Comparison of MicroRNA Expression in Tears of Normal Subjects and Sjögren Syndrome Patients

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Purpose. Deregulated expression of several microRNAs (miRNAs) in sera or salivary glands of patients with Sjögren syndrome (SS) has been reported. However, none have investigated miRNAs in samples that can represent lacrimal glands. We compared the miRNAs expression in the tears of SS patients and healthy controls. Moreover, we investigated the correlation between miRNAs expression and ocular staining score (OSS).

Methods. Individual tear samples were collected from 18 SS patients and 8 age-matched controls. Clinical ophthalmologic assessments included Schirmer I test, tear film breakup time (tBUT), and OSS. The expression of 43 different miRNAs in tears was measured using real-time polymerase chain reaction, and compared between the SS patients and controls. And we also compared between the three groups of control, primary SS, and secondary SS patients. The correlation between the miRNA expression and OSS was evaluated.

Results. The expression levels of miR-16-5p, miR-34a-5p, miR-142-3p, and miR-223-3p were significantly upregulated in patients with SS when compared with those in the control group ($P < 0.05$). The expression of 10 miRNAs (miR-30b-5p, miR-30c-5p, miR-30d-5p, miR-92a-3p, miR-134-5p, miR-137, miR-302d-5p, miR-365b-3p, miR-374c-5p, miR-487b-3p) was significantly downregulated in the SS patients ($P < 0.05$). Eight miRNAs showed statistically significant differences between the three groups of control, primary SS and secondary SS. All 14 miRNAs with significant differences in SS patients and control group were not significantly correlated with OSSs.

Conclusions. The 14 differentially expressed miRNAs may be involved in the pathogenesis of SS, in particular, related to autoimmunity and neuropathy.

Keywords: biomarkers, microRNAs, miRNAs, Sjögren syndrome, tear

Sjögren syndrome (SS) is a gradual and progressive autoimmune disease characterized by the lymphocytic and plasma cell infiltration of exocrine glands such as lacrimal and salivary glands. It is estimated that the worldwide incidence of SS is 7 per 100,000 people, with the highest incidence rates in Europe and Asia, reaching up to 43 per 100,000 people.[1] Lacrimal gland involvement in SS often leads to aqueous deficient dry eye, which is classically associated with a marked decrease in tear production and severe ocular surface inflammation.[2] Dry eye associated with SS is often more severe than non-SS dry eye.[3] Nevertheless, the pathogenesis of dry eye in SS is poorly understood because lacrimal gland biopsy is relatively invasive compared with that of the minor salivary glands.

MicroRNAs (miRNAs) are a group of endogenous small noncoding RNAs, approximately 20 to 25 nucleotides in length, which regulate gene expression posttranscriptionally.[4,5] MiRNAs regulate mRNA degradation or translational interference by binding to a complementary sequence present in the 3′-untranslated regions (UTR) of target gene mRNAs.[6] MiRNAs play significant roles in several cellular processes, including cell differentiation, proliferation, migration, apoptosis, and stem cell maintenance.[7–9] Recent studies demonstrated that miRNAs can be released by cells and tissues in a cell-free form in several biological fluids including serum and tears.[10,11] Circulating miRNAs are extremely stable; they even withstand repetitive freezing/thawing cycles and RNases. Abnormal circulating miRNAs were initially observed in patients with cancers[12–14] and presently their application has been extended to other diseases.

Although several studies on miRNA expression in SS patients have been reported, few of these have investigated the miRNA expression of the dry eye in SS patients. In minor salivary glands of SS patients, deregulated expression of several miRNAs was described and their association with salivary glandular dysfunction was explained.[15] In addition, the elevated expression of miR-146a and miR-155 in peripheral blood mononuclear cells (PBMC) of patients with SS has been reported.[16,17] Nevertheless, little is known about the role of miRNAs in the dry eye of SS patients.

We conducted a preliminary study using a commercial array (miScript miRNA PCR Array Human Pain: Neuropathic & Inflammatory, MIHS-120ZF; Qiagen, Valencia, CA, USA) to compare the miRNA expression in the tear of SS patients and controls. In our preliminary study, we choose miRNAs that were abundantly expressed in the tears and significantly different between the two groups. This study was carried out

using miScript miRNA PCR Array Custom to analyze the aforementioned miRNAs.

Therefore, we investigated the expression of miRNAs in the tears of SS patients and compared them to healthy controls. And we also compared them between the three groups: control, primary SS, and secondary SS patients. Concurrently, we also examined the correlation between miRNA expression and ocular staining scores.

Methods

Patient Selection and Ophthalmologic Examination

We collected 18 tear samples from SS patients (mean age: 47.22 ± 11.56 years, range: 26–62 years) and eight from normal subjects (mean age: 42.5 ± 11.78 years, range: 27–62 years) at the Hanyang University Hospital. Among SS patients, nine patients had primary SS and nine patients had secondary SS. This study followed the tenets of the Declaration of Helsinki. Research protocols were approved by the Institutional Review Board (IRB) of Hanyang University Hospital (IRB number: HYUH 2018-10-013-003) and all patients gave their informed consent. The patients in this study did not use any eye drops or used only preservative-free artificial tears. A rheumatologist diagnosed SS, according to the 2002 American-European Consensus Group international classification criteria. Eight healthy subjects who revealed no symptoms of ocular irritation and had normal tear function (>20 mm on the Schirmer I test) and clear cornea were used as controls.

