

MicroRNA Profiling in Chinese Patients with Primary Sjögren Syndrome Reveals Elevated miRNA-181a in Peripheral Blood Mononuclear Cells

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ABSTRACT. Objective. Characterized by chronic inflammation, dysfunction of exocrine glands, and systemic autoimmunity, primary Sjögren syndrome (pSS) is a common autoimmune disease in elderly women. Our study was performed to explore the potential involvement of microRNA (miRNA) in Chinese patients with pSS.

Methods. Using microarrays, miRNA expression in peripheral blood mononuclear cells (PBMC) was profiled in 4 female patients with pSS and 3 healthy participants, followed by a large-scale study of 33 patients and 10 healthy individuals. Compared to the healthy participants, 202 miRNA were upregulated and 180 were downregulated in the patients with pSS. To confirm this finding, a set of regulated miRNA was further examined in a large patient group, using quantitative reverse transcriptase-PCR assays.

Results. MiR-181a was the miRNA that most profoundly differed between patients with pSS and healthy individuals; however, similar miRNA-181a expression profiles were found in groups with different disease phenotypes. Together, these observations suggested that an elevated miRNA-181a level is a general phenomenon in Chinese patients with pSS.

Conclusion. In addition to the elevated miR-181a levels, our study led to the speculation that elevated miR-181a levels in the PBMC of these patients compromise the maturation of B cells, enabling them to recognize and attack autoantigens and resulting in disease phenotypes. In addition to the regulation of human miRNA, many virus-derived miRNA were unexpectedly upregulated in the patients with pSS, suggesting that viral infection of PBMC plays a role in this disease. (First Release Aug 15 2014; *J Rheumatol* 2014;41:2208–13; doi:10.3899/jrheum.131154)

Key Indexing Terms:

SJÖGREN SYNDROME

MICRORNA-181A

T CELL MATURATION

Sjögren syndrome (SS) is a systemic autoimmune disease characterized by the destruction and dysfunction of exocrine glands, particularly the salivary and lacrimal glands¹. Even though the precise mechanisms underlying this syndrome are still undefined, involvement of the immune system is a well-established fact². One critical hallmark in the development of this syndrome, hypergam-

maglobulinemia, is characterized by increased levels of circulating autoantibodies against ribonucleoproteins (SS-A/Ro and SS-B/La), cellular receptors, and rheumatoid factors^{3,4}. Another major pathology is the infiltration of leukocytes into the exocrine glands, resulting in histologically visible periductal aggregates or even germinal centers, which highlights a fundamental abnormality in terms of an

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exaggerated immune response against self-tissues⁵. This leads to the activation of autoreactive T cells and the production of autoantibody, followed by the destruction of exocrine glands, and ultimately giving rise to the clinical symptoms associated with SS, typically inflammation and dryness of the mouth and eyes⁶. In these patients, substantial accumulation of CD4+ TH17 memory T cells, interleukin 23 (IL-23)-producing macrophages, and dendritic cells promoting the activation of TH17 cells have been identified^{7,8}, highlighting the critical roles of T cells in SS.

MicroRNA (miRNA) are a class of short (20–22 nucleotides) noncoding RNA that function primarily in posttranscriptional gene regulation. Besides their essential functions in biological processes, emerging evidence is beginning to shed light on their roles in various pathogenic processes, such as cancer, cardiac diseases, neurodegenerative diseases, and autoimmune/inflammatory diseases⁹. For example, miRNA profiling in patients with primary SS (pSS) has been reported in a few studies^{1,10,11}. In these, a distinct miRNA expression pattern was identified in the minor salivary glands from patients with pSS compared to healthy controls¹¹. Further, differential miRNA profiles were found to distinguish different disease phenotypes, including low-grade and high-grade inflammation. MiRNA-768-3p and miRNA-574 were found to change in opposite directions in patients with varying focus scores. While the expression of miRNA-768-3p increased in patients with pSS with an increasing focus score, the expression of miRNA-574 decreased, suggesting that miRNA levels might be another class of diagnosis biomarkers for pSS. In contrast to the systemic approach taken in the miRNA study in salivary tissues, the expression of a few candidate miRNA was investigated in peripheral blood mononuclear cells (PBMC) from patients with pSS and healthy controls^{1,10}, including miRNA targeting Ro/SSA and La/SSB ribonucleoproteins and miRNA-146a/b, a promising regulator of the innate immune response¹⁰. Even though the regulated expression of these miRNA has been demonstrated in pSS PBMC, a comprehensive picture, and in particular their involvement in pSS in Chinese patients, is still lacking.

