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Original Article

miR-146a and miR-155 as potential biomarkers for rheumatoid arthritis and disease activity

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Abstract: Objective: The aim of this study was to investigate the clinical value of miR-146a and miR-155 in peripheral blood mononuclear cells (PBMCs) in the diagnosis and treatment of rheumatoid arthritis (RA). Methods: This study retrospectively analyzed medical records of 90 patients treated for RA in the Department of Rheumatology and Immunology of the Fourth Affiliated Hospital of Harbin Medical University, between July 2015 and September 2017. These 90 patients were assigned to the RA group while another 40 healthy normal people, during the same period of time, were assigned to the control group. Disease activity was measured by Disease Activity Score 28 (DAS28). A total of 48 patients had high disease activity (DAS28 value ≥ 5.0) in the RA group. Relative expression levels of miR-146a and miR-155 in PBMCs were measured by RT-PCR. Associations with erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) were analyzed. Results: Relative expression levels of miR-146a and miR-155 in PBMCs in the RA group were higher than those in the control group ($t = 11.460$, $P < 0.001$; $t = 11.360$, $P < 0.001$). Moreover, relative expression levels of miR-146a and miR-155 in patients with high disease activity were higher than those in patients with low disease activity ($t = 12.900$, $P < 0.001$; $t = 17.770$, $P < 0.001$). In the RA group, it was found that relative expression of miR-146a and miR-155 in PBMCs was positively correlated with ESR and CRP (miR-146a, ESR: $r = 0.342$, $P < 0.001$; miR-146a, CRP: $r = 0.271$, $P < 0.001$; miR-155, ESR: $r = 0.328$, $P < 0.001$; miR-155, CRP: $r = 0.274$, $P < 0.001$). Conclusion: Expression of miR-146a and miR-155 was markedly upregulated in the RA group, suggesting that these two microRNAs (miRNAs) may have been involved in occurrence and development of RA. Since upregulation of miR-146a and miR-155 expressions is closely related to RA and disease activity, these two miRNAs may serve as potential biomarkers for early diagnosis of RA and assessment of disease activity.

Keywords: Rheumatoid arthritis, miR-146a, miR-155, erythrocyte sedimentation rate, C-reactive protein

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disorder that can affect the entire body. This disease is primarily characterized by progressive joint damage, erosive arthritis, and joint deformity [1]. Causes of RA remain unclear. There are no typical clinical characteristics during early stages of RA. In the end stage, irreversible pathological changes can take place causing joint damage and deformities, loss of joint function, and even severe disabilities, affecting patient quality of life [2, 3]. RA can not only bring about fever, anemia, and muscle weakness but can also increase risks of pulmonary and blood disorders [4]. The disease has a high prevalence, about 0.5%-1%. RA usually occurs in people over 35 years old. Peak age of onset in women is between 40-55 years while incidence in men increases with age. The male-to-female ratio in RA is around 1:3. Since China has gradually turned into an aging society, incidence of RA has risen each year [5]. At present, there is no cure for this disease. Goals of current treatment methods are mainly to reduce joint inflammation and slow down development of RA [6]. It is believed that an early effective diagnosis and timely intervention are necessary to control the progression of the disease and reduce incidence of disabilities [7]. Therefore, it is of great importance to investigate the pathogenesis of RA, finding biomark-

Table 1. Gene sequences of miR-146a, miR-155, and U6

Gene	Forward primer	Reverse primer
miR-146a	5'-GCAGGGTCCGAGGTATTCG-3'	5'-CGCGTGAGAACTGAATTCCAT-3'
miR-155	5'-GTGCAGGGTCCGAGGT-3'	5'-CGCTTAATGCTAATCGTGATAGG-3'
U6	5'-CTCGCTTCGGCAGCACA-3'	5'-AACGCTTCACGAATTTGCGT-3'

Table 2. Baseline data for the two groups (n (%), mean ± sd)