Clinical ophthalmologic assessments included Schirmer I test, tear film breakup time (tBUT), and ocular staining score (OSS). Tear production was assessed by the Schirmer I test, in which the extent of tear flow down a piece of filter paper inserted into the lateral part of the inferior fornix of the eye was measured over a 5-minute period. Tear breakup time was measured to assess the tear film stability. The OSS was assessed via the Sjögren International Collaborative Clinical Alliance (SICCA) method.[18]

Sample Collection

Basal tear fluid was collected atraumatically from the inferior tear meniscus of both eyes, using micropipettes (Eppendorf, Hamburg, Germany) before the clinical test. Care was taken to avoid touching the corneal and conjunctival surfaces. In the case of few tears in patients with SS, tears were obtained by the same method after instillation of 2.5 μL of distilled water. Tear samples were placed in microtubes and stored at –80°C until further examination.

Total RNA Extraction, Complementary DNA (cDNA) Synthesis, Preamplification, and Real-Time PCR

All collected tear samples were homogenized in reagent (QIazol; Qiagen). Total RNA including small RNAs and miRNAs was isolated from the tear samples, using a serum/plasma kit (miScript; Qiagen) according to the manufacturer's instructions. RNA samples were stored at –80°C until cDNA reaction. The isolated RNAs were reverse-transcribed into cDNA using a serum plasma kit (miScript II RT Kit; Qiagen). Prior to PCR, cDNA samples were preamplified using a PCR kit (miScript PreAMP; Qiagen) as well as a primer mix (miScript; Qiagen). All reactions were performed as specified in the protocols by the manufacturer. The miRNA expression profiling was performed using a customized PCR array (Qiagen) of selected 43 miRNAs of interest. We conducted a preliminary study using a PCR array (Qiagen) to compare the miRNA expression in the tear of SS patients and controls. In our preliminary study, we choose miRNAs which were abundantly expressed in the tears and significantly different between the two groups. This study was carried out using a custom PCR array to analyze the aforementioned miRNAs. Quantitative real-time PCR reactions (qRT-PCR) were carried out via SYBR Green-based RT-PCR with an RT-PCR device (Light Cycler 480; Roche, Basel, Switzerland), following the manufacturer's protocol.

Normalization and Relative Quantification of Tear MiRNA Expression

To eliminate the normalization issue for miRNA expression in the tears, depending on the absence of stable RNA, we used the global mean normalization method for normalizing serum/plasma miRNA expression.[19] The global mean normalization of the miRNA qRT-PCR data was performed via The Gene Global Data Analysis Center (Qiagen, Germantown, MD, USA). The relative expression of miRNAs was calculated using the comparative delta delta Ct (ΔΔCT) method. Fold changes were calculated using the equation $2^{-\Delta\Delta Ct}$.[20,21]

Statistical Analysis

The data are presented as the means and standard deviation (SD). Differences between the control and SS patients were estimated with a two-tailed Mann–Whitney *U*-test. Differences in miRNA levels between the three groups, control, primary SS, and secondary SS patients, were analyzed using Kruskal-Wallis test. Only the candidate miRNAs that showed significant differences between the control group and SS patients ($P < 0.05$) were selected for correlation analysis. Spearman correlation analysis was used for the correlation studies. All analyses were performed using statistical analysis software (SPSS 17.0; SPSS, Inc., Chicago, IL, USA). A value of $P < 0.05$ was considered indicative of statistical significance.

Results

Clinical Characteristics of the Subjects

In the present study, 18 patients with SS (mean age: 47.22 ± 11.56 years; range: 26–62 years) and 8 controls (mean age: 42.5 ± 11.78 years; range: 27–62 years) were included. Of these, 16 were female and 2 were male. No significant differences were observed in the distribution of age and sex between SS patients and controls. Nine patients had primary SS and nine had secondary SS. Among the patients with secondary SS, six had rheumatoid arthritis (RA), and three had systemic lupus erythematosus (SLE). The demographic, clinical, and laboratory data of the SS patients are summarized in Table 1.

Differential Expression of MicroRNAs Between SS Patients and Controls

The miRNA expression levels were compared between the SS patients and control group using the Mann–Whitney *U*-test. We found that four miRNAs were upregulated in SS patients compared with that in the controls and 10 miRNAs were downregulated in SS patients compared with that in the normal controls (Table 2). Expression levels of miR-16-5p, miR-34a-5p, miR-142-3p, and miR-223-3p in patients with SS were significantly higher than those in the controls. Expression levels of miR-30b-5p, miR-30c-5p, miR-30d-5p, miR-92a-3p, miR-134-5p, miR-137, miR-302d-5p, miR-365b-3p, miR-374c-5p,

Table 1. Demographic and Clinical Characteristics of SS Patients

	Mean ± SD
Demographic	
Age, y	47.22 ± 11.56
Sex (*n*), female/male	16/2
Primary SS/secondary SS, *n*	9/9
Clinical features	
Schirmer test, mm	5.53 ± 6.63
tBUT, s	2.40 ± 1.67
OSS (score)	4.00 ± 3.24
	Total, *n* (%)
Rheumatic comorbidity	
SLE	6 (33.3)
RA	3 (16.7)
Laboratory findings	
Anti-Ro/SSA positivity	18 (100)
Anti-La/SSB positivity	9 (50)
Rheumatoid factor positivity	16 (88.9)

and miR-487b-3p in patients with SS were significantly lower than those in controls.

