
Differential expression of miR-17-92 cluster among varying histological stages of minor salivary gland in patients with primary Sjögren's syndrome

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ABSTRACT

Objective. To investigate the differential expression of miR-17-92 cluster, which encodes 6 microRNAs (miRNAs) including miR-17, miR-18a, miR-19a, miR-19b, miR-20a and miR-92a, among varying histological stages of labial minor salivary gland (MSG) tissues in patients with primary Sjögren's syndrome (pSS).

Methods. Fifty-seven pSS patients and 13 healthy volunteers were enrolled in this study. The pSS patients were allocated to 3 subgroups of advanced clinical stages according to the histological findings of the MSG biopsies. Salivary flow rate, Schirmer test and some laboratory indexes were also tested. The expression levels of the 6 miRNAs in MSG were evaluated using quantitative real-time polymerase chain reaction with TaqMan miRNA assay.

Results. The differences between the healthy individuals and the 3 pSS subgroups were statistically significant for positive findings of salivary flow rate, Schirmer test and laboratory indexes. In the labial MSG tissues, we observed that the expression level of miR-18a was significantly up-regulated in patients of the 3 pSS subgroups compared to healthy individuals, while the expression level of miR-92a was significantly down-regulated. We also observed that there was no notable difference in the expression levels of miR-17, miR-19a, miR-19b, and miR-20a. Furthermore, we distinguished that miR-18a was progressively up-regulated along the advanced histological stages of the 3 pSS subgroups, while the miR-92a was progressively down-regulated.

Conclusion. This is the first study on the expression of the miR-17-92 cluster in MSG of pSS patients. The association of increased expression levels of miR-18a and reduced expression levels of miR-92a with advanced clinical

stages of pSS could significantly reduce the substantial subjectivity of scoring inflammatory infiltrates and may aid in the diagnosis of pSS.

Introduction

Sjögren's syndrome (SS) is a chronic autoimmune disorder, characterised by lymphocytic infiltration of exocrine glands, mainly salivary and lacrimal glands, leading to decreased saliva and tear production (1, 2). In general, SS can be divided into primary or secondary SS (pSS or sSS) depending on comorbidity of other autoimmune rheumatic diseases (3). pSS has an overall prevalence rate of 0.06% worldwide, with the overall age of 56.2 years and the female/male ratio of 10.72 (4). Furthermore, a range of autoantibodies can be present in pSS such as anti-SSA/Ro and anti-SSB/La antibodies, rheumatoid factor, cryoglobulins, and antinuclear antibodies (5, 6).

MicroRNAs (miRNAs) are single-stranded, endogenous non-coding RNA of 18 to 25 nucleotides in length that play critical roles in regulating gene expression (7, 8). In recent years, rapidly accumulating evidence has demonstrated that miRNAs are involved in SS (9, 10). For example, distinct miRNA expression profiles have been reported from the minor salivary glands (11) and in peripheral blood mononuclear cells (12) of SS patients compared with healthy controls. In addition, differential expression of miR-768-3p, miR-574, miR-181a, miR-146a, miR-155, miR-16, miR-200b-3p, miR-200b-5p, miR-223, miR-483-5p, miR-34b-3p, miR-4701-5p, miR-609, miR-300, miR-3162-3p and miR-877-3p, etc, has been found in SS patients (11-15). The miR-17-92 cluster, encodes 6 miRNAs including miR-17, miR-18a, miR-19a, miR-19b, miR-20a and miR-92a, has been identified as an oncogene and in-

involved in tumorigenesis initially (16, 17). Recent studies have discovered that miR-17-92 cluster has regulatory functions in rheumatoid arthritis (18-21) and systemic lupus erythematosus (22, 23). However, the role this cluster plays in SS has not been elucidated.

In this study, we provide the first evidence for the differential expression of the miR-17-92 cluster among varying histological stages of minor salivary gland (MSG) tissues from pSS patients compared with healthy volunteers. Additionally, we investigated the potential association between the expressions of these miRNAs with the clinicopathological features, such as histological stages and symptom severity, in patients with pSS.