	RA group (n = 90)	Control group (n = 40)	t/χ^2	P
Gender			0.637	0.500
Male	19 (21.11)	11 (27.50)		
Female	71 (78.89)	29 (72.50)		
Age (year)	49.62 ± 13.62	48.83 ± 12.25	0.314	0.753
ALT (U/L)	63.21 ± 23.24	65.09 ± 22.69	0.428	0.668
γ-GT (U/L)	45.45 ± 18.64	43.19 ± 17.63	0.648	0.517
Glu (mmol/L)	6.09 ± 1.18	5.93 ± 1.14	0.720	0.472
AST (U/L)	20.35 ± 7.63	18.48 ± 7.24	1.310	0.192
TBIL (mol/L)	13.52 ± 6.25	12.52 ± 5.48	0.873	0.384
ESR (mm/H)	18.27 ± 2.39	6.36 ± 2.58	25.590	< 0.001
CRP (mg/L)	11.91 ± 3.88	1.45 ± 0.89	16.820	< 0.001

Note: RA, rheumatoid arthritis; ALT, alanine transaminase; γ-GT, γ-glutamyltransferase; Glu, blood glucose; AST, aspartate aminotransferase; TBIL, total bilirubin; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein.

ers and biological targets related to this disease.

MicroRNAs (miRNAs) are widely distributed in a variety of organs and cells, playing different roles in various physiological processes, including developmental regulation, hematopoiesis, lipid metabolism, neurogenesis, protection against viruses, cell proliferation, apoptosis, and differentiation [8]. It is believed that miRNAs participate in the occurrence and development of many diseases, including cardiovascular diseases, autoimmune diseases, and tumors [9]. Some studies have documented that miRNAs can be expressed in tissues, synovial cells, synovial fluid, chondrocytes, and peripheral blood mononuclear cells (PBMCs) in patients with RA. They are closely associated with occurrence, progression, and prognosis of this disease [10]. It has been reported that a collagen-induced arthritis model cannot be created in miR-155-deficient mice and that inflammatory cell infiltration and bone erosion are not found in the synovial tissue of mice, indicating that miR-155 may play a key role in occurrence of RA [11]. Pauley et al. demonstrated that expression of miR-155 and miR-146a in PBMCs in RA patients are 1.8 and 2.6 times the expression of those in normal healthy people, respectively [12]. In recent years, the roles of miR-146a and miR-155 in RA have become a popular research topic. These two miRNAs may serve as potential biomarkers and biological targets for early diagnosis and treatment of RA.

Expression of miR-146a and miR-155 can vary among patients. However, few studies on the roles of these two miRNAs during the period when RA is active are available. In this present study, expression of miR-146a and miR-155 in PBMCs was analyzed in RA patients. The aim was to investigate the roles of these two miRNAs as potential markers in occurrence, progression, and activity of RA.

Materials and methods

General information

This study retrospectively analyzed medical records of 90 patients treated for RA in the Department of Rheumatology and Immunology of the Fourth Affiliated Hospital of Harbin Medical University, between July 2015 and September 2017. These 90 patients (male 19, female 71, 49.62 ± 13.62 years) were assigned to the RA group while another 40 healthy normal people, during the same period of time, were assigned to the control group (male 11, female 29, 48.83 ± 12.25 years). Assessment of patient RA disease activity was conducted using Disease Activity Score 28 (DAS28). There were 48 patients with high disease activity (DAS28 value ≥ 5.0) and 42 patients with low disease activity (3.2 < DAS28 value < 5.0).

Inclusion and exclusion criteria

This study was approved by the Ethics Committee of the Fourth Affiliated Hospital of Harbin

