR-335-3p, miR-539, miR-323-3p, miR-192-3p, let-7g, let-7d, let-7e, miR-7 1-3p, miR-543, and let-7c), was downregulated in patients with pSS. GALNT1 is responsible for glycosylation of mucin-7 (MUC7), a large, very heavily O-glycosylated salivary protein (150 kDa) containing central mucin domains, rich in serine and threonine residues that covalently bind the O-glycosylation important for salivary viscoelastic behavior and lubrication of the oral mucosa.

Notably, a dramatic reduction in MUC7 glycosylation was found in patients with pSS and it was associated with oral dryness. Chaudhury, et al., in particular, observed that a decrease in the extended, fucosylated core 2 disialylated structure on MUC7 was significantly associated with the perception of oral dryness in patients with pSS.

Several other genes implicated in glycosylation were significantly different between SS and controls including GALNT2, GALNT4, B4GALT5, ST3GAL1, CIGALT1, CIGALT1CI, and GCNT1. A few genes (i.e., EDEM2, GALNT4, and GLBI) tend to be differently expressed in SS patients with LF compared to patients with HF. However, although the pattern of miRNA expression differs between SS patients with LF or HF and associates with altered glycosylation pathway in array analyses, the expression of the major genes that are implicated in glycosylation did not support this difference.

It is possible that we were not able to highlight a significantly different expression in glycosylation genes between the 2 SS subgroups because of different biological biases. First, we know that the miRNA mechanism of action is through either mRNA degradation or suppression of mRNA translation. In particular, regulation of glycogenes (themselves proteins) occurs at multiple steps, including transcriptional, translational, and posttranslational levels. Although transcript (i.e., cellular mRNA) levels are often used as a key indicator of what glycogenes are being translated and the levels of biosynthetic enzymes and glycans that result, the relationship is not straightforward. However, changes in glycan structures upon perturbation do not correlate with changes in glycogene mRNA expression levels. Although part of this discrepancy may be due to difficulties with quantitatively measuring low-abundance glycogene transcript levels, the posttranscriptional regulation of glycogene expression may also control carbohydrate structures. Because the relationship miRNA-glycogenes mRNA is often nonlinear, this may reflect the complexity of the system regulation, although there is in the SS samples a significantly reduced glycogenes mRNA presence (confirming the altered glycosylation in pSS, as reported by other groups). We are aware that the sample size may reflect this assay instability, but the correlation of a cellular pathway as the O-glycosylation pathway may be the link between the miRNA profiling and the already well-known glycosylation decrease on mucins in patients with SS.

Despite these limitations, our study may have several points of strength. First, our experiments are consistent with previous studies on miRNome profiling in pSS. We substantiated previous findings on the deregulation of a large number of microRNA in pSS MSGB. In addition, the use of TLDA for miRNA profiling allowed us to describe a novel list of miRNA and miRNA families potentially implicated in the disease, thus broadening the knowledge of epigenetics contribution in pSS pathogenesis. More remarkably,
our set of descriptive observations indicated that miRNA might be implicated in the deregulated glycosylation and hyperfunction of salivary glands in pSS. However, our research has a number of limitations as well. Given that our findings are based on descriptive and not functional studies, indeed larger functional investigation on MUC7 O-glycosylation should be undertaken to confirm the link between miRNA deregulation, altered mucin glycosylation, and pSS-related hyposalivation. Moreover, owing to the restricted number of samples analyzed, data experiments require validation in a replication cohort.

Nevertheless, our exploratory study still represents a first interesting attempt to explain how the concomitant use of complementary innovative “omics” techniques may be useful to foster the comprehension of complex pathogenetic mechanisms underlying different phenotypic subsets of pSS. From this point of view, deregulated miRNA could represent not only biomarkers for distinct subsets of the disease but also putative targets for future therapies.

ONLINE SUPPLEMENT
Supplementary material accompanies the online version of this article.

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