Upregulated miR-146a Expression in Peripheral Blood Relates to Th17 and Treg Imbalance in Elder Rheumatoid Arthritis Patients

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**Keywords**
Rheumatoid arthritis · miR-146a · Peripheral blood mononuclear cell · Th17 · Treg

**Abstract**
**Background:** The expression level of microRNA-146a (miR-146a) increased in peripheral blood and synovialis tissue of rheumatoid arthritis (RA) patient, and it may play an important role in the pathological process of RA. We investigated its possibility as a diagnostic marker and the correlation with T helper 17 (Th17) and Treg cells in elder RA patients. **Methods:** Blood samples were collected from 38 active RA patients, 38 inactive RA patients, and 40 healthy controls. RNA expression levels of miR-146a were detected from the peripheral blood samples. The proportion of Th17 and Treg cells were analyzed, as well as their cell-specific transcription factor retinoic acid-related orphan receptor variant 2 (RORc) and forkhead box protein 3 (FOXP3). Furthermore, secretion of pre-inflammatory and anti-inflammatory factors was detected. Correlations between miR-146a and these factors were also analyzed. **Results:** Compared with healthy control, expression levels of miR-146a in inactive and active groups were significantly higher, with the highest level in active group. The expression of miR-146a and the RA severity, Th17 cell ratio, RORc expression, IL-17 level showed a significant positive correlation, while it showed a significantly negative correlation with Treg cell ration, FOXP3 expression, and TGF-β1 secretion. **Conclusions:** These results suggested that miR-146a may be used as a disease progression marker in the peripheral blood of elder RA patients.

**Introduction**
Rheumatoid arthritis (RA) is characterized by symmetry, multi-joint, and small joint symptoms [1]. Being a chronic systemic autoimmune disease, its main pathological changes are the immersion of chronic inflammatory cells such as macrophages and T lymphocytes, along with the secretion of many cytokines, which eventually lead to bone damage in the joints and progressive damage to the synovialis tissue. Due to the complicated pathogenesis, there is currently a lack of cure for RA, but early detection of RA can help better control the progression of the disease and relieve symptoms. Therefore, early diagnosis is an important issue in RA treatment.
MicroRNA (miRNA) is a noncoding small RNA that combines with the untranslated region to regulate gene expression. MiRNA has its specific expression spectrum, and it is reported that extracellular miRNAs can circulate in the bloodstream being remarkably stable [2]. They have been studied as markers of many diseases since its discovery, mostly in cancers like pancreatic cancer and breast cancer [3–5]. In recent years, several literatures have reported that microRNA-146 (miR-146) may play an important role in the pathological process of RA and may be a diagnostic marker of RA. It is reported that miR-146a expression in RA patient synovial tissue increases [6], and its expression level in peripheral blood mononuclear cells (PBMCs) of RA patient also increases [7]. Moreover, miR-146a expression in peripheral blood of RA patients shows an increase as well [8], suggesting that miR-146a may be circulating in the bloodstream and could be a diagnostic marker for RA.

On the other hand, the imbalance between the T helper 17 (Th17) and Treg cells with production of pro-inflammatory and anti-inflammatory cytokines is correlated with the disease progression [9]. It has been reported that the imbalances between Th17 and Tregs are closely related to RA. In PBMCs of RA patients, the proportion of Th17 cells increased, while the proportion of Treg cells declined, along with the associated transcription factors and pre-inflammatory factor levels changed accordingly [9–11]. Notably, RA in elder patients, i.e., patients over the age of 60, accounts for a large proportion of patients with RA. Our study specifically looked into the level of miR-146a expression in peripheral blood in elderly RA patients and investigated whether it was related to the imbalance of Th17 and Treg. By collecting samples from RA patients over 60 years and in vitro experiments, we aimed to address this question.

## Material and Methods

### Participants and Groups

**Inclusion Criteria**

Over 60 years old, in line with the RA diagnostic criteria established by the American Academy of Rheumatology and the European Federation for the Prevention and Treatment of Rheumatism in 2010.