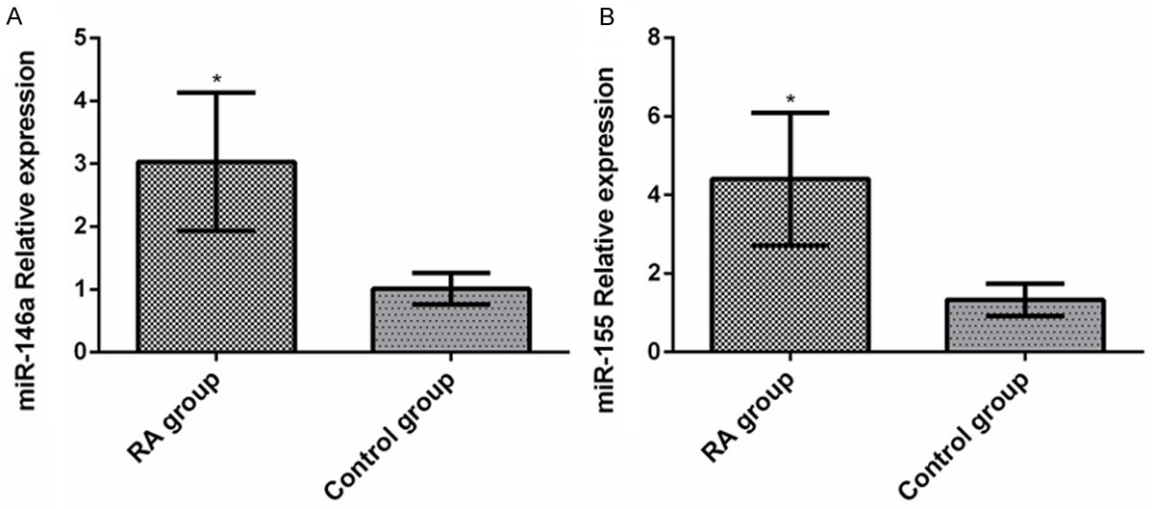

Figure 1. Comparison of relative expression levels of miR-146a and miR-155 in PBMCs in the two groups. Peripheral blood was taken and mononuclear cells were isolated for RNA extraction. Relative expression levels of miR-146a (A) and miR-155 (B) were measured by RT-PCR. A: $^{*}P < 0.001$, t = 11.460 vs. control group; B: $^{*}P < 0.001$, t = 11.360 vs. control group.

Medical University. Informed consent was obtained from all participants.

Inclusion criteria: Patients met classification and diagnostic criteria for RA, as defined by European League against Rheumatism and American College of Rheumatology in 2015 [13].

Exclusion criteria: Patients with other rheumatic diseases, such as osteoarthritis, spinal arthritis, and systemic lupus erythematosus; Patients with severe liver, kidney, and hematopoietic disorders; Patients or patient family members with histories of mental illness; Patients aged ≤ 28 or ≥ 79 years.

Main instruments and reagents

The following instruments and reagents were used for the present study: Applied Biosystems 7300 Real-Time PCR System (Thermo Fisher Scientific (China) Co., Ltd), TRIzol kit (Shanghai Enzyme-linked Biotechnology Co., Ltd), mirVana miRNA isolation kit (Thermo Fisher Scientific (China) Co., Ltd), miR-146a and miR-155 real-time PCR assay kit (Beckman Coulter Inc.), SP-1920 double beam UV-Vis spectrophotometer (Hangzhou Kexiao Chemical Instrument & Equipment Co., Ltd), and miR-146a, miR-155 and U6 control primers (designed and synthesized by Synbio Technologies Co., Ltd, primer sequences are listed in **Table 1**).

RT-PCR

Venous blood samples (5 mL) were collected from each participant using sterile blood collection tubes with anticoagulant EDTA-K2. PBMCs were isolated with lymphocyte separation medium and washed twice in PBS.

RNA extraction: Total RNA extraction was conducted using a TRIzol kit, according to manufacturer instructions. Afterward, RNA purity was verified by 1% agarose gel electrophoresis. Absorbance of RNA samples was measured by UV-Vis spectrophotometer.

cDNA synthesis: Total reaction volume was 20 μL. PCR running parameters were set as follows: 17°C for 25 minutes, 45°C for 25 minutes, and 80°C for 10 minutes. cDNA products were kept at -20°C.

Detection of relative expression of miR-146a and miR-155: Total reaction volume for RT-PCR was 20 μL, including SYBR green mix 10 μL, PCR primer mix 1 μL, diluted cDNA template 5 μL, and RNase-free water 4 μL. PCR cycling and running parameters were: 90°C for 15 minutes, 90°C for 15 seconds, 60°C for 30 seconds, and 72°C for 10 seconds, for a total of 35