MiRNA expression was first assessed in PBMC from several patients with pSS and healthy controls, using a microarray-based gene expression assay. Then, in a large set of patients and healthy controls, the differentially expressed miRNA were further studied by quantitative reverse transcriptase-PCR (RT-PCR), and this led to the identification of elevated miRNA-181a levels in patients with pSS in comparison to healthy controls.

MATERIALS AND METHODS

Ethics statement and patient recruitment. Our study was conducted according to the principles approved by the Ethics Review Board of Peking Union Medical College Hospital (Beijing, China). Written informed consent was provided for sample collection and analysis.

Following the criteria set in the 2002 international diagnostic criteria and the 2012 American College of Rheumatology Society for SS, patients diagnosed with pSS were recruited into the study when no other rheumatology and underlying disease was identified. Age-matched and sex-matched control participants were recruited from healthy volunteers, in whom no pSS diagnostic criteria were met and no immune disease history was found. The criteria for organ involvement were according to the European League Against Rheumatism Sjögren's Syndrome Disease Activity Index (ESSDAI).

Thirty-three patients with pSS (1 man and 32 women; mean age 50 ± 12 yrs) recruited from Peking Union Medical College Hospital between 2012 and 2013 were enrolled. Positive antinuclear antibody (ANA) titers were found in 81.8% (27/33), elevated anti-SS-A levels in 81.8% (27/33), and elevated anti-SS-B in 15.1% (5/33). Based on organ involvement (overlapping cases), patients were divided into 6 groups: 4 patients (12%) without organ involvement, 4 (12%) with renal involvement, 6 (18.2%) with nervous system involvement, 9 (27.3%) with interstitial lung lesions, 19 (57.6%) with blood system involvement, and 3 (9%) with liver involvement. Based on the immunoglobulin G (IgG) level, they were divided into a high IgG group (9 cases, IgG ≥ 16 g/l) and a non-high IgG group (14 cases, IgG < 16 g/l). The ESSDAI score was valued for each patient (average, 5.12 ± 2.19 points).

A cohort of age-matched and sex-matched healthy individuals ($n = 10$; 1 male and 9 females; mean age, 42 ± 9.5 yrs) was recruited from among the volunteer blood donors at Peking Union Medical College Hospital.

Sample acquisition and processing. Whole blood (9 ml) was collected from each patient with pSS and each control participant, and centrifuged for 10 min at 4°C to isolate PBMC. From these, mononuclear cells were obtained and total RNA were extracted using TRIzol reagent (Invitrogen). RNA samples were quantified using a NanoDrop 8000 spectrophotometer (NanoDrop Technologies) and stored at -80°C until use.

Microarray assay and data processing. MiRNA profiling was performed by KangChen Bio-Tech using an LNA human miRNA microarray (Exiqon), comprising probes for 1896 human miRNA and 146 viral miRNA. In each assay, 100 ng total RNA was used for array hybridization, the resulting hybridization signals were quantified using an Axon GenePix scanner (model 4000B), and data extraction and analysis were performed using GenePix Pro6.0 software. Normalization of gene expression was performed by the median normalization method, in which normalized data = (foreground-background)/median, where median is the 50% quantile of microRNA intensity > 50 in all samples after background correction. The threshold value for significance used to define upregulation or downregulation of miRNA was a fold change > 1.5 in expression, and a value of $p < 0.05$ calculated by the t test.