**Exclusion Criteria**

People with severe liver disease, diabetes, kidney disease, thyroid disease, heart failure, cancer, systemic use of high-dose hormones or people with long-term smoking or drinking history, and other autoimmune diseases, etc. RA patients were divided into active group and inactive group, and compared with healthy control.

According to the criteria for determining the active period of RA established by the Rheumatology Branch of the Chinese Medical Association (erythrocyte sedimentation rate ≥30 mm/h, morning stiffness ≥1 h, number of joint swelling and pain ≥3, rheumatoid subcutaneous nodules and rheumatoid factor are higher than normal), patients who meet 3 or more criteria are diagnosed as active RA. According to previous reports [11], selected parameters for patients are shown in Table 1. This research was reviewed and approved by the institutional review board of Wuxi No. 2 People’s Hospital Affiliated to Nanjing Medical University. Written informed consent was obtained from all participants to participate in the study.

### Plasma IL-17 and TGF-β1 Detection

The levels of plasma IL-17 and TGF-β1 were measured by enzyme-linked immunosorbent assay (ELISA), using human IL-17 ELISA kit (ab100556) (coefficient of variation: 14%) and TGF-β1 ELISA kit (ab100647) (coefficient of variation: 16%) from Abcam (Cambridge, MA, USA), following the manufacturer’s instructions. All samples were measured in duplicate.

### Th17 and Treg Cell Isolation and Detection

PBMCs were isolated from blood. Flow cytometry was used for sorting and analysis. WOLF G2 Cell Sorter and Diva software were used for sorting and analysis.

### RT-qPCR

Isolation of RNA from blood followed the protocol from the manufacturer using RiboPure™ RNA Purification Kit (Thermo Fisher). QIAGEN OneStep RT-PCR Kit (Qiagen, Valencia, CA, USA) was used for detection of the mRNA level, following the manufacturer’s standard protocol. For the detection of miR-146a, U6 was used as the housekeeping gene, and for retinoic acid-related orphan receptor variant 2 (ROrC), β-actin was used as the housekeeping gene. Primers are listed as follows: forward (TTTCTGTGATGCACATCCCCACCA) and reverse (CCGACGATGCTCAGGGCTTG) for FOXP3; forward (GTGGGGACAGTGTCGTTG) and reverse (AGTCGTCGATCGTTTCCG) for RORC; forward (TGGCATCCGGCACTTGGGAA) and reverse (CTAAGCTTATCTTGAGCTTGG) for β-actin; forward (CAGTGCATTGGAATTTACCA) and reverse (CCGACGATGCTCAGGGCTTG).

### Sample Collection

All study subjects were collected 12 mL of whole blood in the early morning after fasting overnight and used dipotassium ethylenediaminetetraacetate for anticoagulation, of which 6 mL was used for the separation and identification of monocytes, 2 mL was used for serum pre-inflammatory factor detection, and the rest of 4 mL was used for the detection of miRNA and mRNA in the blood.

### Plasma IL-17 and TGF-β1 Detection

The levels of plasma IL-17 and TGF-β1 were measured by enzyme-linked immunosorbent assay (ELISA), using human IL-17 ELISA kit (ab100556) (coefficient of variation: 14%) and TGF-β1 ELISA kit (ab100647) (coefficient of variation: 16%) from Abcam (Cambridge, MA, USA), following the manufacturer’s instructions. All samples were measured in duplicate.
(GCCTGAGACTCTGCCTTCTG) for miR-146a; forward (CTCGCTTCACGAATTTG) and reverse (AACGCTTCACGAATTTT) for U6.

Statistical Analysis
The data were expressed as mean ± standard deviation or violin plot. We used the software GraphPad Prism version 5.01 (San Diego, CA, USA) for data analysis. Anderson-Darling test, D’Agostino and Pearson test, Shapiro-Wilk test, Kolmogorov-Smirnov test were used for normality tests. Comparisons among groups were performed with a one-way ANOVA followed by a Dunn’s multiple comparison test, χ² test, or Fisher’s exact test. Wilson/Brown test was used for ROC analysis, and the 95% confidence interval was applied. Pearson correlation analysis was used to calculate the coefficient. In all comparisons, p < 0.05 was considered as statistically significant.

