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Overexpression of miR-31 in Peripheral Blood Mononuclear Cells (PBMC) from Patients with Ankylosing Spondylitis

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Background: miRNAs play vital roles in regulating immunologic functions and autoimmunity. However, the levels of miR-31, miR-155, miR-16, and miR-181a have not been explored in AS, but were verified to play vital roles in other immunological diseases. The aim of our study was to examine whether the expressions of miR-31, miR-155, miR-16, and miR-181a are abnormal in AS.

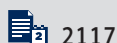
Material/Methods: Real-time transcription-polymerase chain reaction analysis (RT-PCR) was used to determine the expression of miR-31, miR-155, miR-16, and miR-181a in peripheral blood mononuclear cells (PBMC) from 40 patients with AS and 40 healthy controls.

Results: The expression of miR-31 was increased in AS patients compared with healthy controls ($p=0.001$). Furthermore, we detected no significant differences in the expressions of miR-155, miR-16, and miR-181a between AS patients and healthy controls. However, the expression levels of the 4 miRNAs were all significantly different between less active AS and more active AS, with higher levels in more active AS. Moreover, no significant correlations were found between the 4 miRNAs levels with the clinical characteristics in the patients with AS. Interestingly, the expression levels of miR-31, miR-155, and miR-16 in PBMCs were significantly positively correlated with the ESR in new AS patients but not old AS patients.

Conclusions: Our results suggest that miR-31 is overexpressed in PBMCs of AS patients. Furthermore, miR-31, miR-155, miR-16 and miR-181a may be associated with AS disease activity.

MeSH Keywords: **Autoimmune Diseases • MicroRNAs • Spondylitis, Ankylosing**

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Background

Ankylosing spondylitis (AS) is a common inflammatory rheumatic disease that mainly affects sacroiliac joints and axial skeleton, causing characteristic inflammatory back pain, and can lead to structural and functional impairments and a decrease in quality of life [1]. The prevalence of AS is about 0.86% in whites [2], 0.10–1.40% in Europe [1], and 0.20–0.54% in China [3]. Chronic joint diseases affect millions of people worldwide and impose a high burden on society [4,5]. It has been confirmed that human leucocyte antigen (HLA)-B27 is strongly associated with susceptibility to AS [6]. However, the disease mechanisms of AS are still unknown. AS is a multifactorial process that involves autoimmune responses and is influenced by genetic and environmental factors and immunologic abnormality [7].

MicroRNAs (miRNAs) belong to a class of small (approximately 22 nucleotides) noncoding RNA molecules and regulate the activities of target mRNAs by binding at sites in the 3'untranslated region of the mRNAs [8,9]. They act as key post-transcriptional regulators of gene expression [10,11]. miRNAs play crucial roles in diverse biologic processes, including the regulation of immunologic functions and autoimmunity [12–15]. Altered expression of miRNAs has been described under various pathological conditions, including autoimmune diseases [16,17]. With respect to autoimmune diseases, the abnormal expression of miRNAs has attracted increasing attention owing to the important roles that miRNAs play in the regulation of the immune system. Many studies have demonstrated that miRNAs in peripheral blood mononuclear cells (PBMCs) from patients with autoimmune diseases have altered expression [18–20]. miR-31 controls osteoclast formation and bone resorption by targeting RhoA, which are responsible for bone loss and destruction of joints in rheumatoid arthritis [21]. Through the study of psoriasis, miR-31 has been reported as a new regulatory factor in the activation of NF- κ B pathway and the production of IL-2, which play critical roles in inflammatory responses [22–24]. Previous studies suggest that miR-31 is involved in immune regulation. Up-regulation of miR-155 has been previously identified in peripheral blood-derived mononuclear cells (PBMC) from rheumatoid arthritis patients [18]. Lai et al. studied miRNA-regulated immunologic functions and autoimmunity and found that miR-16 was elevated in AS, but the expression level of miR-16 was not associated with Bath Ankylosing Spondylitis Radiology Index (BASRI) of the lumbar spine [25]. Another study demonstrated that blood miR-181a might serve as a new marker in individuals with inflammatory responses [26]. In conclusion, miR-31, miR-155, miR-16, and miR-181a are reportedly abnormal expressed in autoimmune diseases, especially rheumatoid arthritis. We hypothesized that in AS, as a common autoimmune and articular inflammatory disease, those 4 miRNAs might represent aberrant expression in PBMCs of AS patients.