Differential Expression of MicroRNAs Between Controls, Primary, and Secondary SS Patients

We included 18 patients: 9 with primary SS and 9 with secondary SS. We compared miRNA expression between the three groups of control, primary and secondary SS groups (Table 3). Eight miRNAs showed statistically significant differences between the three groups using the Kruskal-Wallis test. Comparison between the two groups using Mann-Whitney *U*-test revealed significant decreases in the expression of miR-30b-5p, miR-30c-5p, miR-30d-5p, miR-92a-3p, and miR-365b-3p and significant increase in the expression of miR-142-3p in primary SS patients compared with the controls. There was a significant difference in five miRNAs between the secondary SS patients and the control group. In patients with secondary SS, mir-16-5p, and miR-142-3p were upregulated and miR-137, miR-302d-5p, and miR-374c-5p were downregulated compared to the controls. There was no significant difference between the primary and secondary SS patients except for miR-146a-5p ($P = 0.027$, data not shown).

Correlation Between OSS and the miRNA Level in SS Patients

The correlation between the OSS score and level of miRNAs that were significantly different in SS group compared to those in the control group was analyzed (Table 4). All miRNAs were not significantly correlated with the OSS scores.

Discussion

SS is characterized by systemic autoimmunity, inflammation, and dysfunction in the exocrine organs, primarily the salivary and lacrimal glands.[22] Patients with SS present several symptoms including oral, ocular, and extraglandular manifestations. Of these, about 25% patients initially present with extraglandular symptoms including arthralgia, myalgia, and fatigue; 25% reveal ocular discomfort, and 50% show oral symptoms such as xerostomia and dental decay.[23] In addition, the severity of symptoms and signs varies widely among patients with SS. However, it is not known why SS patients have different symptoms and varying degrees of these symptoms.

Several studies on miRNA expression in patients with SS have been reported. Some studies reported the overexpression of miR-146a and miR-155 in the PBMC of patients with primary SS.[24–26] Gourzi et al.[27] identified miRNAs suspected to target Ro/SSA and La/SSB mRNAs in primary SS as follows: let-7b, miR-16, miR-181a, miR-200b-3p, miR-200b-5p, miR-223, and miR-483-5p. The overexpression of miR-16 in minor salivary glands (MSGs), miR-200b-3p in salivary gland epithelial cells (SGECs), and miR-223 collectively with miR-483-5p in PBMCs of SS patients compared to those in the sicca complaining controls has been reported.[27] Several studies have examined miRNA expression in sera, salivary glands, or SGECs of SS patients; however, none have investigated miRNAs in samples that can represent lacrimal glands.

Tear fluid, as part of the lacrimal functional unit, contains various proteins, lipids, and metabolites in a dynamic state. Tears act as biomarkers for ocular diseases such as dry eye syndrome because it is relatively difficult to perform a biopsy on the lacrimal glands. Tear is an obvious source for a noninvasively obtainable biomarker in SS since it is a direct product of the lacrimal gland. To the best of our knowledge, this is the first study in which the profiles of miRNAs in tears of SS patients were analyzed and compared with those in healthy controls.

In this study, we found 14 miRNAs with significantly different expression in SS patients compared to that in the controls. The expression of miR-16-5p, miR-34a-5p, miR-142-3p, and miR-223-3p was increased, and the expression of miR-30b/c/d-5p, miR-92a-3p, miR-134-5p, miR-137, miR-302d-5p, miR-365b-3p, miR-374c-5p, miR-487b-3p was decreased in the tears of the SS patients compared with those in the controls. There were significant differences in eight miRNAs between the three groups of the control, primary and secondary SS patients. MiRNAs that differed significantly from the control group were different between the primary and secondary SS groups, but there were similar trends of increase and decrease. And there was no significant difference between primary and secondary SS patients except for miR-146a-5p. This suggests that the pathogenesis of primary and secondary SS may be similar or that miR-146a-5p may play an important role in the pathogenesis of SS. Further studies with more subjects are needed.

SS is a multifactorial syndrome, involving environmental factors, genetic predisposition, and hormonal factors in the presence of the innate and acquired immune system costimulation.[28,29] Although the pathogenesis of SS remains unclear, the autoimmunity is considered to be the key player in the syndrome development. Cytokine production, T lymphocytes, B-cell activating factor (BAFF), and autoantibodies secreted by B lymphocytes were found in the target tissue of SS and the salivary and lacrimal glands.[29] Recently, the emerging theory states that autoimmunity is linked to chronic neuropathy in SS.[30] The theory of the relationship between the neural and immune mechanisms of SS manifestations states that lesions of the autonomic and peripheral neural system reduce the threshold for inflammatory and noxious events and disturb the balance of inflammatory mediators.[30]

MiR-16, miR-142, and miR-223 are highly expressed in the hematopoietic cells and are crucial during hematopoietic lineage differentiation. MiR-16 and miR-142 substantially altered the T lymphoid lineage differentiation as one of the most abundant miRNAs in T cells.[31,32] MiR-223 is required for differentiation of various myeloid cells including neutrophils, basophils, and mast cells from common myeloid precursor cells and enhanced T cell differentiation.[32] The decrease in the miR-17-92 cluster was associated with accumulation of mature

Table 2. Expression of Tear microRNAs in Patients With SS as Compared to Control Group