Quantitative RT-PCR (qRT-PCR) assay. SuperScript II (Invitrogen) reagent was used to synthesize cDNA. Briefly, 15- μl RT reactions contained 0.5 μg total RNA, 3 μl RT primer, 1.5 μl deoxyribonucleoside triphosphate (dNTP; 10 mM each), 4 μl 5 \times first-strand buffer, 0.5 μl RNase inhibitor (40 U/ μl), and 1 μl TIANScript M-MLV (2000 U/l). For RT, the mixture was incubated on ice for 5 min, then at 16°C for 30 min, 42°C for 30 min, and finally 85°C for 5 min. A TaqMan microRNA assay kit (ABI, series no. 4499312) was used to perform quantitative real-time RT-PCR. A 20- μl PCR reaction included 2 μl RT product, 2 μl 10 \times HotMaster Taq buffer, 1 μl dNTP (2.5 mM each), 1 μl TaqMan microRNA probe, and 0.15 μl HotMaster Taq DNA polymerase (2.5 U/ μl). The reactions were carried out at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Relative gene expression was calculated by the $2^{-\Delta\Delta\text{CT}}$ method, using snRNA U6 as an internal control.

Statistical analysis. Data of qRT-PCR analysis was performed using SPSS for Windows, version 17.0, followed by the independent samples Mann-Whitney U test. Spearman's rank correlation test was used for correlation analysis¹². A $p < 0.05$ was considered statistically significant. A Bonferroni correction was calculated for the 11 comparisons and led to a corrected p of 0.0045.

RESULTS

To explore the involvement of miRNA in Chinese patients with pSS, miRNA expression in PBMC was systematically studied. The patients fulfilled the criteria set in the 2002 international diagnostic criteria and the 2012 American College of Rheumatology for SS^{13,14}. In total, 33 patients with pSS, and 10 age-matched and sex-matched healthy individuals were involved in the study. Detailed information on these patients with pSS is presented in a supplementary table available from the authors on request. In both groups, whole-blood samples were collected to isolate PBMC, as described. RNA samples were then extracted and subjected to miRNA expression analyses.

MiRNA profiling assay. Using a commercial miRNA profiling platform, miRNA expression profiles were initially determined in 4 patients with pSS and 3 controls.

A threshold of 1.5-fold change in expression and a p value cutoff of 0.05 (t test) were used to identify 382 miRNA that were differentially expressed in the 2 groups. Compared to healthy controls, 202 miRNA were upregulated and 180 were downregulated in the patients with pSS (Figure 1). In a high-throughput gene expression platform, it is not surprising to identify hundreds of differentially regulated genes. To make this high-throughput data compatible with downstream low-throughput assays, a rational selection approach was used to identify a subgroup of function-relevant miRNA. For this purpose, a literature search was performed to collect miRNA involved in immunology or immune disease. Taking together the expression pattern revealed in microarray assay, and the potential physiological and pathological functions found in

the literature search, a subgroup of 11 miRNA was identified for further study: miRNA-let-7b, miRNA-142-3p, miRNA-142-5p, miRNA-146a, miRNA-148b, miRNA-155, miRNA-18b, miRNA-181a, miRNA-223, miRNA-23a, and miRNA-574-3p. Using qRT-PCR assay, the expression levels of these miRNA were further measured in the original samples used in the microarray assay, as well as in an enlarged patient and control dataset. In addition to confirming the microarray data, this study further demonstrated that miRNA-146a, miRNA-155, and miRNA-181a were the most highly regulated miRNA between patients with pSS and healthy participants. Therefore, further study was performed on these 3 miRNA.

Elevated miR-181a levels in patients with pSS. Using qRT-PCR, the expression levels of the 3 miRNA were measured in 33 patients with pSS and 10 healthy controls (Figure 2). Compared to the healthy controls, downregulation of miRNA-155, and upregulation of miRNA-146a and miRNA-181a were confirmed in patients with pSS (Figure 2).