In the present study, we aimed to examine the expression levels of these same microRNAs and to determine if results can be validated in AS patients. Furthermore, we explored the relationships between their expression levels and the clinical characteristics of AS patients.

Material and Methods

Study subjects

We performed a case-control study. Ethical approval for the use of clinical specimens was granted by the Ethics Committees of Anhui Medical University. Informed consent was obtained from all patients or their guardians. The study was conducted according to the principles of the Declaration of Helsinki. Patients who met the following criteria were included: (1) patients were diagnosed according to the 1984 modified New York criteria [27]; (2) patients completed the structured questionnaire including demographics and clinical characteristics. Furthermore, cases were excluded if they were complicated with pulmonary tuberculosis, rheumatoid arthritis, systemic lupus erythematosus, psoriatic arthritis, psoriasis, or other chronic inflammatory or immune diseases. Eight patients were excluded because they failed to complete questionnaire or had any of the above-mentioned diseases. From July 2014 to March 2015, 40 eligible patients, including 13 new cases and 27 old cases, diagnosed with AS were recruited from the Department of Rheumatology and Immunology, the First Affiliated Hospital of Anhui Medical University, Hefei, China. New cases were those who were diagnosed recently and did not receive any drug treatment, and old cases were those who had received regular treatment for some time. The Bath AS Disease Activity Index (BASDAI) was used to evaluate disease activity of patients with AS, with a BASDAI score of ≥ 3 indicating more active AS. More active AS means more severe disease progression. Subsequently, 40 healthy controls matched by sex, age, and ethnicity were selected from the Center Blood Station of Hefei City, China. All healthy controls had no family history of autoimmune diseases and finished the structured questionnaire.

Sample handling

First, we collected peripheral blood from the subjects, and the samples were stored in tubes treated with EDTA-2K. Then, according to the manufacturer's protocol, mononuclear leukocytes were separated using Lympholyte-H (Cedarlane, Burlington, NC, USA). Prior to RNA isolation, the PBMCs were washed twice in sterile phosphate-buffered saline (PBS).

Table 1. Clinical characteristics of the patients with ankylosing spondylitis (AS) and healthy controls.

Variables	Ankylosing spondylitis patients (40)	Controls (40)
Age, yrs	28.53±8.133*	27.42±7.551 *
Sex (male)	30 (75%)	29 (73%)
HLA-B27+	36 (90.0%)	
ESR, mm/h	21.35 (7–22)**	
CRP, mg/L	21.69 (2.38–24)**	
BASDAI	1.59±1.44*	
BASFI	0.05 (0–1.67)**	
Disease duration, yrs	3.0 (0–19)	

* Mean ±SD. ** Data were presented as median (Q₁–Q₃). ESR – erythrocyte sedimentation rate; CRP – C-reactive protein; BASDAI – Bath Ankylosing Spondylitis Disease Activity Index; BASFI – Bath Ankylosing Spondylitis Functional Index.

miRNA processing

Total RNA was separated from the newly acquired PBMCs using TRIzol according to the instructions of the manufacturer (Invitrogen, USA). RNA concentration and quality, including optical density (OD) 260/OD 280 and OD 260/OD 230 measurements, were determined by use of a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The reverse transcription of total miRNA was performed using the All-in-One™ miRNA qRT-PCR Detection Kit to convert miRNA to cDNA. The samples of cDNA were stored at –20°C until use.

