

Oleuropein as An Effective Suppressor of Inflammation and MicroRNA-146a Expression in Patients with Rheumatoid Arthritis

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Abstract

Objective: Rheumatoid arthritis (RA) is a common progressive autoimmune disorder that causes chronic inflammation of the joints and damage to other organs. Previous studies have reported the important role of miRNA-146a in the pathogenesis of RA. In addition, the anti-inflammatory and modulatory effects of oleuropein (OLEU) on the expression pattern of microRNAs (miRNAs) have been shown in different diseases. Therefore, this study aimed to evaluate both the sensitivity and specificity of miRNA-146a and determine the potential effects of OLEU on the expression levels of miRNA-146a and tumour necrosis factor-alpha (TNF- α) in RA patients.

Materials and Methods: The participants in this experimental study were divided into 2 groups: RA (n=45) and healthy controls (n=30). The isolated peripheral blood mononuclear cells (PBMCs) were treated with different concentrations of OLEU; and the level of TNF- α expression, anti-citrullinated protein, and miRNA-146a were determined using enzyme-linked immunoassay and real-time polymerase chain reaction, respectively. In addition, the receiver operating characteristic (ROC) curve analysis evaluated the sensitivity and specificity of miRNA-146a in RA patients.

Results: Results revealed a positive correlation between the levels of miRNA-146a expression with the serum levels of C-reactive protein (CRP) and rheumatoid factor (RF) in RA patients. In addition, OLEU treatment decreased the levels of TNF- α and miRNA-146a expression in treated PBMCs samples compared with untreated cells. The ROC curve analysis showed an 85% sensitivity and 100% specificity of miRNA-146a in RA patients.

Conclusion: Therefore, miRNA-146a can be used as a useful biomarker for RA diagnosis, particularly for early detection. In addition, OLEU could suppress inflammation in RA patients through the regulation of miRNA-146a.

Keywords: Anti-Citrullinated Protein, miRNA-146a, Oleuropein, Rheumatoid Arthritis, Tumour Necrosis Factor-Alpha

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Introduction

Rheumatoid arthritis (RA) is a progressive chronic autoimmune disease (1). Chronic inflammatory reactions at the cellular and molecular levels are major causes of bone and cartilage degeneration in this disease (2). Deposition of immunoglobulin (Ig) IgM-IgG complexes in joints, infiltration of inflammatory cells, and production of proinflammatory cytokines and chemokines have been proposed as the most important causes of inflammation (3). In addition, during the inflammation process, an autoantibody called anti-citrullinated protein (anti-CCP) antibody is produced after enzymatically converting arginine amino acid residues into citrulline in both rheumatoid factor (RF)-positive and RF-negative RA patients. Studies have shown that tissue degradation and the development of anti-CCP antibodies are caused by the

local abnormal citrullination of various proteins, such as fibrins, in RA joints. In the very early phases of RA disease, these antibodies were identified with 95% specificity and >70% sensitivity (4).

According to previous studies, microRNAs (miRNAs) may play a significant role in the etiology of several disorders, including autoimmune diseases (5, 6). miRNAs are a type of short noncoding RNA molecule that regulate the posttranscriptional expression of proteins in different physiological and pathophysiological conditions (7, 8). It is well established that miRNAs are able to regulate different immune responses by suppressing the expression of many molecules, such as transcriptional factors, cytokines, chemokines, and key signaling proteins at the posttranscriptional level (7, 9, 10). However, several

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studies reported the association between the expression level of different miRNAs with various autoimmune disorders such as RA (7, 11-13). Numerous studies have also examined the function of miRNA-146a in the regulation of the immune system, particularly inflammatory responses (14-16). Taganov et al. (17) study revealed that treatment of THP-1 cells, a human leukemia monocytic cell line, with microbial lipopolysaccharide (LPS), interleukin (IL)-1 β , and tumour necrosis factor-alpha (TNF- α) increased the expression levels of mature miR-146a via activation of nuclear transcriptional factor (NF)- κ B. Besides, Pauley et al. (9) reported the association between the upregulation of miR-146a with suppression of IL-1 receptor-associated kinase 1 (IRAK-1) and TNF receptor-associated factor 6 (TRAF6)-2 key molecules in the induction of proinflammatory cytokines IL-1 and TNF- α .