Materials and methods

Patients

Fifty-seven patients (5 males, 52 females) fulfilling the American-European consensus criteria for pSS (3) were enrolled in this study consecutively. They were recruited from the inpatients in the Department of Rheumatology and Immunology, Affiliated Hospital of Nanjing University of Chinese Medicine, where they received regular follow-up treatment. None of the pSS patients had evidence of other connective tissue disorders, lymphoma, sarcoidosis, essential mixed cryoglobulinaemia, or infection by hepatitis-B, hepatitis-C, or human immunodeficiency virus. Thirteen healthy volunteers (1 males, 12 females) were served as controls. The study was approved by the ethic committee of the Affiliated Hospital of Nanjing University of Chinese Medicine, and all subjects provided signed informed consent.

Laboratory analysis

Patients' and healthy volunteers' blood samples were collected for routine biochemical tests, including rheumatoid factor (RF), antistreptolysin-O (ASO), C-reactive protein (CRP), Anti-SS-A and Anti-SS-B antibodies, in the hospital's laboratory. Their whole unstimulated saliva was collected in a sterilised plastic tube over a period of 15 minutes. The subjects were instructed not to eat or drink for 2 hours before

Table I. ID and sequence of TaqMan miRNAs assay used in qRT-PCR.

Assay Name	Assay ID	Mature miRNA Sequence
hsa-miR-17	002421	ACUGCAGUGAAGGCACUUGUAG
hsa-miR-18a	002422	UAAGGUGCAUCUAGUGCAGAUAG
hsa-miR-19a	000395	UGUGCAAAUCUAUGCAAAACUGA
hsa-miR-19b	000396	UGUGCAAAUCCAUGCAAAACUGA
hsa-miR-20a	000580	UAAAGUGCUUAUAGUGCAGGUAG
hsa-miR-92a	000431	UAUUGCACUUGUCCCGGCCUGU GTGCTCGCTTCGGCAGCACATATACTAAAATTGGAACGATA
U6 snRNA	001973	CAGAGAAGATTAGCATGGCCCTCGCGCAAGGATGACACGC CAAATTCGTGAAGCGTCCATATTTT (Control Sequence)

saliva collection. All collections were performed under resting conditions in a quiet room between 9 a.m. and 10 a.m. A volume of <1.5 ml of saliva in 15 minutes was considered abnormal (24). The Schirmer test was performed without anesthesia by placing a standardised strip of filter paper in the lateral canthus away from the cornea of each eye and left in place for 5 minutes, with the subject keeping the eyes closed. Outcomes were recorded in millimeters of wetting for 5 minutes. A wet length of <5 mm in 5 minutes was considered abnormal (25).

Histological assessment of MSG biopsies

The labial MSG biopsies were obtained from all patients and healthy volunteers at the time of inclusion in the study. These tissues were fixed in 10% formalin overnight, dehydrated in a series of graded ethanol solutions and embedded in paraffin. For histological evaluation, the sections were cut at a thickness of 4 µm. Then the slides were stained with haematoxylin and eosin for conventional histological examination by a well-trained pathologist in a blinded manner. The degree of lymphocytic infiltration in each section was judged by focus scoring. A lymphocytic foci was defined as an aggregate of >50 lymphocytes in each 4-mm² area of MSG tissue (26). The focus score (FS) was classified in: FS=0: no lymphocytic infiltration; FS=1: less than 1 lymphocytic focus; FS=2: less than 2 lymphocytic foci; FS=3: two or more lymphocytic foci (27).

RNA extraction

Total RNA were extracted from the un-

stained formalin fixed paraffin embedded (FFPE) labial MSG tissues. The tissues were sectioned to 10-µm thickness and placed into xylene to remove paraffin, followed by ethanol series to remove xylene. RNA was extracted with the miRNeasy FFPE kit (Qiagen) according to the manufacturer's instructions. Briefly, samples were suspended in buffer PKD and then lysed with proteinase K digestion followed by heat treatment. After centrifugation, the supernatant was treated with DNase booster buffer and DNase stock solution. After mixing with buffer RBC and ethanol, the mixture was passed through an RNeasy MinElute spin column where total RNA was bound. RNA was washed with buffer RPE to remove impurities and eluted with RNase-free water. The RNA was quantified by using a NanoDrop 2000 Spectrophotometer (Thermo Scientific) and immediately stored at -80°C until further use.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Quantitative-RT-PCR was performed using the TaqMan microRNA assay (Applied Biosystems). The total RNA extracted from the FFPE tissues was reverse-transcribed with the TaqMan MicroRNA Reverse Transcription Kit (Life Technologies Inc.) according to the manufacturer's instructions. In brief, the reverse transcription reaction was performed in a total volume of 15 µL mixture containing 5 µL of RNA sample, 3 µL of 5× RT primer (Applied Biosystems), 0.15 µL of 100 mM dNTPs with dTTP, 1.00 µL of 50 U/µL MultiScribe reverse transcriptase, 1.50 µL of 10× reverse transcription buffer, 0.19 µL of 20 U/µL RNase inhibitor,

Table II. Demographic and clinical characteristics of healthy volunteers and pSS patients.