Quantitative real-time PCR (qRT-PCR)

miRNA qRT-PCR was conducted using the SYBR Green miRNA assay (AceQ™ qPCR Kit, Vazyme Biotech Co., Ltd., China). The PCR reaction was processed in 20 µl for each of the samples, including AceQ™ qPCR SYBR® Green Master Mix (Vazyme biotech co., Ltd., China), 10 µM each primer, 50×ROX Reference Dye 2, and cDNA. The PCR procedure was as follows: 95°C 5 min; 40 cycles x (95°C for 10 s, 65°C for 40 s); 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. RT-PCR was performed using the ABI ViiA7 real-time PCR system (Applied Biosystems, Carlsbad, CA, USA). The expressions of miR-31, miR-155, miR-16, and miR-181a were evaluated in triplicate and the level of U6 was also detected as an internal control.

Statistical analysis

SPSS version 13.0 for Windows (SPSS Inc., Chicago, IL) was used for all statistical analyses. The Mann-Whitney test was applied to assess the difference in miRNAs level between patients and controls. Spearman's rank correlation analysis was conducted to calculate the correlation coefficient and significance. A probability level less than 0.05 was accepted to indicate a significant difference.

Results

Clinical features of the subjects

A total of 80 samples (from 40 AS patients and 40 healthy controls) were analyzed. Their demographic and clinical characteristics are summarized in Table 1. As shown in Table 1, the average age of the patients and controls were 28.53 (years) ±8.133 and 27.42 (years) ±7.551, respectively. The frequency of males was 30 (75%) in AS patients and 29 (73%) in controls. There was no significant difference between the 2 groups in age ($t=0.627$, $P=0.533$) or sex ($\chi^2=0.251$, $P=0.617$). We found that 90% of patients were HLA-B27-positive, the range of disease duration was 0–19 years, and the median disease duration was 3.00 years. The median of erythrocyte sedimentation rates (ESR) was 21.35, with the Q₁–Q₃ from 7 to 22. The median C-reactive protein (CRP) was 21.69 with the Q₁–Q₃ from 2.38 to 24.0. The mean BASDAI score was 1.59±1.44. The median BASFI score was 0.05 with the Q₁–Q₃ from 0.00 to 1.67.

Quantification of miRNAs expression in PBMC from AS patients and healthy controls by real-time RT-PCR

RT-PCR analysis was performed to detect the levels of miR-31, miR-155, miR-16, and miR-181a in PBMCs from AS patients and healthy controls. As shown in Figure 1A, compared with healthy controls, miR-31 was significantly overexpressed in patients with AS (0.497, IQR 0.103 to 3.656, versus 0.03, IQR 0.012 to 0.293, $P=0.001$), and miR-31 relative expression level was significantly higher in more active AS cases. Moreover, the relative expression of miR-155, miR-16, and miR-181a was higher in patients with AS than in healthy controls, but the difference was not significant. Notably, the relative expressions of miR-155, miR-16, and miR-181a were significantly higher in more active AS cases (Figure 1B–1D).