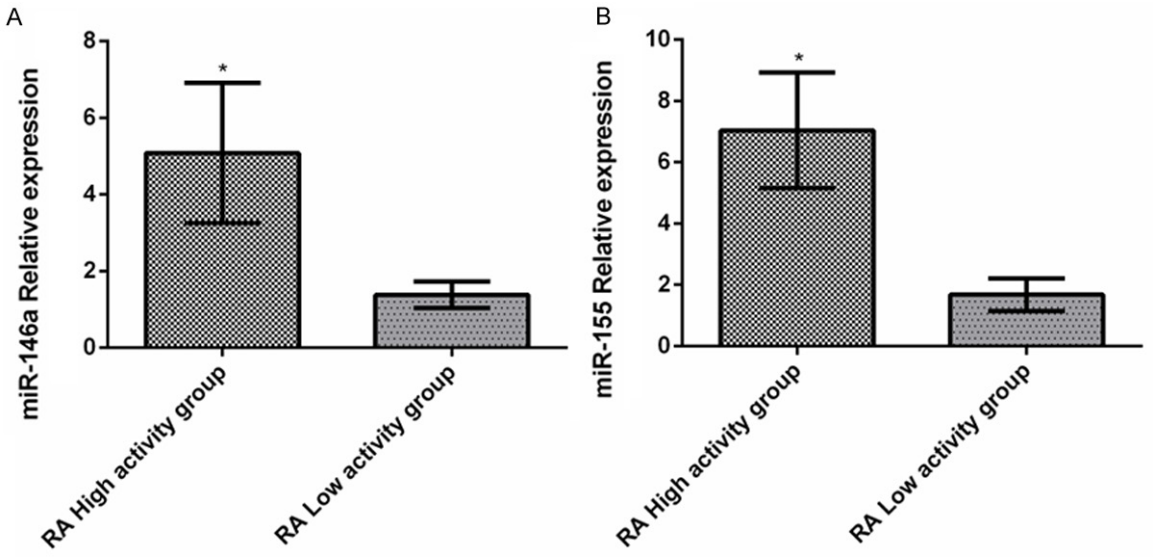

Figure 2. Comparison of relative expression levels of miR-146a and miR-155 in PBMCs in patients with high disease activity and patients with low disease activity. Peripheral blood was taken and mononuclear cells were isolated for RNA extraction. Relative expression levels of miR-146a (A) and miR-155 (B) were measured by RT-PCR. A: $^{*}P < 0.001$, t = 12.900 vs. patients with low disease activity; B: $^{*}P < 0.001$, t = 17.770 vs. patients with low disease activity.

cycles. Amplified products were analyzed using PCR software with U6 as an internal control gene. Results were calculated using $2^{-\Delta CT}$ method.

Statistical analysis

Statistical software SPSS 17.0 was applied for data analysis. Measurement data are expressed as mean ± standard deviation and comparison between the groups was performed by t-test. Count data in different groups were compared by χ^2 test. Pearson's correlation coefficient was used to analyze association between variables of relative expression levels of miR-146a and miR-155 in PBMCs and variables of erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP). $P < 0.05$ is considered statistically significant.

Results

Baseline data in the two groups

There were no intergroup differences in terms of age, gender, alanine transaminase (ALT), γ-glutamyltransferase (γ-GT), blood glucose (Glu), aspartate aminotransferase (AST), and total bilirubin (TBIL) (all $P > 0.05$). ESR and CRP values in the RA group were much higher than those in the control group (both $P < 0.001$) as shown in **Table 2**.

Relative expression levels of miR-146a and miR-155 in PBMCs in the two groups

Relative expression levels of miR-146a and miR-155 in PBMCs in the RA group were higher than those in the control group (miR-146a: 3.03 ± 1.10 vs. 1.01 ± 0.25, t = 11.460, $P < 0.001$; miR-155: 4.41 ± 1.69 vs. 1.33 ± 0.41, t = 11.360, $P < 0.001$) as shown in **Figure 1**.

Relative expression levels of miR-146a and miR-155 in PBMCs in patients with high disease activity and patients with low disease activity

In the RA group, patients with high disease activity had higher relative expression levels of miR-146a and miR-155 in PBMCs than patients with low disease activity (miR-146a: 5.08 ± 1.83 vs. 1.38 ± 0.34, t = 12.900, $P < 0.001$; miR-155: 7.04 ± 1.89 vs. 1.68 ± 0.53, t = 17.770, $P < 0.001$) as shown in **Figure 2**.