An increase of 2.81-fold ($p = 0.001$) was identified for the expression of miRNA-181 in patients with pSS, which is statistically significant after Bonferroni correction ($pc = 0.045$). Individual measurement of miR-181a levels in isolated B cells and T cells indicated that the elevated miR-181a level was contributed by B cell population. This, together with published studies^{15,16}, emphasizes its potential role in the development of pSS. A hematopoietic miRNA, miR-181a is preferentially expressed in mouse thymus, and plays a critical role in B and T cell differentiation¹⁵. A study of its expression during T cell development found that,

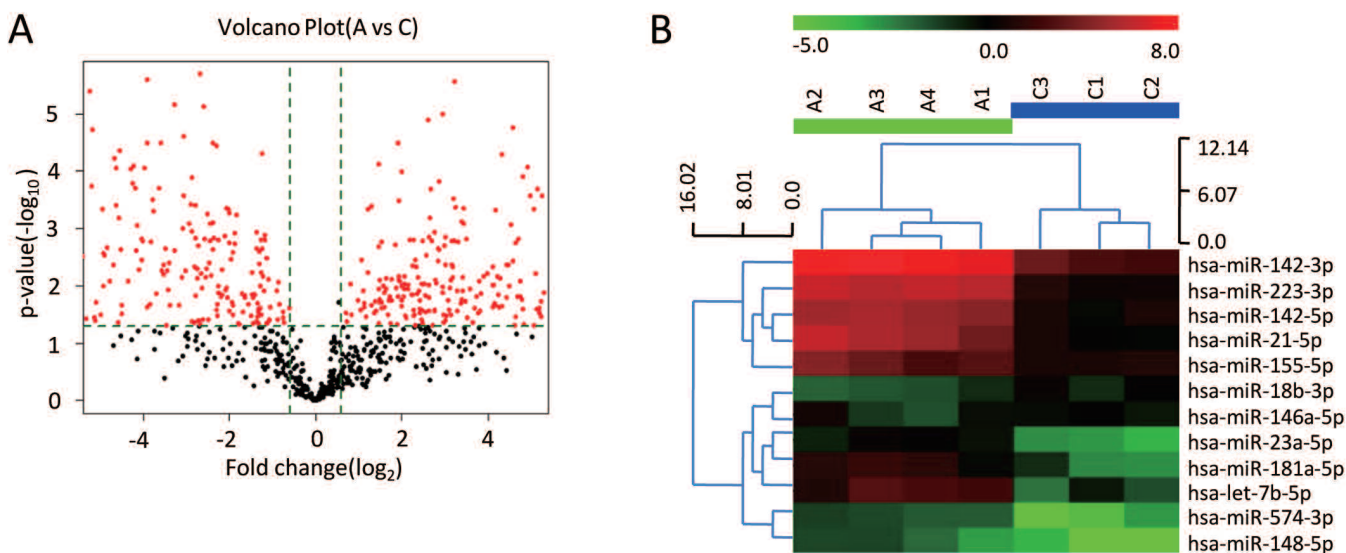


Figure 1. Microarray data. A. Volcano plot presenting the expression changes of microRNA (miRNA; 1.5-fold increase or decrease) versus p, as detected by microarray in patients with pSS relative to healthy controls. B. Distinct expression of a set of microRNA in patients with pSS (A1-A4) and healthy controls (C1-C3). Green, low expression levels; red, high expression levels. pSS: primary Sjögren syndrome.