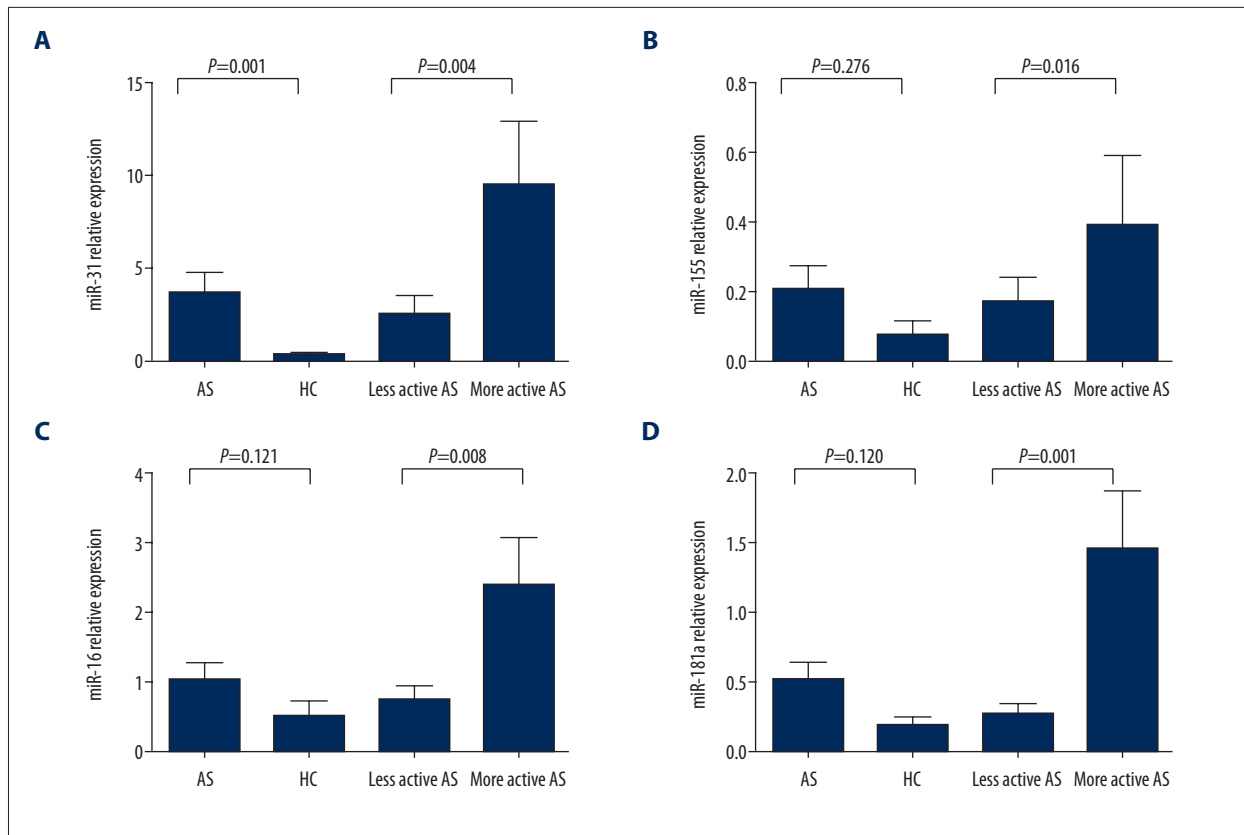


Figure 1. Comparison of miRNAs expression levels in PBMC between patients with AS and healthy controls. Real-time RT-PCR was performed to quantify the expression of miR-31 (A), miR-155 (B), miR-16 (C), and miR-181a (D) in AS patients (n=40) and healthy controls (n=40). There was a significant increase in miR-31 expression in AS patients compared with healthy controls (p=0.001). Columns represent the expression of miR-31 (A), miR-155 (B), miR-16 (C), and miR-181a (D). miR-31 – microRNA-31; miR-155 – microRNA-155; miR-16: microRNA-16; miR-181a – microRNA-181a; AS – ankylosing spondylitis. All error bars represent standard error.

Table 2. Correlations of miRNAs relative expression levels with clinical characteristics of the patients AS (n=40).

	ESR		CRP		BASDAI		BASFI		Disease duration	
	r_s	p	r_s	p	r_s	p	r_s	p	r_s	p
miR-31	0.08	0.69	-0.09	0.66	0.05	0.74	-0.16	0.32	-0.01	0.98
miR-155	0.09	0.64	-0.18	0.38	-0.01	0.95	-0.17	0.31	0.04	0.82
miR-16	0.15	0.43	0.04	0.86	0.02	0.91	-0.15	0.37	-0.04	0.82
miR-181a	0.04	0.81	-0.07	0.73	0.10	0.54	-0.12	0.45	-0.05	0.79

ESR – erythrocyte sedimentation rate; CRP – C-reactive protein; BASDAI – Bath Ankylosing Spondylitis Disease Activity Index; BASFI – Bath Ankylosing Spondylitis Functional Index; AS – ankylosing spondylitis.