In addition, a previous study investigated that the level of miR-146a expression was related to a decrease in TNF- α production and disease activity of patients with RA (18). It has been also found that miRNA-146a stimulates the differentiation of Th17 cells by regulation of IRAK-1 and suppressor of cytokine signaling 1 in RA patients (19). Overexpression of miR-146a regulates FAS-associated factor 1 and inhibition of CD4⁺ T-cells apoptosis in patients with RA (20). Inhibition of apoptosis may be leading to inflammation progress and increased severity of RA. As a result, alteration in the expression levels of miR-146a could be related to inflammatory immune responses and the pathogenesis of RA. On the other hand, it was revealed that diverse natural/herbal products are a growing source of new medications that exhibit effective and therapeutic effects against a variety of disorders by controlling the expression of miRNAs.

There is a growing interest in the pharmacological and medical uses of oleuropein (OLEU), a nontoxic polyphenolic chemical found in olive tree leaves and olive oil because of its anti-inflammatory and antioxidant properties (21, 22). The results of our previous study revealed the positive effects of OLEU in shifting CD4⁺ T cells to CD4⁺ CD25⁺ FoxP3 regulatory T cells (Tregs) in RA patients in a dose-dependent manner (23). Moreover, various studies revealed the modulation effects of OLEU on the pattern of miRNAs expression in several disorders such as cancer (24, 25). These findings suggest the important impacts of OLEU on the expression level of miR-146a in RA patients. This study aimed to evaluate the sensitivity and specificity of miRNA-146a in RA patients. In addition, the possible relationship between the expressions of miRNA-146a with TNF α was evaluated. Finally, the effects of OLEU on the expression pattern of these factors were investigated.

Material and Methods

Participants

This experimental study was conducted at the Shahroud

University of Medical Sciences, Shahroud, Iran, from November 2020 to July 2021. The minimum sample size of 41 was considered for the study group and 25 cases for the control using statistical tools. In this study, the medical histories of 45 RA cases referred to the rheumatology clinic were screened. The Disease Activity Score 28 (DAS-28) for these patients with RA was finalized based on clinical symptoms and the C-reactive protein (CRP) value.

Inclusion criteria

The study included RA patients who were over 18 and below 60 years of age at the time of diagnosis with active disease, had a disease duration of more than a year, and fell under the 1987 Revised Criteria for Disease Classification of the American Rheumatism Association.

Exclusion criteria

Participants under 18 years with a disease duration of <1 year who were also taking common immunosuppressants, such as infectious illnesses, immunodeficiency, uncontrolled blood pressure, and hepatic or renal disorders, were excluded from the study. A control group of 30 healthy people with no signs of disease or a family history of autoimmune disorders was used in this study. Inflammatory conditions of any kind, RF positivity, and increased CRP were all considered exclusion criteria for the control group. The pregnant participants who had a special diet or were breastfeeding were also excluded from the study.

Blood sampling and peripheral blood mononuclear cells isolation

Blood samples were taken from both types of patients with RA and HCs, preserved in 2 serum-separating tubes and dipotassium (K2) ethylene diamine tetraacetic acid (EDTA) tubes to separate serum samples and peripheral blood mononuclear cells (PBMCs), respectively. After centrifuging, the serum sample was separated and maintained at -20°C until evaluation. PBMCs were also separated from whole blood samples using the standard Density Gradient Centrifugation (DGC) technique and Ficoll-Paque (GE Healthcare®, Buckinghamshire, UK) (26). The percentage of live cells was assessed using a trypan blue exclusion assay. PBMCs were suspended in an RPMI-1640 (Gibco, USA) medium supplemented with 100 ug/ml penicillin-streptomycin (Gibco, USA) and 10% fetal bovine serum (Gibco, USA). 5 \times 10⁵ cells of isolated PBMCs were transferred to each well of 24-well tissue culture plates and incubated for 72 hours at 95% humidity and 37°C after stimulation with appropriate concentrations of LPS (Gibco, USA) to stimulate cell proliferation and enhance the total number of cells. The results were compared with the cell culture of the control group, both with and without LPS stimulation.