Variables	Control (n=13)	Group I (n=16)	<i>p</i> -value	Group II (n=20)	<i>p</i> -value	Group III (n=21)	<i>p</i> -value
<i>Demographic</i>							
Age (years), mean±SD (range)	38.4 ± 6.0 (28-47)	60.1 ± 15.7 (22-79)	<0.001	54.2 ± 13.1 (15-77)	<0.001	50.2 ± 13.8 (28-72)	0.007
Gender (Female/Male)	12/1	13/3	NS	19/1	NS	20/1	NS
<i>Clinical</i>							
Salivary flow rate (mL/15min)	9.19 ± 8.40	1.15 ± 0.73	<0.001	1.18 ± 0.89	0.005	1.05 ± 0.91	<0.001
Schirmer test (mm/5min)							
OD	14.94 ± 7.13	3.74 ± 2.26	<0.001	3.35 ± 2.08	<0.001	3.38 ± 2.33	<0.001
OS	15.33 ± 8.10	3.61 ± 2.29	<0.001	3.15 ± 2.18	<0.001	2.38 ± 1.94	<0.001
<i>Laboratory</i>							
RF (U/mL)	20.0 ± 0.0	105.9 ± 106.3	0.007	159.3 ± 161.9	0.001	249.7 ± 292.7	0.008
ASO (IU/mL)	31.3 ± 5.0	55.4 ± 28.9	0.006	57.0 ± 36.7	0.006	57.8 ± 33.8	0.009
CRP (mg/L)	1.60 ± 0.60	8.16 ± 10.48	0.033	7.53 ± 9.51	0.012	11.34 ± 14.89	0.026
Anti-SS-A antibodies, n (%)	0 (0.0)	9 (56.2)	<0.001	8 (40.0)	<0.001	12 (57.1)	<0.001
Anti-SS-B antibodies, n (%)	0 (0.0)	1 (6.2)	<0.001	0 (0.0)	-	7 (33.3)	<0.001

The data were expressed as numbers (frequencies) unless otherwise mentioned. *p* refers to the comparison between the 3 pSS subgroups and the control group. *p*<0.05: statistically significant.

OD: right eye; OS: left eye; RF: rheumatoid factor; ASO: anti-streptolysin O; CRP: C-reactive protein.

and 4.16 µL of Nuclease-free water. The reaction mixture was incubated at 16°C for 30 min, then 42°C for 30 min, finally 85°C for 5 min, and then held at 4°C. The real-time RT-PCR was then carried out on the ABI 7900 HT Real-Time PCR System (Life Technologies Inc.) using TaqMan probes (Applied Biosystems) according to the manufacturer's instructions with minor modifications. Briefly, 2.0 µL of RT product was added to 1.0 µL of probe, 10 µL of TaqMan Universal Master Mix II (2×, No UNG) and 7 µL of Nuclease-free water for a total reaction mix of 20 µL. The thermal cycling conditions were as follows: an initial denaturation step at 95°C for 10 min, 40 cycles of PCR amplification at 95°C for 15 s, 60°C for 1 min. All the samples were performed in triplicates. The relative expression levels of miRNAs were normalised to U6 snRNA as internal control, which was selected based on literatures and our pre-experiments suggesting its stable expression in cells and tissues (28, 29), using the $\Delta\Delta CT$ method in which $\Delta CT = CT(\text{miRNA}) - CT(\text{snU6})$ and CT represented the threshold cycle number. The fold changes in miRNAs were calculated by the equation $2^{-\Delta\Delta CT}$ (30). ID and sequence of TaqMan miRNAs as shown in Table I.

Statistical analyses

Unless otherwise stated, values in this study were expressed as mean±standard

deviation (SD). Comparisons of continuous variables between groups were performed using either Student's *t*-test or one-way analysis of variance (ANOVA). Categorical variables were compared by the Chi-square tests. Two-tailed *p*-values less than 0.05 were considered to be statistically significant (**p*<0.05; ***p*<0.01). All statistical analyses were conducted using SPSS software, v. 18.0 (Chicago, Illinois, USA).