Correlation between variables of relative expression levels of miR-146a and miR-155 in PBMCs and variables of ESR and CRP

In the RA group, both relative expression levels of miR-146a and miR-155 in PBMCs were positively correlated with ESR and CRP (miR-146a, ESR: r = 0.342, $P < 0.001$; miR-146a, CRP: r = 0.271, $P < 0.001$; miR-155, ESR: r = 0.328, P <

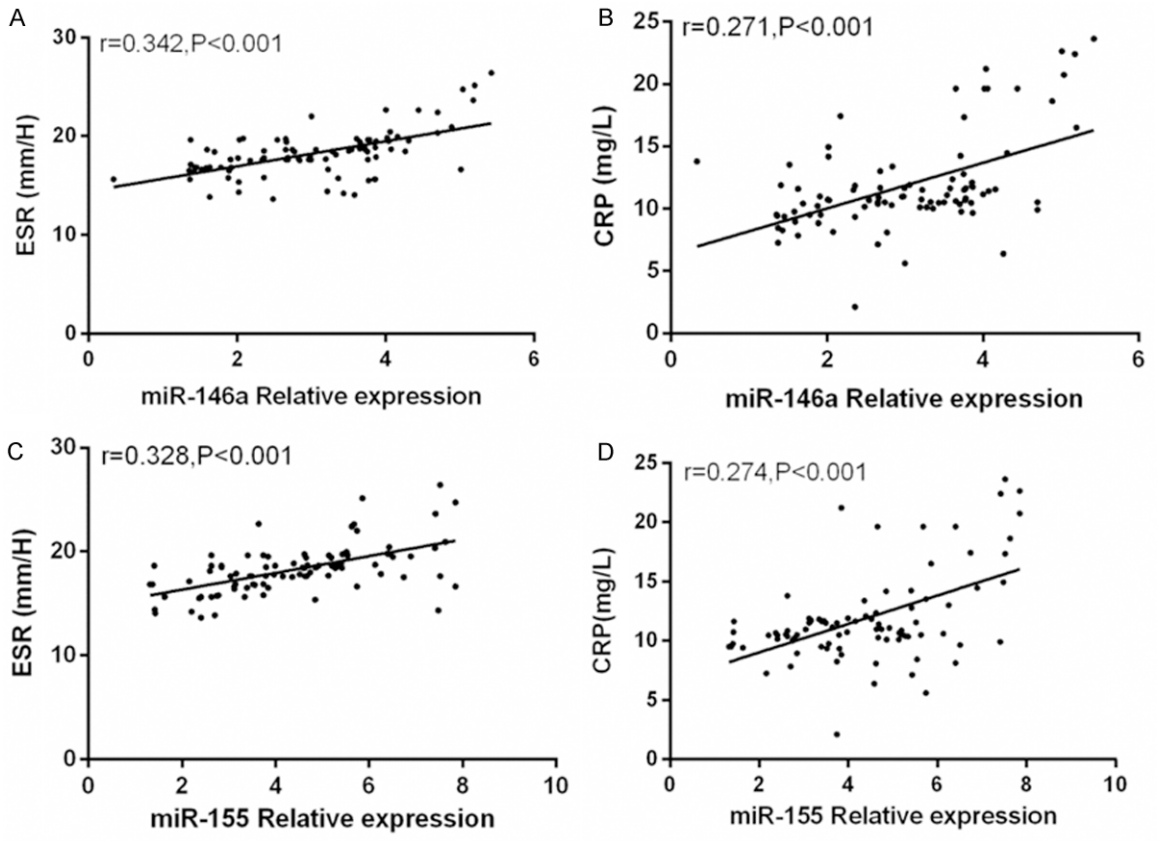

Figure 3. Association between variables of miR-146a and miR-155 relative expression in PBMCs and variables of ESR and CRP in the RA group. A and B: Pearson's correlation analysis showed that relative expression levels of miR-146a in PBMCs were positively correlated with ESR and CRP ($r = 0.342$, $P < 0.001$; $r = 0.271$, $P < 0.001$); C and D: Pearson's correlation showed that relative expression levels of miR-155 in PBMCs were positively correlated with ESR and CRP ($r = 0.328$, $P < 0.001$; $r = 0.274$, $P < 0.001$).

0.001; miR-155, CRP: $r = 0.274$, $P < 0.001$) as shown in **Figure 3**.