Results

MiR-146a Was Upregulated in Peripheral Blood from RA Patients
According to the enrollment criteria, the study included active and inactive RA patient group, 38 patients for each group, with 40 healthy volunteers as control group (Table 1). First, analysis of miR-146a expression in peripheral blood of elder RA patients was tested by RT-qPCR (Fig. 1a), and U6 was used as the internal reference. Compared with healthy control, expression level of miR-146a in inactive group was significantly higher, while it showed the highest level in active group. Subsequently, the correlation between the expression of miR-146a and the RA severity score DAS28 was analyzed (Fig. 1b). This analysis included data from all RA patients, showing a significant positive correlation. ROC analysis was also carried out to explore the diagnostic value of miR-146a in RA and active RA, to find the best diagnostic threshold. Comparing RA patients with healthy people, the miRNA index can distinguish RA patients from healthy people very well (Fig. 1c, cut off = 1.258, sensitivity = 86.84%, specificity = 87.50%, AUC = 0.8938, p < 0.0001), and it can further distinguish between active group and inactive group of RA patients (Fig. 1d, cut off = 2.235, sensitivity = 65.79%, specificity = 84.21%, AUC = 0.7528, p < 0.0001). These results suggested that miR-146a in the peripheral blood of RA patients was correlated with disease progression.

### Table 1. Demographic and clinical characteristics of RA patients and healthy controls enrolled in the study

| Characteristics          | Study group | p value |
|--------------------------|-------------|---------|
|                          | active RA (n = 38) | inactive RA (n = 38) | healthy control (n = 40) |
| Gender, n (%)            |             |         |                   |
| Male                     | 5 (13.2)    | 7 (18.4) | 10 (25)           | 0.4088 |
| Female                   | 33 (86.8)   | 31 (81.6) | 30 (75)          |
| Age, years               | 68.54±5.31  | 66.73±4.86 | 64.91±4.39       | 0.1654 |
| ESR, mm/h                | 37.86±12.21 | 15.83±8.93 | 8.95±4.17        | <0.0001 |
| CRP, mg/dL               | 26.38±7.82  | 18.28±7.25 | 3.44±2.05        | <0.0001 |
| Disease duration, years  | 11.32±5.16  | 12.47±7.01 | –                |       |
| RF positivity, n (%)     | 32 (84.2)   | 28 (73.7)  | –                |       |
| DAS28                    | 5.5±1.2     | 3.4±0.8   | –                |       |
| Therapy, n (%)           |             |         |                   |
| MTX                      | 20 (52.6)   | 18 (47.4)  | –                |       |
| Leflunomide              | 3 (7.9)     | 2 (5.3)    | –                |       |
| Glucocorticoids          | 13 (34.2)   | 10 (26.3)  | –                |       |
| Antimalarial drug        | 31 (81.6)   | 29 (76.3)  | –                |       |
| Anti-TNF therapy         | 8 (21.1)    | 11 (28.9)  | –                |       |

Values were expressed as n (%) or mean±SD. RA, rheumatoid arthritis; RF, rheumatoid factor; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; SD, standard deviation; MTX, methotrexate. p values were derived from unpaired t test or one-way ANOVA test. χ² test and Fisher’s exact test were used for assessing distribution of observations.
miR-146a was upregulated in peripheral blood from elderly RA patients. a Expressions of miR-146a were measured by RT-qPCR. **p < 0.01, ***p < 0.001 from one-way ANOVA followed by a Dunn’s multiple comparison test. b The correlation analysis of expressions of miR-146a in peripheral blood and the disease activity score (DAS28). Data analyzed with Spearman correlation analysis. c ROC analysis of expressions of miR-146a in peripheral blood to diagnose RA from healthy controls. d ROC analysis of expressions of miR-146a in peripheral blood to diagnose active RA from inactive RA.