MicroRNA	SS ΔCt* (mean ± SD)	Control ΔCt* (mean ± SD)	Fold Changes†	*P* Value
miR-15b-5p	1.55 ± 1.25	1.78 ± 0.89	1.17	0.541
miR-16-5p	−0.24 ± 1.22	0.98 ± 0.56	2.34	0.012‡
miR-17-5p	3.97 ± 1.07	3.73 ± 0.93	−1.19	0.617
miR-20a-5p	2.15 ± 0.82	1.94 ± 0.80	−1.16	0.505
miR-20b-5p	4.35 ± 0.98	5.35 ± 0.94	1.99	0.075
miR-21-5p	−5.58 ± 1.76	−4.82 ± 1.13	1.69	0.120
miR-23b-3p	−0.52 ± 1.57	−0.96 ± 0.46	−1.36	0.267
miR-25-3p	2.01 ± 1.17	1.42 ± 0.75	−1.5	0.120
miR-29a-3p	−0.57 ± 1.28	−0.33 ± 0.79	1.18	0.617
miR-30a-3p	4.24 ± 1.09	3.99 ± 1.12	−1.18	0.470
miR-30b-5p	2.10 ± 1.15	1.01 ± 0.73	−2.14	0.020‡
miR-30c-5p	4.07 ± 1.61	2.26 ± 1.75	−3.5	0.020‡
miR-30d-5p	2.35 ± 0.96	1.45 ± 0.68	−1.87	0.026‡
miR-34a-5p	1.85 ± 1.43	2.99 ± 2.18	2.21	0.046‡
miR-92a-3p	0.26 ± 1.07	−0.53 ± 0.55	−1.74	0.011‡
miR-93-5p	3.51 ± 1.03	3.33 ± 0.50	−1.13	0.739
miR-99a-5p	2.88 ± 1.11	2.26 ± 1.17	−1.54	0.149
miR-100-5p	2.54 ± 1.02	2.14 ± 1.15	−1.32	0.291
miR-103a-3p	4.03 ± 1.44	4.90 ± 0.79	1.82	0.149
miR-124-3p	4.54 ± 1.53	3.91 ± 0.74	−1.55	0.085
miR-125b-5p	0.48 ± 1.49	−0.31 ± 0.56	−1.73	0.165
miR-127-3p	1.62 ± 2.19	1.36 ± 0.32	−1.19	0.059
miR-132-3p	3.84 ± 1.03	4.42 ± 1.05	1.5	0.165
miR-134-5p	6.48 ± 1.63	5.75 ± 0.72	−1.66	0.046‡
miR-137	6.65 ± 1.71	5.75 ± 0.72	−1.86	0.035‡
miR-142-3p	2.88 ± 2.11	4.76 ± 1.42	3.69	0.012‡
miR-142-5p	5.96 ± 1.50	5.13 ± 0.91	−1.78	0.067
miR-143-3p	5.65 ± 1.47	5.42 ± 0.58	−1.18	0.317
miR-145-5p	2.82 ± 1.39	2.17 ± 0.71	−1.57	0.096
miR-146a-5p	0.10 ± 1.62	0.59 ± 1.81	1.41	0.470
miR-146b-5p	4.58 ± 1.57	4.42 ± 1.52	−1.12	0.912
miR-152-3p	3.22 ± 1.25	3.10 ± 0.58	−1.09	0.956
miR-155-5p	5.54 ± 2.18	5.50 ± 0.58	−1.03	0.437
miR-182-5p	4.75 ± 1.34	4.46 ± 1.04	−1.22	0.505
miR-183-5p	3.89 ± 1.24	3.93 ± 1.43	1.03	0.824
miR-203a-3p	−0.53 ± 1.51	−1.72 ± 1.35	−2.28	0.067
miR-221-3p	1.98 ± 1.26	2.30 ± 1.09	1.25	0.541
miR-223-3p	−2.25 ± 2.34	−0.90 ± 1.04	2.55	0.040‡
miR-302d-5p	6.42 ± 1.59	5.46 ± 0.60	−1.94	0.013‡
miR-338-5p	1.97 ± 1.52	1.32 ± 0.56	−1.57	0.085
miR-365b-3p	2.74 ±1.07	1.84 ± 1.00	−1.86	0.040‡
miR-374c-5p	6.41 ± 1.93	4.58 ± 1.34	−3.56	0.020‡
miR-487b-3p	6.59 ± 1.68	5.75 ± 0.72	−1.78	0.040‡

* ΔCt; delta threshold cycle; Ct (gene of interested)–Ct (housekeeping gene).
† Fold changes; log2 fold change; positive values indicate elevated and negative values reduced levels in SS samples, respectively.
‡ Indicating a value of $P < 0.05$.

B cells and pro-B cells with a marked reduction of pre-B events that have been linked to lymphoproliferative disease and autoimmunity.[33,34] In our study, miR-16-5p, miR-142-3p, and miR-223-3p levels were significantly increased in patients with SS compared to those in the control, and miR-92a-3p level was significantly decreased, and these miRNAs would be involved in the pathogenesis of SS by participating in the development and differentiation of immune cells.

In previous studies, several miRNAs have been identified as biomarkers with a difference in their expression in autoimmune diseases. MiR-16-5p and miR-223-3p were found to be dysregulated in patients with RA and SS.[27,35–37] Moreover, the changes observed in miR-16-5p and miR-223-3p expression significantly correlated with those observed for clinical parameters and inflammatory parameters.[38] MiR-142 was overexpressed in SLE, systemic sclerosis, and psoriasis.[39–41] MiR-92a was found to be dysregulated in systemic sclerosis and multiple sclerosis.[42,43] MiR-34a was found to be upregulated in active MS lesions and contributed to MS pathogenesis by targeting CD47 to release macrophages.[43] The potential contribution of other miRNAs in autoimmunity remains unclear and further investigation is required.