Anti-citrullinated protein measurement

The serum level of anti-CCP was measured

using enzyme-linked immunoassay (ELISA) kits (ichromx Anti-CCP Plus, Boditech, South Korea, Lot No: ACREA15E) for all HCs and RA patients according to manufacturer's protocol. In brief, 100 μ L of reference controls, Calibrators, and each sample were transferred into appropriate wells and incubated for 60 ± 10 minutes at 18°C to 25°C . Each well was washed 4 times with 300 μ L of wash buffer. In addition, 100 μ L of conjugated antibody was added to each well and incubated at 18°C to 25°C for 30 ± 5 minutes. The plates were washed 4 times as described previously. Then, 100 μ L of substrate solution was added to each well and the plates were incubated at 18°C to 25°C for 30 ± 5 minutes. Then, 100 μ L of stop solution was added to wells to stop the reactions. The optical density (OD) of each sample was determined at 450 nm using an ELISA reader.

MiRNA-146a extraction

Extraction of miRNA146a from serum or isolated PBMCs was performed using a TBioFACT kit (Korea, Cat No: Rp101-050/RP101-100) according to manufactures instructions. Briefly, 10 μ L of 2ME buffer, 350 μ L of RB buffer, and 5 μ L of protein kinase K were added to the 250 μ L of serum, or the cells list was vortexed for 30 seconds and incubated at 56°C for 10 minutes. The samples were centrifuged at 1400 rpm for 3 minutes at 4°C , followed by transferring supernatants to a new Microtube. In addition, 250 μ L of ethanol (100%) was added to each Microtube and vortexed for 30 seconds at RT. The prepared solution was pipetted to the spin columns that were placed into collection tubes and centrifuged at 1400 rpm for 30 seconds at 4°C . The columns were transferred to a new collection tube, and 500 μ L of RNA washing solution (RW buffer) was added to each spin column and centrifuged for 30 seconds at 1400 rpm at 4°C . This step was repeated twice. The columns were transferred to an RNase and DNase free Microtube, and 100 μ L of RNase-free water was added to each column and incubated for 1 minute at RT, followed by centrifugation for 1 minute at 1400 rpm and 4°C . Then, fluids containing RNA were collected. Finally, the concentration of RNAs was determined using nanodrop and maintained at -80°C until use.

cDNA synthesis

The cDNA synthesis was conducted using the Ana micro-RNA Detection kit (Norgen Biotek, Canada, Cat No: MI001) according to the manufacturer's protocols. Two Microtubes were prepared for each sample. Moreover, 1 μ L of extracting mine was transferred into each Microtube. Appropriate concentrations of RT stem-loop housekeeping and RT stem-loop miRNA146a primers were added into tubes. The prepared final volume of each solution was at 14.5 μ L using RNase-free distilled water. Microtubes were incubated at 70°C for 5 minutes and then spun quickly. Each Microtube was placed in the cool box; and 4 ml of the 5X RT reaction buffer, 1 μ L of dNTP mix (10 mm), and 0.5 μ L of RT enzyme were added at a final volume of 20 μ L. The tubes were placed in a

thermocycler, and the synthesis of cDNA was performed according to the following protocol: 60 minutes at 37°C and 5 minutes at 70°C .

Real-time quantitative polymerase chain reaction

Real-time quantitative polymerase chain reaction (PCR) was performed according to previous studies (27). The procedure of this method is defined in a Table 1. Briefly, 2 μ L of miRNA146a and the housekeeping gene were added to separate the strip and then 10 μ L of Sybr Green was pipetted to the strips. Besides, 0.8 μ L of forward and reverse primers were added to the strips, adjusting the final volume to 20 μ L with distilled water. Finally, the strips were lidded, and a real-time PCR reaction was carried out using the BIORad instrument according to the manufactory's protocol. The cycle threshold of the samples was calculated using the $2^{-\Delta\Delta\text{CT}}$ formula.

OLEU preparation

To prepare a stock solution of OLEU, Cayman Chemical Company's powdered olive leaf extract (Sigma Aldrich, USA, Cat: 12247-10MG) was dissolved in dimethyl sulfoxide (DMSO), which was then stored at -20°C or -80°C until use.

Table 1: Real-time polymerase chain reaction procedure

Material	miRNA strip (μ L)	HK strip (μ L)
cDNA	2	2
2X QPCR Master mix (Sybr Green)	10	10
miRNA 146a-specific forward primer (10 μ m)	0.8	-
HK-specific forward primer (10 μ m)	-	0.8
Revers primer (10 μ m)	0.8	0.8
dH ₂ O	Up to 20	Up to 20
Final volume	20	20

Investigation of the cytotoxic effects of OLEU on PBMCs

The cytotoxic effects of various concentrations of OLEU on PBMCs were assessed using the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) test based on the previous studies (23). The wells with only OLEU solvent (DMSO) and LPS were selected as controls. The results were determined as a percentage of OD in both treated and control cells. Using linear regression, the half-maximal inhibitory concentration (IC_{50}) was evaluated. A suitable concentration (100 μ g/ml) of OLEU was established to continue the study.