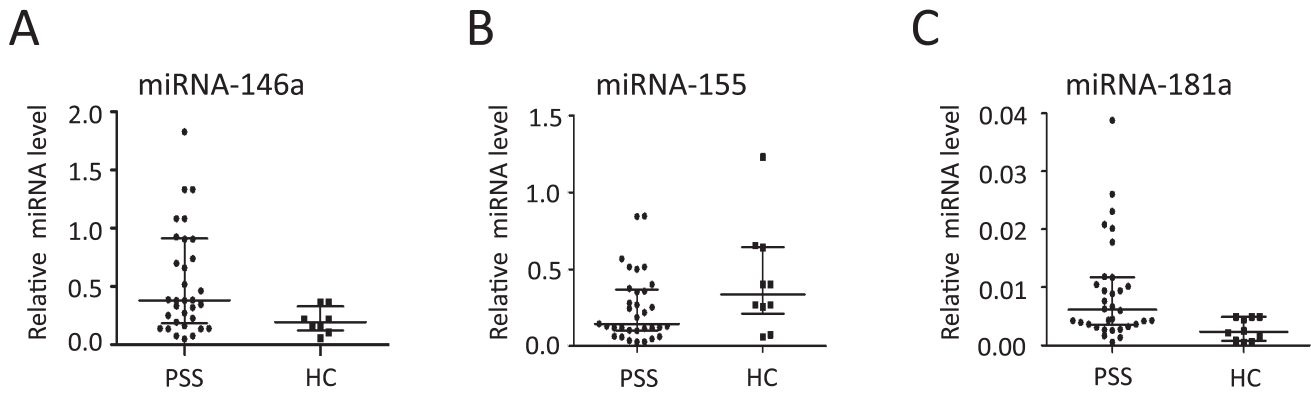


Figure 2. Differential microRNA (miRNA) expression in patients with pSS and healthy controls. A. Expression levels of miRNA-146a in patients with pSS and healthy controls. B. Expression levels of miRNA-155. C. Expression levels of miRNA-181a. pSS: primary Sjögren syndrome.

while increasing miR-181a expression in mature T cells augments their sensitivity to peptide antigens, inhibiting its expression in immature T cells reduces the sensitivity and impairs both positive and negative selection¹⁶. It was also reported that T cell sensitivity to autoantigen is quantitatively regulated by managing the expression levels of miR-181a, so as to enable mature T cells to recognize antagonists (inhibitory peptide antigens) as agonists. Their study revealed a correlation between higher miR-181a levels and greater T cell sensitivity in immature T cells, which likely explains the elevated miR-181a levels in patients with pSS.

To further characterize the relevance of miR-181a in pSS, statistical analyses were performed in patient groups classified by various laboratory tests (organ affected, ANA titer, erythrocyte sedimentation rate, high-sensitivity C-reactive protein, and IgG level; Figure 3). Except for a positive correlation between miRNA-181a levels and ANA titer (Figure 3D, $r = 0.416$, $p = 0.031$), no statistical difference was found in the other phenotype groups. These lines of evidence indicate that miR-181a levels in PBMC can be used to distinguish patients with pSS from healthy individuals, but not different disease phenotypes. Considering the well-established functions of miRNA in cell differentiation, this finding is not surprising because a compromised immune system is a common feature in all patients with pSS, irrespective of their phenotype.

Taken together, ours and other studies lead to the hypothesis that elevated miR-181a levels compromise the functions of B cells, particularly their antigen sensitivity. This enables them to recognize and attack autoantigen, and leads to the dysfunction of exocrine glands in pSS.

Elevated viral miRNA in pSS PBMC. In addition to these findings, we unexpectedly found that many virus-derived miRNA were upregulated in the PBMC of patients with pSS. Among the 146 viral miRNA included in the microarray, 13 were upregulated (ebv-miR-BART17-3p,

ebv-miR-BART19-5p, ebv-miR-BART13, ebv-miR-BART8*, hcmv-miR-UL148D, hsv1-miR-H3*, hsv1-miR-H18, hsv1-miR-H2, hsv1-miR-H7*, hsv2-miR-H25, hsv2-miR-H3, hsv2-miR-H24, and kshv-miR-K12-10a). In agreement with a study reporting elevated Epstein-Barr virus (EBV) miRNA in American patients with pSS¹⁷, our results showed that the expression of EBV-derived miR-BART17-3p, miR-BART19-5p, and miR-BART8* was upregulated from 2- to 58-fold in samples from Chinese patients. Our study further revealed that miR-K12-10a was upregulated 300-fold (both findings in supplementary table available from the authors on request). MiR-K12-10a is coded by Kaposi sarcoma-associated herpes virus, a human cancer virus causing Kaposi sarcoma in AIDS¹⁸, primary effusion lymphoma¹⁹, and multicentric Castelman disease. Even though the involvement of this virus in pSS needs further investigation, the results highlight this possibility.