As aforementioned, the pathogenesis of SS is associated with the neuropathic mechanism as well as autoimmunity. The basal and reflex wetting of the mouth and the ocular surface provided, respectively, by saliva and tears are directly controlled by the autonomic nervous system and respond to sensorial stimuli of taste and vision and general sense nerves.[44–47] Among miRNAs that are significantly expressed in SS, some are reported to be associated with neuropathy. MiR-134 level was decreased under neuropathic conditions. MiR-34 and miR-16 were downregulated in the dorsal root ganglia upon inflammatory induction.[48–50] MiR-223 was upregulated in the prefrontal cortex during inflammation and downregulated

Table 3. Expression of Tear microRNAs in Patients With Primary and Secondary SS as Compared to Control Group

MicroRNA	Control ΔCt* (mean ± SD)	Primary SS ΔCt* (mean ± SD)	Secondary SS ΔCt* (mean ± SD)	*P* Value†	Control vs. Primary SS		Control vs. Secondary SS	
					Fold Changes‡	*P* Value§	Fold Changes‡	*P* Value§
miR-15b-5p	1.78 ± 0.89	1.91 ± 1.14	1.27 ± 1.32	0.542	−1.09	0.916	1.42	0.286
miR-16-5p	0.98 ± 0.56	0.10 ± 1.32	−0.51 ± 1.12	0.027||	1.85	0.059	2.82	0.016||
miR-17-5p	3.73 ± 0.93	4.00 ± 0.92	3.95 ± 1.23	0.876	−1.21	0.674	−1.17	0.657
miR-20a-5p	1.94 ± 0.80	2.25 ± 0.55	2.07 ± 1.01	0.780	−1.25	0.462	−1.09	0.657
miR-20b-5p	5.35 ± 0.94	4.41 ± 0.85	4.30 ± 1.12	0.199	1.91	0.141	2.06	0.110
miR-21-5p	−4.82 ± 1.13	−5.56 ± 1.36	−5.60 ± 2.11	0.251	1.67	0.401	1.71	0.076
miR-23b-3p	−0.96 ± 0.46	−0.09 ± 1.37	−0.86 ± 1.70	0.348	−1.83	0.208	−1.07	0.477
miR-25-3p	1.42 ± 0.75	2.35 ± 1.32	1.74 ± 1.03	0.263	−1.90	0.208	−1.24	0.155
miR-29a-3p	−0.33 ± 0.79	−0.39 ± 1.11	−0.70 ± 1.45	0.780	1.05	0.916	1.30	0.477
miR-30a-3p	3.99 ± 1.12	4.63 ± 0.95	3.92 ± 1.14	0.249	−1.55	0.172	1.05	1.000
miR-30b-5p	1.01 ± 0.73	2.33 ± 1.07	1.93 ± 1.24	0.047||	−2.49	0.027||	−1.89	0.062
miR-30c-5p	2.26 ± 1.75	4.39 ± 1.54	3.80 ± 1.69	0.044||	−4.40	0.027||	−2.92	0.062
miR-30d-5p	1.45 ± 0.68	2.71 ± 0.96	2.06 ± 0.90	0.042||	−2.40	0.012||	−1.53	0.155
miR-34a-5p	2.99 ± 2.18	1.89 ± 1.16	1.81 ± 1.67	0.134	2.14	0.059	2.26	0.110
miR-92a-3p	−0.53 ± 0.55	0.72 ± 0.84	−0.10 ± 1.13	0.009||	−2.38	0.002||	−1.35	0.155
miR-93-5p	3.33 ± 0.50	3.73 ± 1.05	3.33 ± 1.03	0.637	−1.32	0.462	1.00	0.929
miR-99a-5p	2.26 ± 1.17	2.88 ± 0.90	2.88 ± 1.31	0.339	−1.54	0.141	−1.53	0.286
miR-100-5p	2.14 ± 1.15	2.55 ± 0.75	2.52 ± 1.24	0.498	−1.33	0.172	−1.30	0.594
miR-103a-3p	4.90 ± 0.79	4.03 ± 0.81	4.03 ± 1.84	0.339	1.82	0.074	1.82	0.424
miR-124-3p	3.91 ± 0.74	4.45 ± 1.46	4.62 ± 1.66	0.217	−1.46	0.208	−1.64	0.091
miR-125b-5p	−0.31 ± 0.56	0.90 ± 1.63	0.14 ± 1.36	0.290	−2.32	0.172	−1.37	0.286
miR-127-3p	1.36 ± 0.32	1.46 ± 2.00	1.75 ± 2.43	0.152	−1.07	0.172	−1.31	0.062
miR-132-3p	4.42 ± 1.05	4.17 ± 0.92	3.57 ± 1.09	0.217	1.19	0.529	1.81	0.091
miR-134-5p	5.75 ± 0.72	6.41 ± 1.63	6.55 ± 1.71	0.126	−1.57	0.115	−1.73	0.062
miR-137	5.75 ± 0.72	6.41 ± 1.63	6.85 ± 1.84	0.078	−1.57	0.115	−2.13	0.041||
miR-142-3p	4.76 ± 1.42	3.20 ± 1.38	2.62 ± 2.59	0.036||	2.95	0.027||	4.41	0.033||
miR-142-5p	5.13 ± 0.91	6.13 ± 1.58	5.83 ± 1.51	0.152	−2.00	0.093	−1.62	0.131
miR-143-3p	5.42 ± 0.58	5.53 ± 1.55	5.74 ± 1.49	0.588	−1.09	0.600	−1.25	0.248
miR-145-5p	2.17 ± 0.71	2.58 ± 1.34	3.01 ± 1.47	0.249	−1.33	0.208	−1.80	0.110
miR-146a-5p	0.59 ± 1.81	0.77 ± 1.58	−0.45 ± 1.52	0.125	−1.13	1.000	2.06	0.248
miR-146b-5p	4.42 ± 1.52	4.81 ± 1.67	4.39 ± 1.54	0.603	−1.32	0.674	1.02	0.859
miR-152-3p	3.10 ± 0.58	3.73 ± 1.16	2.80 ± 1.21	0.188	−1.55	0.345	1.23	0.374
miR-155-5p	5.50 ± 0.58	5.85 ± 1.72	5.29 ± 2.55	0.719	−1.28	0.529	1.15	0.477
miR-182-5p	4.46 ± 1.04	4.97 ± 1.31	4.57 ± 1.40	0.669	−1.42	0.462	−1.08	0.657
miR-183-5p	3.93 ± 1.43	4.11 ± 1.31	3.71 ± 1.22	0.915	−1.13	0.916	1.16	0.657
miR-203a-3p	−1.72 ± 1.35	−0.32 ± 1.47	−0.71 ± 1.60	0.152	−2.64	0.074	−2.02	0.155
miR-221-3p	2.30 ± 1.09	2.22 ± 1.20	1.79 ± 1.33	0.661	1.06	0.916	1.43	0.374
miR-223-3p	−0.90 ± 1.04	−2.43 ± 1.92	−2.11 ± 2.72	0.120	2.88	0.059	2.31	0.091
miR-302d-5p	5.46 ± 0.60	6.30 ± 1.62	6.51 ± 1.65	0.045||	−1.79	0.052	−2.07	0.021||
miR-338-5p	1.32 ± 0.56	1.76 ± 1.00	2.14 ± 1.88	0.202	−1.36	0.208	−1.77	0.091
miR-365b-3p	1.84 ± 1.00	3.01 ± 1.10	2.53 ± 1.04	0.098	−2.24	0.016||	−1.60	0.214
miR-374c-5p	4.58 ± 1.34	5.87 ± 2.01	6.85 ± 1.84	0.031||	−2.44	0.141	−4.81	0.013||
miR-487b-3p	5.75 ± 0.72	6.41 ± 1.63	6.73 ± 1.80	0.104	−1.57	0.115	−1.97	0.051