Tumour necrosis factor-alpha measurement

The concentration of TNF- α was measured in the serum of patients with RA and HCs as well as cell culture supernatants from both untreated and treated PBMCs with 100 $\mu\text{g}/\text{mL}$ OLEU (optimal dose after MTT assay). The serum samples and supernatants were collected, spun down at 1500 rpm for 5 minutes, and cell-free samples were stored at -80°C until use. The immunoassays technique was performed in duplicate using human TNF- α commercial ELISA kits (Bioassay Technology Laboratory, China) according to the kit instructions. The OD of serum samples and supernatants of each well was determined by the ELISA reader at 450 nm and the concentration of TNF- α was calculated using a standard curve and dilution factor.

Statistical analysis

The serum levels of RF and CRP in RA patients compared with healthy individuals presented as mean \pm SEM. A t test was used to analyze serum levels of TNF- α and anti-CCP between patients and the control group. The expression of miRNA-146a was analyzed as the $2^{-\Delta\Delta\text{Ct}}$ method. $P < 0.05$ was considered statistically significant. The correlation between anti-CCP with miRNA-146a was determined using linear regression. The sensitivity and specificity of anti-CCP and miRNA-146a were determined by receiver operating characteristic (ROC) curve analysis.

Ethics approval and consent to participate

The study protocol was approved by the local Ethics Committee (IR.SHMU.REC.1398.100) of Shahroud University of Medical Sciences, Shahroud, Iran. All the experimental methods followed the guidelines of the Declaration of Helsinki (2008), as stated in the guidelines of Iran's Medical Ethics Committee, Ministry of Health. The samples were collected after receiving informed consent from every participant.

Results

Demographic, clinical, and laboratory information

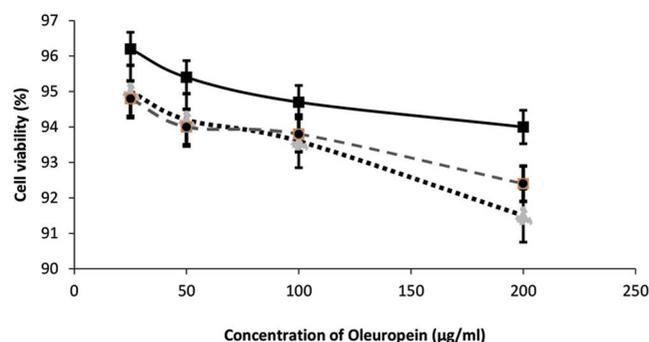
Table S1 (See Supplementary Online Information at www.celljournal.org) shows the demographic, clinical, and laboratory parameters of RA patients and healthy controls (HCs). All patients were under treatment with sulfasalazine, hydroxychloroquine, leflunomide, methotrexate, and azathioprine either alone or in combination with prednisone. The duration of RA disease in all patients was more than 1 year, and the disease activities were performed according to the DAS28 score. The DAS-28 < 2.6 : remission; the DAS-28 ≥ 2.6 and ≤ 3.2 : low disease activity; the DAS-28 > 3.2 and ≤ 5.1 : moderate disease activity; and the DAS-28 > 5.1 : high disease activity. All HCs were RF- and CRP-negative. Blood samples were collected from each RA patient to analyze the main diagnostic

factors, including RF, CRP, and anti-CCP.

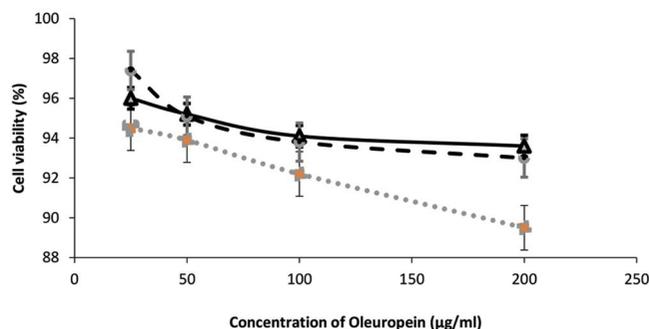
Cytotoxicity effects of OLEU

Based on the IC_{50} value, treatment of PBMCs with various concentrations of OLEU (50-200 $\mu\text{g}/\text{mL}$) for 24, 48, and 72 hours did not reveal a significant effect on the percentage of cell viability at 50 and 100 $\mu\text{g}/\text{mL}$ in all times of the treatment. Therefore, treatment of PBMCs with 100 $\mu\text{g}/\text{mL}$ of OLEU for 24 hours was selected to continue the study (Fig.1).