Results

Baseline characteristics and clinicopathologic parameters

The pSS patients were allocated to 3 subgroups according to the histological findings of the MSG biopsies. Group I: FS=1, n=16; Group II: FS=2, n=20; Group III: FS=3, n=21. Thirteen healthy volunteers, FS=0, were served as control group. The representative pictures of MSG biopsies that belonged to the control and 3 pSS subgroups, as classified by the grade of FS, were shown in Figure 1.

The demographic and clinical characteristics of the study subjects were summarised in Table II. The mean age of healthy volunteers was younger than the pSS patients (38.4±6.0 vs. 54.4±14.4 years; *p*<0.001). The prevalence of gender was comparable between pSS patients and controls, where most of the study subjects were females. The differences between the control group and the 3 pSS subgroups were statis-

tically significant for positive findings of salivary flow rate, Schirmer test, RF, ASO and CRP, as well as the frequencies of anti-SS-A and anti-SS-B antibodies.

Differentially expressed miRNAs among varying histological stages of MSG tissues

In the labial MSG tissues, we observed that the expression level of miR-18a was significantly up-regulated in patients of the 3 pSS subgroups compared to control group (*p*<0.05), while the expression level of miR-92a was significantly down-regulated (*p*<0.05). We also observed that there was no notable difference in the expression levels of miR-17, miR-19a, miR-19b, and miR-20a between pSS patients and controls (*p*>0.05). Furthermore, we distinguished the differential expression levels of the two miRNAs, miR-18a and miR-92a, among varying histological stages of tissues. As shown in Figure 2, miR-18a was progressively up-regulated along the advanced histological stages, while the miR-92a was progressively down-regulated.

Discussion

To our knowledge, this is the first study on the expression of the miR-17-92 cluster in labial MSG tissues of pSS patients. Our data provide the first evidence that the expression levels of the miR-17-92 cluster, or its members,