* ΔCt; delta threshold cycle; Ct (gene of interested)–Ct (housekeeping gene).
† Comparison of 3 groups using Kruskal-Wallis 1-way ANOVA.
‡ Comparison of each 2 groups using Mann-Whitney *U*-test
§ Fold changes; log2-fold change; positive values indicate elevated and negative values reduced levels in SS samples, respectively.
|| Indicating a value of $P < 0.05$.

in patients with fibromyalgia.[51,52] In our study, miR-16-5p, miR-34a-5p, and miR-223-3p levels were increased in the SS patients, whereas miR-134-5p level was decreased, suggesting that neuropathy may be involved in the pathogenesis of SS. Further studies are required to address this issue.

Inflammation is crucial in the pathogenesis of dry eye and the OSS is known to represent the severity of inflammation in dry eye.[44,53–55] Hence, if the severity of inflammation in dry eye is clearly identified and controlled, dry eye treatment will be possible. In this study, we analyzed the correlation between OSS and miRNA expression level; however, no miRNA revealed a significant correlation with the OSS. Alevizos et al.[34] reported that the expression of miR-768-3p and miR-574 was inversely correlated to the focus score representing the degree of inflammation of the salivary glands and confirmed them as potential biomarkers. We will further investigate the correlation between ocular surface inflammation as determined by OSS and tear cytokines as well as the expression of various miRNAs.

Our study had several limitations. First, the research was limited by a small sample size. Although we aimed to include a larger sample size, the prevalence of SS is not high enough. Second, this study only verified the expression levels of miRNAs in the tears. In future studies, we intend to investigate and compare the expression of miRNAs in PBMCs, salivary gland tissues, and saliva simultaneously in a large cohort of

Table 4. Correlation Between OSS and miRNA Expression in SS Patients

MicroRNA	r*	P
miR-16-5p	−0.176	0.485
miR-30b-5p	0.271	0.277
miR-30c-5p	0.170	0.501
miR-30d-5p	0.198	0.431
miR-34a-5p	−0.304	0.220
miR-92a-3p	0.331	0.179
miR-134-5p	−0.042	0.870
miR-137	−0.165	0.514
miR-142-3p	−0.030	0.905
miR-223-3p	0.032	0.899
miR-302d-5p	0.041	0.873
miR-365b-3p	0.200	0.426
miR-374c-5p	−0.268	0.283
miR-487b-3p	−0.314	0.205

* Spearman correlation coefficients (*r*).

patients. In addition, the correlation between the degree of inflammation and microRNA expression level could not be accurately investigated. Inflammatory cytokine analysis of the tears may reveal the precise mechanism.

Conclusion

In conclusion, we expect that tear miRNAs which can be obtained noninvasively will provide clues as to the pathogenesis of lacrimal gland dysfunction in patients with SS. Further study with more subjects and further exploration of the predicted molecular pathways associated with tear reduction in SS are necessary to get the insight into the pathophysiology of SS.