Treg cells was downregulated (Fig. 2b) in RA patients as the disease worsened. With correlation analysis, the expression of miR-146a was significantly positively correlated with Th17 cell ratio (Fig. 2c), but negatively correlated with the ratio of Treg cells (Fig. 2d). These results suggested that miR-146a expression level may indicate the immune status of RA patients.

Analysis of Transcription Factors RORc and FOXP3

Th17 and Treg cells are characterized by the specific expression of different transcription factors. RORc is the characteristic transcriptional factor of Th17 cells, and FOXP3 is the characteristic transcriptional factor of Treg cells. In order to detect these immune cells in RA patients from the gene expression level, the transcripts of RORc and FOXP3 in the peripheral blood from all three groups and the correlation analysis with miR-146 level were tested. It showed that RORc was upregulated with disease progression (Fig. 3a), while FOXP3 was the opposite, with downregulated expression with disease progression (Fig. 3b). Furthermore, there was a significant positive correlation for RORc with the expression of miR-146a.
(Fig. 3c, $r = 0.5424, p < 0.0001$), while the expression of FOXP3 and miR-146a showed a significant negative correlation (Fig. 3d, $r = 0.3690, p = 0.0010$). These results suggested that Th17 and Treg cells were changed from the transcription level and correlated with miR-146 expression differentially.

Detection of Secreted Pre-Inflammatory and Anti-Inflammatory Factors

Although the number of Th17 and Treg cells and transcription levels were correlated with RA disease progression and miR-146a expression level, next we analyzed the difference of pre-inflammatory and anti-inflammatory factors secreted by Th17 and Treg cells. The concentration of the two factors (IL-17 and TGF-β1) in the serum was tested by ELISA. Similar to previous findings [10], we found that IL-17, the cytokine secreted by Th17, was significantly upregulated in the peripheral blood of RA patients as the disease progressed (Fig. 4a), while the cytokine TGF-β1 secreted by Treg cells decreased along with the disease progression (Fig. 4b). Through correlation analysis, it was found that the expression of IL-17 and miR-146a was significantly positively correlated (Fig. 4c), and the expression of TGF-β1 and miR-146a was significantly negatively correlated (Fig. 4d). Therefore, the miR-146a expression level may also reflect the secretion level of these secreted factors.

**Fig. 2.** Flow cytometry analysis of Th17 (a) and Treg (b) lymphocyte subpopulations in peripheral blood from control, inactive RA, and active RA. **p < 0.01, ***p < 0.001 from one-way ANOVA followed by a Dunn’s multiple comparison test. The correlation analysis of expressions of miR-146a and Th17 (c) and Treg (d) lymphocyte subpopulations in peripheral blood from RA patients. Data analyzed with Pearson correlation analysis.
Discussion

It is important to diagnose early and accurate for RA patients in clinic. Due to the lack of sensitive and reliable diagnostic marker, the rate of misdiagnosis and missed diagnosis of RA is high, which often leads to joint deformities and loss of function in most RA patients [12]. This is even worse in elderly RA patients. Therefore, our study specifically focused on the population of elderly RA patients.

In our study, we found a 2.5-fold change increased expression of miR-146a in the peripheral blood of RA patient, which is consistent with a previous report [13]. ROC analysis showed the diagnostic value of miR-146a. Moreover, experimental results showed that miRNA can be used as a stable marker based on blood testing regardless of whether plasma or serum samples are tested [14]. There were many studies reported that miRNAs regulated immune pathways in cancer, and they mediated regulation of immune cells in the tumor microenvironment and can also be potential target for immunotherapies [15]. Compared with protein markers, the abnormal expression of miRNA appears earlier, thus making it a prognostic biomarker in early stage of RA progression.