A



B



C

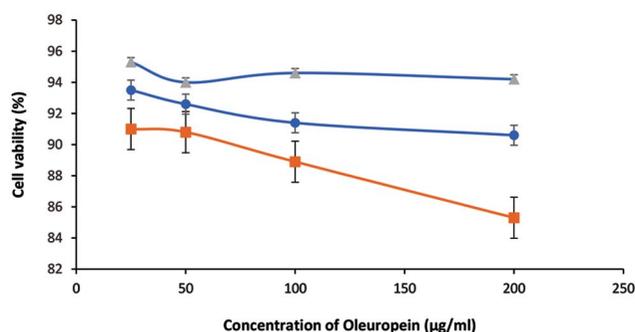


Fig.1: Effects of various concentrations of OLEU on the cell viability (%) at time intervals. **A.** 24 hours, **B.** 48 hours, and **C.** 72 hours. The IC_{50} value showed that OLEU did not have cytotoxicity effects on the percentage of cell viability in different concentrations of 25, 50, and 100 $\mu\text{g}/\text{mL}$ at three examined time points. OLEU; Oleuropein, IC_{50} ; Half-maximal inhibitory concentration, LPS; Lipopolysaccharides, DMSO; Dimethyl sulfoxide, and h; Hours,

Expression levels of tumour necrosis factor-alpha

Higher levels of TNF- α were detected in the serum of RA patients compared with HCs ($P < 0.0001$, Fig.2). Treatment of cultured PBMCs with an optimal concentration of OLEU significantly decreased the expression levels of this proinflammatory cytokine in supernatants of treated cells compared with untreated cells after 24 hours of incubation ($P < 0.0001$, Fig.2A, B).