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References

1. Qin B, Wang J, Yang Z, et al. Epidemiology of primary Sjogren's syndrome: a systematic review and meta-analysis. *Ann Rheum Dis*. 2015;74:1983–1989.
2. Kassan SS, Moutsopoulos HM. Clinical manifestations and early diagnosis of Sjogren syndrome. *Arch Intern Med*. 2004; 164:1275–1284.
3. Tsubota K, Toda I, Yagi Y, Ogawa Y, Ono M, Yoshino K. Three different types of dry eye syndrome. *Cornea*. 1994;13:202–209.
4. Peters L, Meister G. Argonaute proteins: mediators of RNA silencing. *Mol Cell*. 2007;26:611–623.
5. Chen K, Rajewsky N. The evolution of gene regulation by transcription factors and microRNAs. *Nat Rev Genet*. 2007;8: 93–103.
6. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*. 2004;116:281–297.
7. Brennecke J, Hipfner DR, Stark A, Russell RB, Cohen SM. bantam encodes a developmentally regulated microRNA that controls cell proliferation and regulates the proapoptotic gene hid in Drosophila. *Cell*. 2003;113:25–36.
8. Li X, Carthew RW. A microRNA mediates EGF receptor signaling and promotes photoreceptor differentiation in the Drosophila eye. *Cell*. 2005;123:1267–1277.
9. Hatfield SD, Shcherbata HR, Fischer KA, Nakahara K, Carthew RW, Ruohola-Baker H. Stem cell division is regulated by the microRNA pathway. *Nature*. 2005;435:974–978.
10. Turchinovich A, Tonevitsky AG, Burwinkel B. Extracellular miRNA: a collision of two paradigms. *Trends Biochem Sci*. 2016;41:883–892.
11. Weber JA, Baxter DH, Zhang S, et al. The microRNA spectrum in 12 body fluids. *Clin Chem*. 2010;56:1733–1741.
12. Huang Z, Huang D, Ni S, Peng Z, Sheng W, Du X. Plasma microRNAs are promising novel biomarkers for early detection of colorectal cancer. *Int J Cancer*. 2010;127:118–126.
13. Fang C, Zhu DX, Dong HJ, et al. Serum microRNAs are promising novel biomarkers for diffuse large B cell lymphoma. *Ann Hematol*. 2012;91:553–559.
14. Hayes J, Peruzzi PP, Lawler S. MicroRNAs in cancer: biomarkers, functions and therapy. *Trends Mol Med*. 2014; 20:460–469.
15. Alevizos I, Alexander S, Turner RJ, Illei GG. MicroRNA expression profiles as biomarkers of minor salivary gland inflammation and dysfunction in Sjogren's syndrome. *Arthritis Rheum-Us*. 2011;63:535–544.
16. Shi H, Zheng LY, Zhang P, Yu CQ. miR-146a and miR-155 expression in PBMCs from patients with Sjogren's syndrome. *J Oral Pathol Med*. 2014;43:792–797.
17. Zilahi E, Tarr T, Papp G, Griger Z, Sipka S, Zeher M. Increased microRNA-146a/b, TRAF6 gene and decreased IRAK1 gene expressions in the peripheral mononuclear cells of patients with Sjogren's syndrome. *Immunol Lett*. 2012;141:165–168.
18. Whitcher JP, Shiboski CH, Shiboski SC, et al. A simplified quantitative method for assessing keratoconjunctivitis sicca from the Sjogren's Syndrome International Registry. *Am J Ophthalmol*. 2010;149:405–415.
19. Mestdagh P, Van Vlierberghe P, De Weer A, et al. A novel and universal method for microRNA RT-qPCR data normalization. *Genome Biol*. 2009;10:R64.
20. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res*. 2001;29:e45.
21. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\delta\delta CT}$ method. *Methods*. 2001;25:402–408.
22. Fox RI. Sjogren's syndrome. *Lancet*. 2005;366:321–331.
23. Fox RI, Howell FV, Bone RC, Michelson P. Primary Sjogren syndrome: clinical and immunopathologic features. *Semin Arthritis Rheum*. 1984;14:77–105.
24. Pauley KM, Stewart CM, Gauna AE, et al. Altered miR-146a expression in Sjogren's syndrome and its functional role in innate immunity. *Eur J Immunol*. 2011;41:2029–2039.
25. Chen JQ, Zilahi E, Papp G, Sipka S, Zeher M. Simultaneously increased expression of microRNA-155 and suppressor of cytokine signaling 1 (SOCS1) gene in the peripheral blood mononuclear cells of patients with primary Sjogren's syndrome. *Int J Rheum Dis*. 2017;20:609–613.
26. Zilahi E, Tarr T, Papp G, Griger Z, Sipka S, Zeher M. Increased microRNA-146a/b, TRAF6 gene and decreased IRAK1 gene expressions in the peripheral mononuclear cells of patients with Sjogren's syndrome. *Immunol Lett*. 2012;141:165–168.
27. Gourzi VC, Kapsogeorgou EK, Kyriakidis NC, Tzioufas AG. Study of microRNAs (miRNAs) that are predicted to target the autoantigens Ro/SSA and La/SSB in primary Sjogren's syndrome. *Clin Exp Immunol*. 2015;182:14–22.