MiRNA plays a key role in many physiological processes, including the development and function of the im-

Fig. 3. The mRNA expressions of RORc (a) and FOXP3 (b) in peripheral blood from control, inactive RA, and active RA. **p < 0.01, ***p < 0.001 from one-way ANOVA followed by a Dunn’s multiple comparison test. The correlation analysis of expressions of miR-146a and mRNA expressions of RORc (c) and FOXP3 (d) in peripheral blood from RA patients. Data analyzed with Pearson correlation analysis.
mune system. It is not surprising for us to find its relationship with immune cells, as miR-146a was discovered in a systematic study aimed at identifying miRNAs that play a potentially important role in the innate immune response to microbial infections [16]. In another study, miR-146a was found mediating the TLR4 pathway and adaptive immune response [17]. In our study, transcription factors, secretions, and ratio of cell numbers all showed the correlation between Th17 and Treg in RA, Th17 is positively correlated with RA disease development, Treg is negatively correlated with RA disease development, and miRNA-146a showed a positive correlation with Th17 and a negative correlation with Treg. It is agreed with previous study, which has shown that upregulation of miR-146a plays an important role in enhancing immune suppression through increasing the regulatory T cell population. There was study found that miRNA-146a expressing in IL-17-producing T cells in RA patients. Besides, miR-146a, let-7a, miR-26, miR-146b, miR-150, and miR-155 were also significantly upregulated in the IL-17-producing T cells. Moreover, double staining revealed that miR-146a is expressed in IL-17 expressing cells, and thus miR-146a was associated with IL-17 expression in the PBMC and synovium in RA patients [18]. Thus, there is possibility that miR-146a participates in the regulation of IL-17 expression.
More evidences have suggested miR-146a participated in immune function. It has been reported that dysregulated miRNA was involved in multiple sclerosis and functioned in Th17 cell differentiation by regulating CD4+ T cell differentiation into Th17 cells [19]. There also reported that miR-146a could modulate Th17 cell differentiation and regulate organ-specific autoimmunity [20]. In cancer, miR-146a participates in the regulation of the function of Th17 cell differentiation, in modulation of cell growth and apoptosis in cervical cancer via NF-κB signaling [21]. It also limited tumorigenic inflammation in colorectal cancer, preventing destructive colonic inflammation and associated tumorigenesis via modulation of IL-17 responses, decreasing the inflammatory IL-17 production mediated by myeloid cell [22]. In primary Sjögren’s syndrome (pSS) patients, miR-146a expression was significantly upregulated in PBMCs, and through targeting and negatively regulating ADAM17, it could promote Th17 cell differentiation [23]. Therefore, the previous studies support the idea that miR-146a correlated with Th17 in RA patient.

Except its function in regulating Th17 cells, miR-146a also functions in controlling Treg cell-mediated regulation of Th1 responses. It is one of the miRNAs commonly expressed in Treg cells, and it can regulate the suppressive function of Treg cells. That is why we saw the negative correlation of miR-146a with Treg cells in our results. Deficiency of miR-146a in Treg cells leads to the destruction of immune tolerance. In this case, fatal IFNγ-dependent immune-mediated diseases occur in multiple organs. This may be because the signal transducer and activator transcription 1 is the direct target of miR-146a, and its enhanced expression and activation lead to the occurrence of diseases [24]. Thus, other than being a diagnostic marker, miR-146a could possibly be a therapeutic target, which needs further investigation for the mechanism. Further experiments are needed to clarify the molecular mechanism, and preclinical study for targeting miR-146a in RA animal model would be meaningful for its potential clinical use.

Statement of Ethics

This research was reviewed and approved by the institutional review board of Wuxi No. 2 People’s Hospital Affiliated to Nanjing Medical University (No. 20190407wx24). Written informed consent was obtained from all participants to participate in the study.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Investigation: Menglan Liu and Zhi Lin; methodology: Minhui Hua; project administration: Minhui Hua; resources: Minhui Hua and Tianli Ren; software: Tianli Ren and Menglan Liu; supervision: Minhui Hua; writing – original draft: Menglan Liu; writing – review and editing: Menglan Liu, Tianli Ren, Zhi Lin, and Minhui Hua. All authors reviewed the results and approved the final version of the manuscript.

Data Availability Statement

Further inquiries of all data generated or analyzed during this study in this article can be obtained upon reasonable request to the corresponding author.

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