Discussion

RA is an autoimmune disease characterized by synovial hyperplasia, synovitis, and cartilage or bone damage and deformities [14]. Pathogenesis of RA remains unclear. Missed diagnosis or misdiagnosis often occurs, as it is quite difficult to diagnose RA in early stages [15]. This disease has a poor prognosis. Medicines and biological agents used in treating RA are only effective in some of the patients. Some patients are not sensitive to these drugs. Moreover, antirheumatic drugs have severe side effects and cannot relieve the condition completely [16]. Patients with RA usually have a higher mortality than normal healthy people. Complications such as cardiovascular disease and other systemic problems still pose great challenges to clinical treatment [17]. Clinically, patients are often categorized according to clinical manifestations for treatment. At present, there is still a lack of biomarkers for early diagnosis and prognosis of this disease [18]. Therefore, it is of great clinical significance to discover biomarkers and treatment targets for RA.

miRNAs are a type of small single stranded non-coding RNA molecules containing about 19-25 nucleotides. They can regulate gene expression through the level of transcription, thereby participating in biological processes such as cell proliferation, apoptosis, differentiation, metabolism, and death [19]. Transcriptional change of miRNAs plays an important role in physiological and pathological process in human bodies, affecting expression of target genes [20]. Abnormal expression of miRNAs in synovial fluid, PBMC, synovium, synovioblast,

and specific cells in RA patients has been observed in some research. It has been reported that miRNAs participate in regulating differentiation and growth of inflammatory cells and the proliferation and differentiation of T lymphocytes in pathways in innate and acquired immune systems. MicroRNAs, including miR-146a and miR-155, can be expressed abnormally at different stages of RA [21]. Gysler et al. documented that miR-146a and miR-155 are closely related to the growth of adaptive and innate immune cells and have abnormal expression in inflammatory and immune system disorders. These two miRNAs can downregulate downstream signaling pathways of IL-1 and TLR, thus regulating inflammatory and immune responses [22]. Bogunia-Kubik et al. found that patients with RA had higher relative expression levels of miR-146a in synovium than patients with osteoarthritis. Relative expression of miR-146a was upregulated by the stimulation of TNF-α and IL-1 in synovioblast [23]. Kurowska-Stolarska et al. found that expression of macrophages in synovial tissue and synovial fluid of RA patients was significantly upregulated. This increase in expression of miR-155 in synovial fluid $CD14^+$ cells was related to a decrease in expression of miR-155 target gene SHIP-1. Generation of TNF-α was reduced by suppressing expression of miR-155 in synovial fluid $CD14^+$ cells [24]. This finding provides new insight into RA treatment. Results of the present study displayed that patients in the RA group had much higher relative expression levels of miR-146 and miR-155 in PBMCs than patients in the control group. Additionally, in the RA group, patients with high disease activity had higher expression levels of miR-146a and miR-155 than those with low disease activity. This indicates that these two miRNAs may have participated in occurrence and development of RA and may be potential biomarkers for RA disease activity.

CRP, a globulin made by the liver, is a reactive protein produced in response to inflammation or injuries in the body. When inflammation or infection occurs, CRP synthesis will be accelerated, reaching a peak within 50 hours [25]. Bacterial infections, chronic inflammatory response, acute trauma, burn injuries, and viral infections can all result with an increase in CRP. CRP levels are positively associated with severity of the condition. With RA, there is persistent chronic inflammation with repeated attacks. Mild to moderate increase in CRP expression levels can occur. Both CRP and ESR are good indicators reflecting the inflammatory state of RA [26]. Present study results demonstrated that relative expression levels of miR-146a and miR-155 in PBMCs were positively correlated with ESR and CRP in the RA group, suggesting that upregulation of expression of these two miRNAs is closely associated with RA activity.

There were no intergroup differences in age, gender, ALT, AST, γ-GT, Glu, and TBIL, suggesting reliable results for this study. Peripheral blood was chosen as the specimen since it is more easily collected and more accepted by the participants. Relative expression of miRNAs in PBMCs can reflect changes and progression of RA and can be monitored continuously. Since detailed mechanisms regarding miR-146a and miR-155 in RA were not investigated in this study, future studies are necessary to explore mechanisms of miR-146a and miR-155 regulation, further verifying the present results.

In conclusion, noticeable upregulation of miR-146a and miR-155 expression was observed in patients with RA, suggesting that these two miRNAs may be involved in occurrence and development of RA. Upregulation of miR-146a and miR-155 expression is closely related to disease activity, supporting the idea that these two miRNAs can serve as potential biomarkers in early diagnosis of RA and assessment of disease activity. Therefore, measuring relative expression of miR-146a and miR-155 has high clinical value in the diagnosis and treatment of RA.

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Disclosure of conflict of interest

None.

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