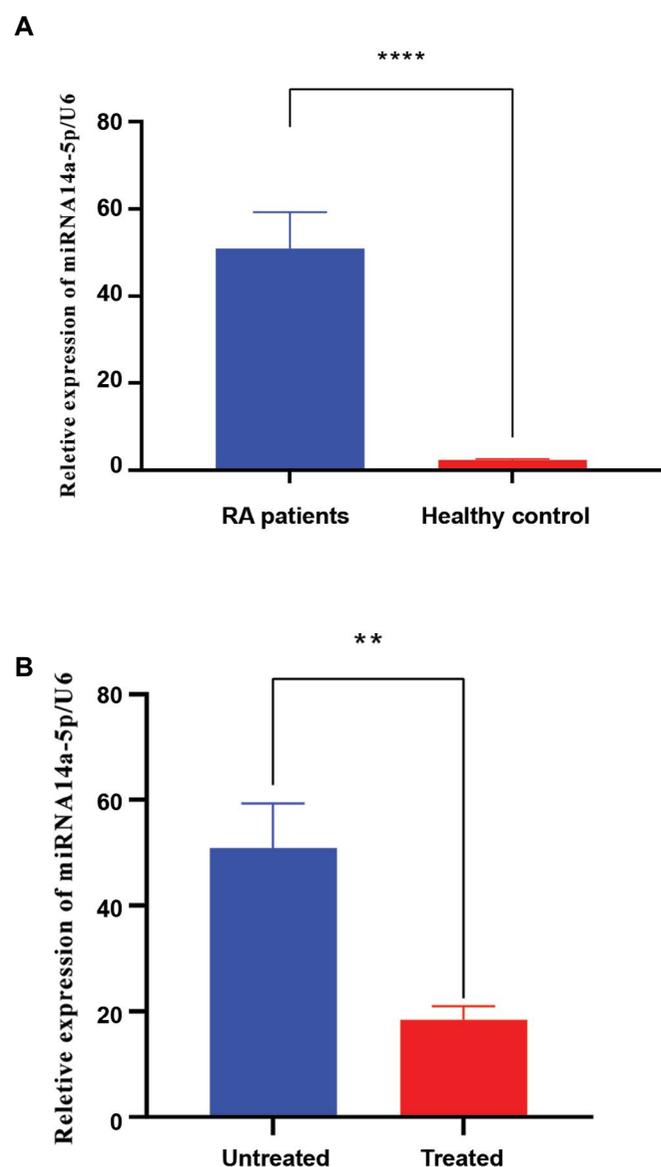


Fig.2: The levels of TNF- α . **A.** The serum levels of TNF- α were significantly higher in RA patients than in healthy controls. **B.** Whereas, a significant reduction in the amount of TNF- α was observed in treated PBMC samples with OLEU compared to untreated samples. TNF- α ; Tumor necrosis factor alpha, RA; Rheumatoid Arthritis, PBMCs; Peripheral blood mononuclear cells, OLEU; Oleuropein, **, The significance level, and ****, $P < 0.0001$.

Relative expression of miRNA-146a

The relative expression of miRNA-146a in the serum of patients with RA and HCs as well as treated PBMCs with 100 $\mu\text{g/mL}$ of OLEU is depicted in Figure 2. The results of data analysis revealed higher levels of miRNA-

146a in patients with RA compared with HCs ($P < 0.0001$, Fig.3A). However, the expression level of miRNA-146a was significantly lower in treated PBMCs than untreated samples ($P < 0.006$, Fig.3B).

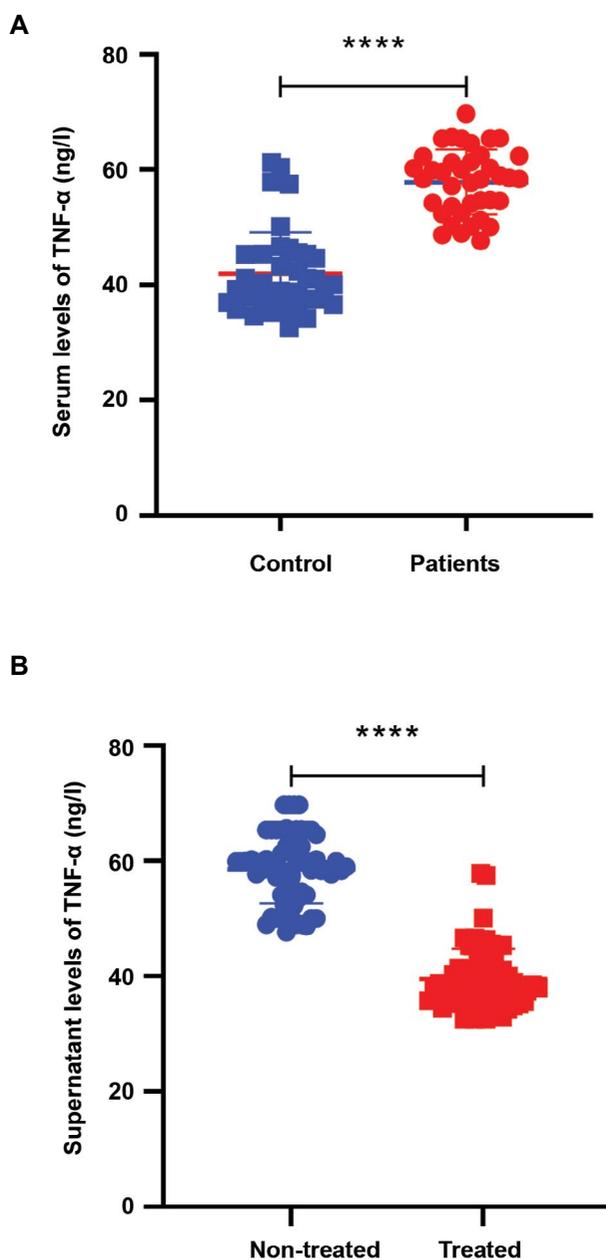


Fig.3: The levels of miRNA-146a. **A.** The results of qRT-PCR revealed higher levels of miRNA-146a in RA patients compared to the healthy controls. **B.** Significantly lower expression of miRNA-146a was identified in treated PBMCs samples with OLEU than in untreated samples. qRT-PCR; Quantitative real-time reverse transcription-polymerase chain reaction, PBMCs; Peripheral blood mononuclear cells, OLEU; Oleuropein, TNF- α ; Tumor necrosis factor alpha, and ****, $P < 0.0001$.