DISCUSSION

SS is a systemic autoimmune disease causing the dysfunction and destruction of exocrine glands, typically the salivary and lachrymal glands¹. Critical hallmarks in the development of pSS include the production of autoantibodies and the infiltration of leukocytes into the affected glands, forming histologically visible aggregates or germinal centers. Even though the precise causes and mechanisms of the disease have not been defined, dysfunction of the immune system and the involvement of B cells are well characterized⁷. A study reported that the development of T cells is regulated by miR-181a¹⁵. While increasing miR-181a expression in mature T cells augments their sensitivity to peptide antigens, inhibiting its expression in immature T cells reduces sensitivity and impairs both positive and negative selection, therefore emphasizing the importance of miRNA in the regulation of immune cell development.

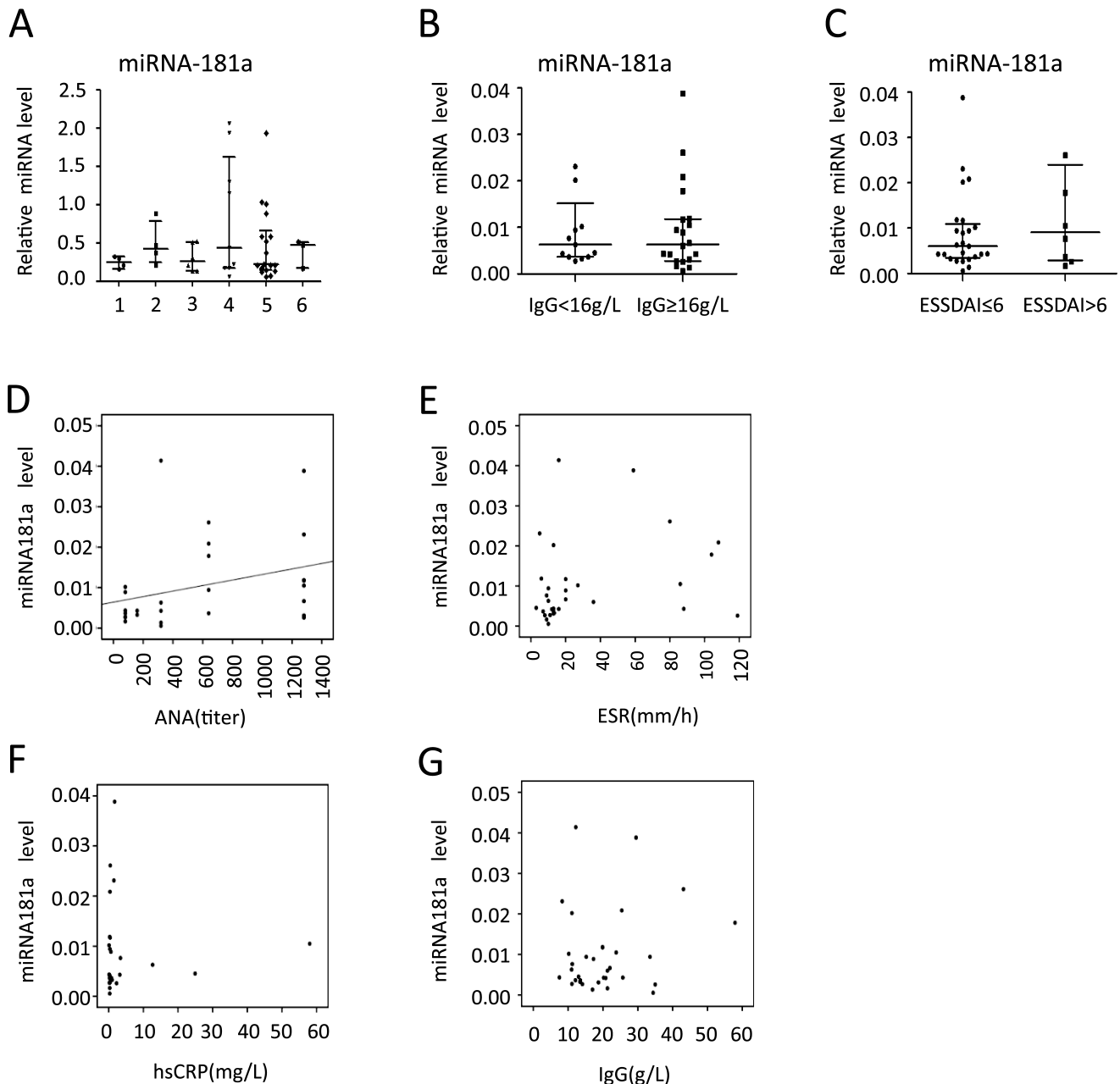


Figure 3. Relative expression levels of miR-181a in different disease phenotype groups. A. miRNA-181 levels in patients with pSS classified by affected organs, in addition to the exocrine glands. (1) no other organ affected, 4 cases; (2) kidney affected, 4 cases; (3) nervous system affected, 6 cases; (4) interstitial lung affected, 9 cases; (5) blood system affected, 19 cases; (6) liver affected, 3 cases. X-axis, patient groups; Y-axis, miRNA levels relative to that of U6. B. Comparison of miR-181a levels between the high-globulin group (IgG ≥ 16 g/l, 19 cases) and the low-globulin group (IgG < 16 g/l, 14 cases). C. Comparison of miR-181a levels between the low (≤ 6) and high (> 6) ESSDAI score groups. D. Relative miR-181a levels in patient groups classified by ANA pattern. E. Erythrocyte sedimentation rate (ESR). F. High-sensitivity CRP. G. IgG level. Data analyses were performed using Spearman's correlation method, 2-tailed test. pSS: primary Sjögren syndrome; IgG: immunoglobulin G; miRNA: microRNA; ESSDAI: European League Against Rheumatism Sjögren's Syndrome Disease Activity Index; ANA: antinuclear antibody.