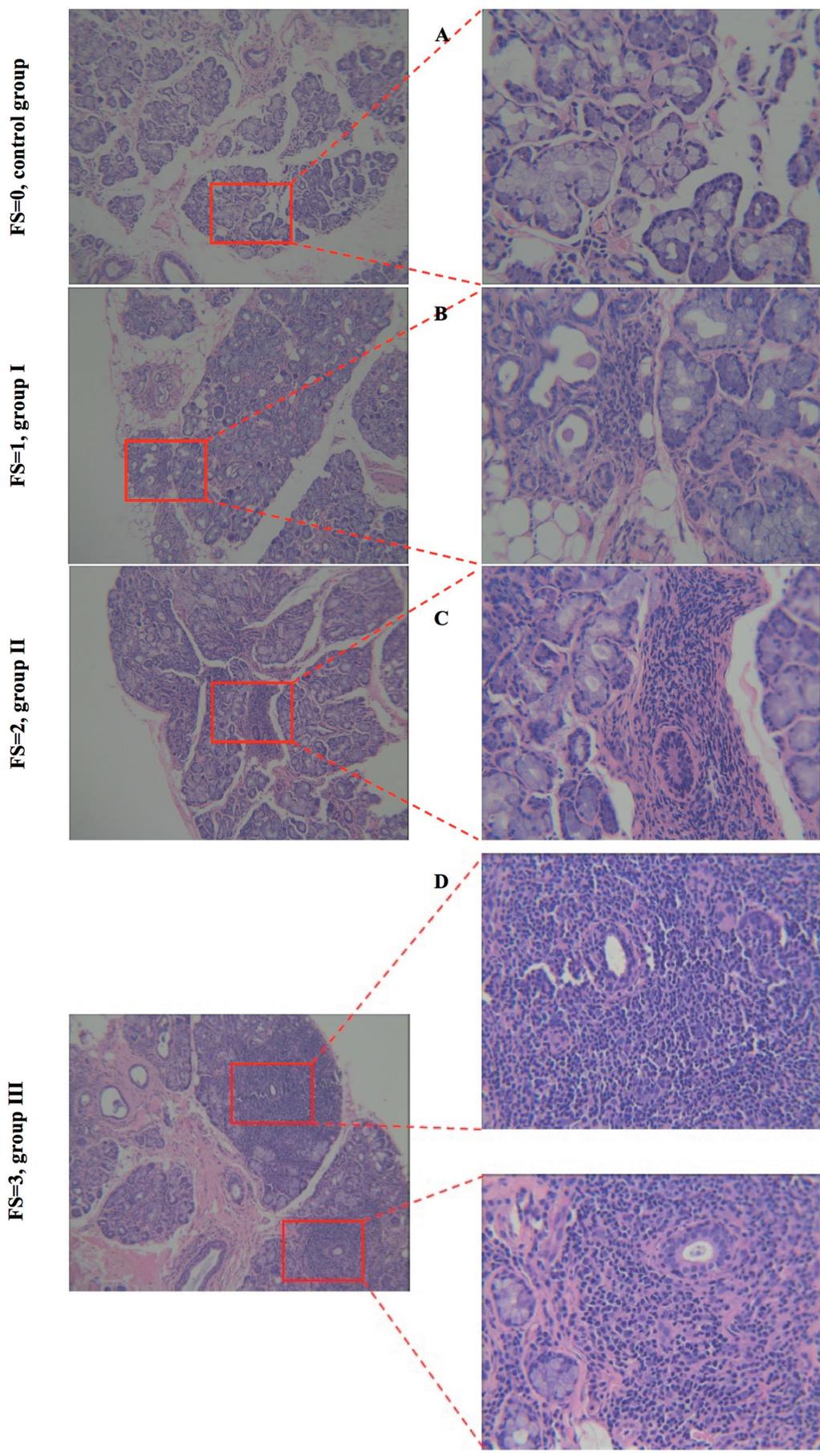


Fig. 1. Representative pictures of labial MSG biopsies. Sections were stained with haematoxylin and eosin. Photomicrographs showing the different grades of FS were based on the presence of lymphocytic foci consisting of >50 lymphocytes per 4 mm²: (A) FS=0: no lymphocytic infiltration, in control group; (B) FS=1: less than 1 lymphocytic focus, in group I; (C) FS=2: less than 2 lymphocytic foci, in group II; (D) FS=3: two or more lymphocytic foci, in group III. The left column showed larger area of the tissue samples while enlarged area of the portions marked were shown in the right column. Original magnification: left column ×10, right column ×40. MSG: minor salivary gland; FS: focus score.