Analysis of relationships between miRNA expression and clinical characteristics in patients with AS

Association of the 4 miRNAs with demographic characteristics and clinical characteristics were analyzed. We examined the levels of miR-31, miR-155, miR-181a, and miR-16 in PBMCs from AS cases, but no significant associations were found between

the 4 miRNAs levels with Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) score, Bath AS Function Index (BASFI) score, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and disease duration (Table 2). However, we found that the expression levels of miR-31, miR-155, and miR-16 in PBMCs of new AS patients were positively associated with ESR, but the correlations were not significant in patients with old AS (Figure 2).

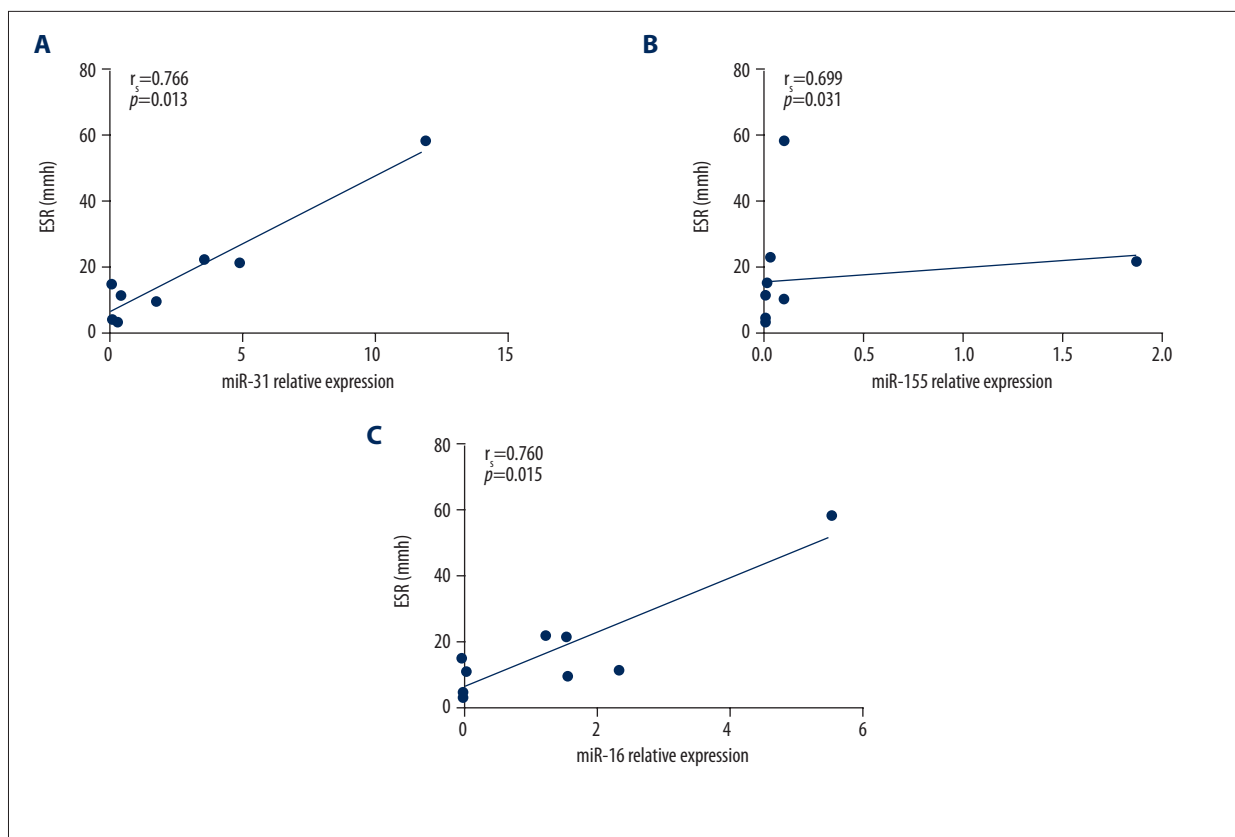


Figure 2. Correlation between ESR and miRNAs expression levels in new AS patients (n=13). **(A)** Correlation of expression of miR-31 with ESR (mm/h). **(B)** Correlation of expression of miR-155 with ESR (mm/h). **(C)** Correlation of expression of miR-16 with ESR (mm/h). miR-31 – microRNA-31; miR-155 – microRNA-155; miR-16: microRNA-16; ESR – erythrocyte sedimentation rate. Columns represent the level of ESR in AS patients.