To assess the involvement of miRNA in lymphocyte regulation in Chinese patients with pSS, changes of miRNA expression were first profiled in PBMC. Compared to healthy subjects, profiling miRNA expression in patients with pSS identified a set of differentially regulated miRNA;

202 were upregulated and 180 downregulated. Using qRT-PCR, the expression levels of a subset of the regulated miRNA was investigated in a large patient group. This, on the one hand, confirmed the microarray assay, and on the other hand, identified 3 of the most highly regulated miRNA

between patients with pSS and healthy participants: miRNA-146a, miRNA-155, and miRNA-181a. We further examined the association between the expression levels of the 3 miRNA and disease status with additional samples. Even though downregulation of miRNA-155 and upregulation of miRNA-146a and miRNA-181a were confirmed, the only change with statistical significance was found for miRNA-181a, emphasizing its potential role in the immune dysfunction in pSS. Taken together with published studies, our data led to the hypothesis that in patients with pSS, elevated miR-181a in PBMC compromises B cell maturation, causing them to recognize and attack autoantigen in exocrine glands, leading to the onset of pSS.

In addition to the elevated miR-181a levels, an unexpected finding was that multiple virus-derived miRNA were significantly upregulated in patients with pSS. Although viruses have long been thought to initiate pSS, direct evidence and mechanisms are still elusive. To this end, identification of elevated viral miRNA in PBMC suggests that viral infection might play a role in the dysfunction of the immune system in patients with pSS.

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