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28. Nair JJ, Singh TP. Sjogren's syndrome: review of the aetiology, pathophysiology & potential therapeutic interventions. *J Clin Exp Dent*. 2017;9:e584–e589.
29. Nikolov NP, Illei GG. Pathogenesis of Sjogren's syndrome. *Curr Opin Rheumatol*. 2009;21:465–470.
30. de Oliveira FR, Fantucci MZ, Adriano L, et al. Neurological and inflammatory manifestations in Sjogren's syndrome: the role of the kynurenine metabolic pathway. *Int J Mol Sci*. 2018;19: E3953.
31. Wu H, Neilson JR, Kumar P, et al. miRNA profiling of naive, effector and memory CD8 T cells. *PLoS One*. 2007;2:e1020.
32. Chen CZ, Li L, Lodish HF, Bartel DP. MicroRNAs modulate hematopoietic lineage differentiation. *Science*. 2004;303:83–86.
33. Rauniyar VK, Wu W, Yang H. MicroRNAs in autoimmune disease. *Auto Immun Highlights*. 2011;2:59–65.
34. Alevizos I, Illei GG. MicroRNAs in Sjogren's syndrome as a prototypic autoimmune disease. *Autoimmun Rev*. 2010;9: 618–621.
35. Chen JQ, Papp G, Szodoray P, Zeher M. The role of microRNAs in the pathogenesis of autoimmune diseases. *Autoimmun Rev*. 2016;15:1171–1180.
36. Kriegsmann M, Randau TM, Gravius S, et al. Expression of miR-146a, miR-155, and miR-223 in formalin-fixed paraffin-embedded synovial tissues of patients with rheumatoid arthritis and osteoarthritis. *Virchows Arch*. 2016;469:93–100.
37. Kapsogeorgou EK, Gourzi VC, Manoussakis MN, Moutsopoulos HM, Tzioufas AG. Cellular microRNAs (miRNAs) and Sjogren's syndrome: candidate regulators of autoimmune response and autoantigen expression. *J Autoimmun*. 2011; 37:129–135.
38. Filkova M, Aradi B, Senolt L, et al. Association of circulating miR-223 and miR-16 with disease activity in patients with early rheumatoid arthritis. *Ann Rheum Dis*. 2014;73:1898–1904.
39. Dai Y, Huang YS, Tang M, et al. Microarray analysis of microRNA expression in peripheral blood cells of systemic lupus erythematosus patients. *Lupus*. 2007;16:939–946.
40. Makino K, Jinnin M, Kajihara I, et al. Circulating miR-142-3p levels in patients with systemic sclerosis. *Clin Exp Dermatol*. 2012;37:34–39.
41. Zibert JR, Lovendorf MB, Litman T, Olsen J, Kaczkowski B, Skov L. MicroRNAs and potential target interactions in psoriasis. *J Dermatol Sci*. 2010;58:177–185.
42. Sing T, Jinnin M, Yamane K, et al. microRNA-92a expression in the sera and dermal fibroblasts increases in patients with scleroderma. *Rheumatology (Oxford)*. 2012;51:1550–1556.
43. Tufekci KU, Oner MG, Genc S, Genc K. MicroRNAs and multiple sclerosis. *Autoimmune Dis*. 2010;2011:807426.
44. Willcox MDP, Argueso P, Georgiev GA, et al. TFOS DEWS II tear film report. *Ocul Surf*. 2017;15:366–403.
45. Meng ID, Kurose M. The role of corneal afferent neurons in regulating tears under normal and dry eye conditions. *Exp Eye Res*. 2013;117:79–87.
46. Perez P, Rowzee AM, Zheng C, Adriaansen J, Baum BJ. Salivary epithelial cells: an unassuming target site for gene therapeutics. *Int J Biochem Cell Biol*. 2010;42:773–777.
47. Dartt DA. Neural regulation of lacrimal gland secretory processes: relevance in dry eye diseases. *Prog Retin Eye Res*. 2009;28:155–177.
48. Bai G, Ambalavanar R, Wei D, Dessem D. Downregulation of selective microRNAs in trigeminal ganglion neurons following inflammatory muscle pain. *Mol Pain*. 2007;3:15.
49. Ni J, Gao Y, Gong S, Guo S, Hisamitsu T, Jiang X. Regulation of mu-opioid type 1 receptors by microRNA134 in dorsal root ganglion neurons following peripheral inflammation. *Eur J Pain*. 2013;17:313–323.
50. Kusuda R, Cadetti F, Ravanelli MI, et al. Differential expression of microRNAs in mouse pain models. *Mol Pain*. 2011;7:17.
51. Poh KW, Yeo JF, Ong WY. MicroRNA changes in the mouse prefrontal cortex after inflammatory pain. *Eur J Pain*. 2011; 15:801–812.
52. Bjersing JL, Lundborg C, Bokarewa MI, Mannerkorpi K. Profile of cerebrospinal microRNAs in fibromyalgia. *PLoS One*. 2013; 8:e78762.
53. De Paiva CS, Chotikavanich S, Pangelinan SB, et al. IL-17 disrupts corneal barrier following desiccating stress. *Mucosal Immunol*. 2009;2:243–253.
54. Chen YT, Nikulina K, Lazarev S, et al. Interleukin-1 as a phenotypic immunomodulator in keratinizing squamous metaplasia of the ocular surface in Sjogren's syndrome. *Am J Pathol*. 2010;177:1333–1343.
55. De Paiva CS, Villarreal AL, Corrales RM, et al. Dry eye-induced conjunctival epithelial squamous metaplasia is modulated by interferon-gamma. *Invest Ophthalmol Vis Sci*. 2007;48:2553–2560.

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