Discussion

The role of miRNAs in autoimmune diseases has attracted increasing attention, and recent discoveries have indicated the dysregulation of various miRNAs in AS [28].

In the present study, we detected the expression levels of miR-31, miR-155, miR-181a, and miR-16, which are thought to be crucial regulators in the immune system of AS patients. Our result revealed that miR-31 was up-regulated in PBMCs of AS patients compared with healthy controls, but the other 3 miRNAs were not significantly different between 2 groups. Moreover, there were no significant associations between the relative expressions of miR-155, miR-181a, and miR-16 and the demographic features and clinical characteristics of AS patients. However, we found a significant association between ESR and miR-31, miR-155, and miR-16 when compared with new cases.

The human miRNA miR-31 gene is located at chromosome 9p21.3 [29]. Immune system homeostasis is mediated by regulatory T (Treg) cells [30]. miR-31 can negatively regulate pT_{reg}-cell generation by directly targeting Gprc5a, suggesting that

miR-31 and its target, Gprc5a, play a vital role in autoimmune diseases [31]. Impaired T_{reg}-cell proliferative potential and reduced cloning frequency were verified in autoimmune disease [32]. Thus, miR-31, as a regulator of T_{reg}-cell generation, is of great interest in autoimmune diseases. Previous studies have indicated that miR-31 is expressed abnormally in autoimmune diseases. For example, miR-31 is significantly decreased in T cells of lupus patients in contrast to healthy controls [20]. miR-31 is also overexpressed in keratinocytes of patients with psoriasis, a chronic autoimmune disease characterized by the formation of itchy, silvery scales on the skin [22,23]. Our study showed that miR-31 was significantly overexpressed in PBMCs of AS patients, consistent with previous studies. However, it is not clear what factors cause the up-regulation of miR-31 expression in AS. Further research is required to validate the overexpression of miR-31 in AS and to investigate its possible mechanisms.

The expression levels of miR-155, miR-16, and miR-181a in PBMCs of AS patients and healthy controls were also examined in our study. The results showed that the expressions of the 3 miRNAs in AS PBMCs were not significantly different

from expression in healthy controls. However, compared with less active AS cases, the relative expression of miR-31, miR-155, miR-16, and miR-181a were significantly higher in more active AS cases. Thus, we concluded that because the sample size was relatively small, the differences in overexpression levels of the 3 miRNAs between AS and healthy controls were not significant. It is possible that the 4 miRNAs were correlated with disease activity. Moreover, the result of correlation analysis showed that there were no significant associations between the levels of the 4 miRNAs with clinical characteristics in the patients with AS, possible due to the heterogeneity of AS cases. Further research with larger samples will be needed to verify our results.

Notably, the expression of miR-31, miR-155, and miR-16 was related to the erythrocyte sedimentation rate (ESR) in new cases but not in old cases. Similar to BASDAI, erythrocyte sedimentation rate (ESR) usually reflects the disease activity of patients [33]. Thus, the results may strengthen the speculation that these studied miRNAs are correlated with AS disease activity. Given the effect of drug treatment, we analyzed the correlation in new AS patients and the conclusion may be reliable. The change of correlation between the 3 miRNAs and ESR after drug treatment would be of great interest in further studies.

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Conclusions

miR-31 is overexpressed in AS and the 4 miRNAs may be associated with AS disease activity.

Acknowledgments

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Conflict of Interest

None.

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