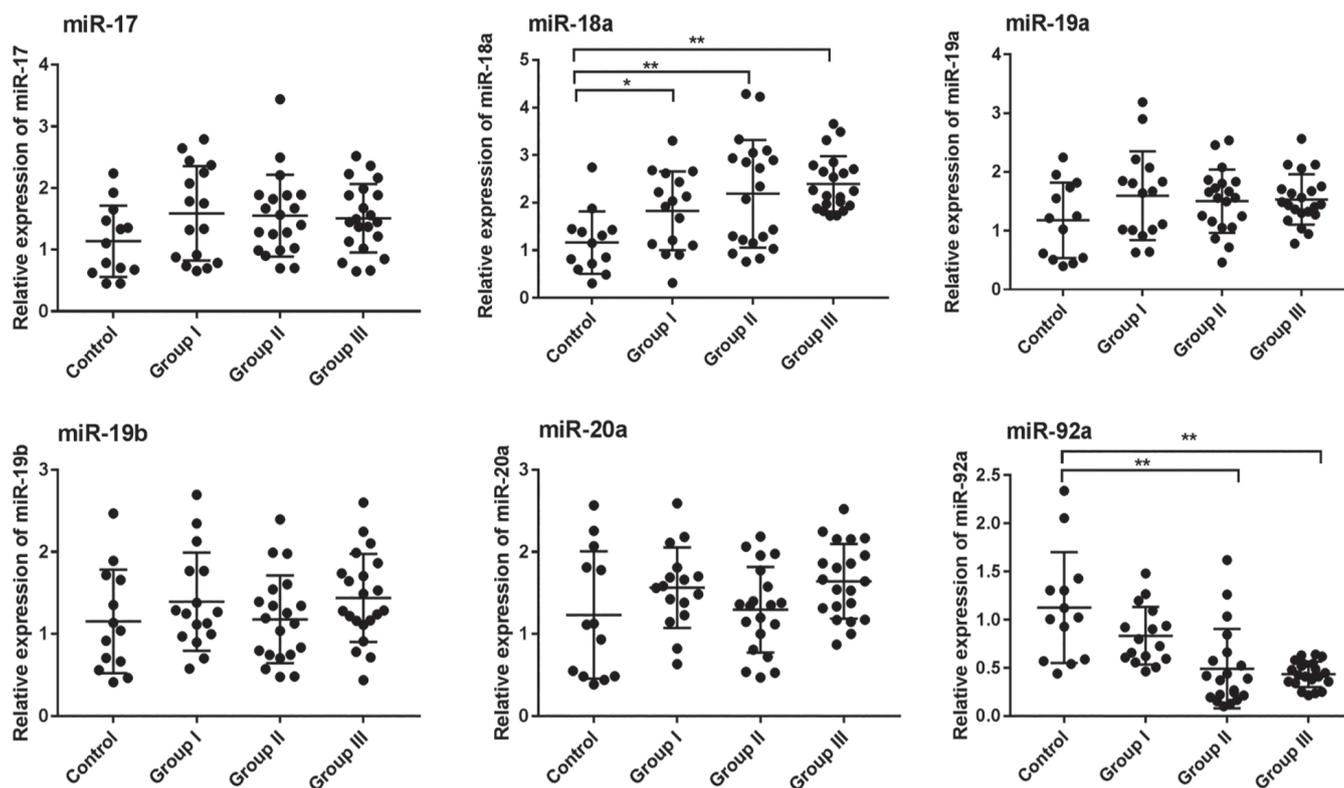


Fig. 2. Differentially expressed miRNAs in labial MSG tissues. The expression of miR-17, miR-18a, miR-19a, miR-19b, miR-20a and miR-92a relative to that of U6 small nuclear RNA was determined by quantitative polymerase chain reaction in labial MSG tissues from pSS patients of group I (FS=1, n=23), group II (FS=2, n=23), group III (FS=3, n=23) and control group (healthy individuals, FS=0, n=23). Data are presented as the mean±standard deviation. * $p<0.05$ vs. controls. ** $p<0.01$ vs. controls.

miR-18a and miR-92a, can clearly separate non-SS control subjects from all patients with pSS.

MiR-18a is the most highly expressed miRNAs in the miR-17-92 cluster, which has been found to be significantly up-regulated in various human cancers (31, 32). The results of the current study demonstrated that the expression of MSG miR-18a was significantly higher in patients with pSS than in healthy control individuals. Furthermore, a strong association was observed between higher miR-18a expression and pSS compared with healthy individuals. In addition, significantly higher expression of MSG miR-18a was detected in pSS patients with more aggressive lymphocytic infiltration and of a more advanced histological stage, suggesting that miR-18a expression may correlate with progression of the disease.

The downregulated miRNA, miR-92a, which has been demonstrated the contributing effect on tumourigenesis in a variety of experiments (33), exhibited lower levels in the labial MSG of pSS

patients than in healthy individuals. MiR-92a is the last and least described member of the miR-17-92 cluster (34). So far, no investigation of miR-92a has been carried out in tissue samples from pSS patients. Our, hitherto, investigations demonstrated the pSS-specific decrease in levels of miR-92a. In addition, the decreased levels of miR-92a were also associated with progressive lymphocytic infiltration, indicating that miR-92a might be involved in pSS progression. It had been reported that the expression of miR-92a was regulated negatively by oxidative stress and associated with cell viability, pre-miR-92a treatment had protective effects against oxidative stress-induced apoptosis and antisense inhibition of miR-92a could induce cell apoptosis (35-37). On the other hand, the involvement of oxidative stress and apoptosis was thought to be an important mechanism in the pathogenesis and development of SS (38, 39). Thus, the miR-92a might be the upstream factor to influence the oxidative stress and apoptosis in pSS and

more studies need to be performed to identify more downstream targets and pathways.

In conclusion, the current study provided, for the first time, an important link between miR-17-92 cluster and pSS. We found the association of increased expression levels of miR-18a and reduced expression levels of miR-92a with advanced clinical stages of pSS, as indicated by the advanced clinicopathologic features. These results further support the view that the approach of miRNAs assay could significantly reduce the substantial subjectivity of scoring inflammatory infiltrates and may aid in the diagnosis of pSS (6). However, larger, more precise and problem-oriented researches should be carried out to validate whether the miR-17-92 cluster or miR-18a and miR-92a can be used as marker for the diagnosis of pSS. Moreover, in-depth studies are required to understand the precise mechanisms behind the up-regulation of miR-18a and down-regulation of miR-92a in pSS.

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