Association between CRP, RF, and anti-CCP levels with miRNA-146a

The linear regression analysis showed a positive correlation between different concentrations of CRP and RF with the expression levels of miRNA-146a (Fig.4A, B). However,

a negative association was identified between the levels of miRNA-146a expression with anti-CCP in RA patients (Fig.4C).

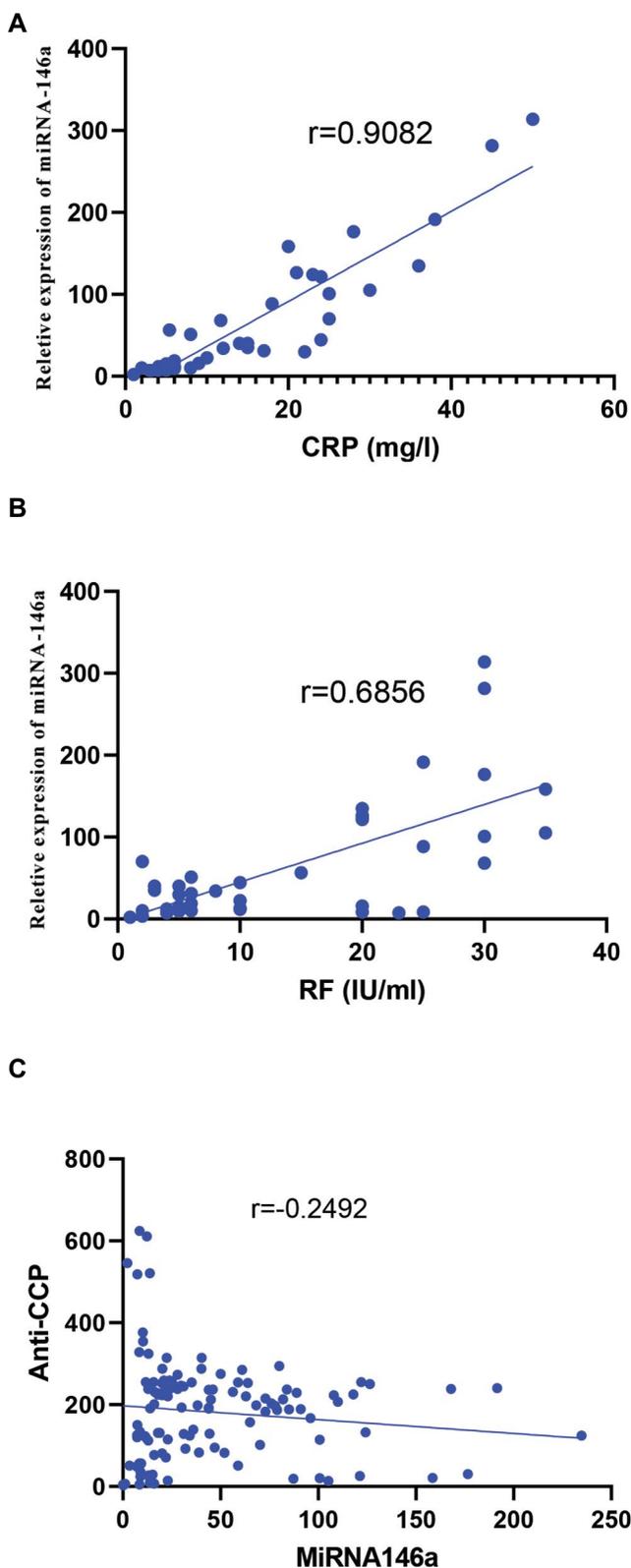


Fig.4: Correlation between CRP, RF, and anti-CCP with miRNA-146a. A positive association were identified between different concentrations of **A.** CRP, and **B.** RF with miRNA-146a. **C.** While anti-CCP showed a negative association with expression levels of miRNA-146a. CRP; C-reactive protein, RF; Rheumatoid factor, and anti-CCP; Anti-citrullinated protein antibody.

Sensitivity and Specificity of anti-CCP and miRNA-146a

The ROC analysis was performed to determine the sensitivity and specificity of anti-CCP and miRNA-146a in patients with RA. The sensitivity and specificity of anti-CCP were determined at 76 and 96%, respectively (area under the ROC curve [AUC]: 0. 9733 at a cutoff value of >28 U/mL) (Fig.5A). In addition, the results showed an 85% sensitivity and 100% specificity of miRNA-146a in these patients (AUC: 0.9958 at a cutoff value of >2.19) (Fig.5B).

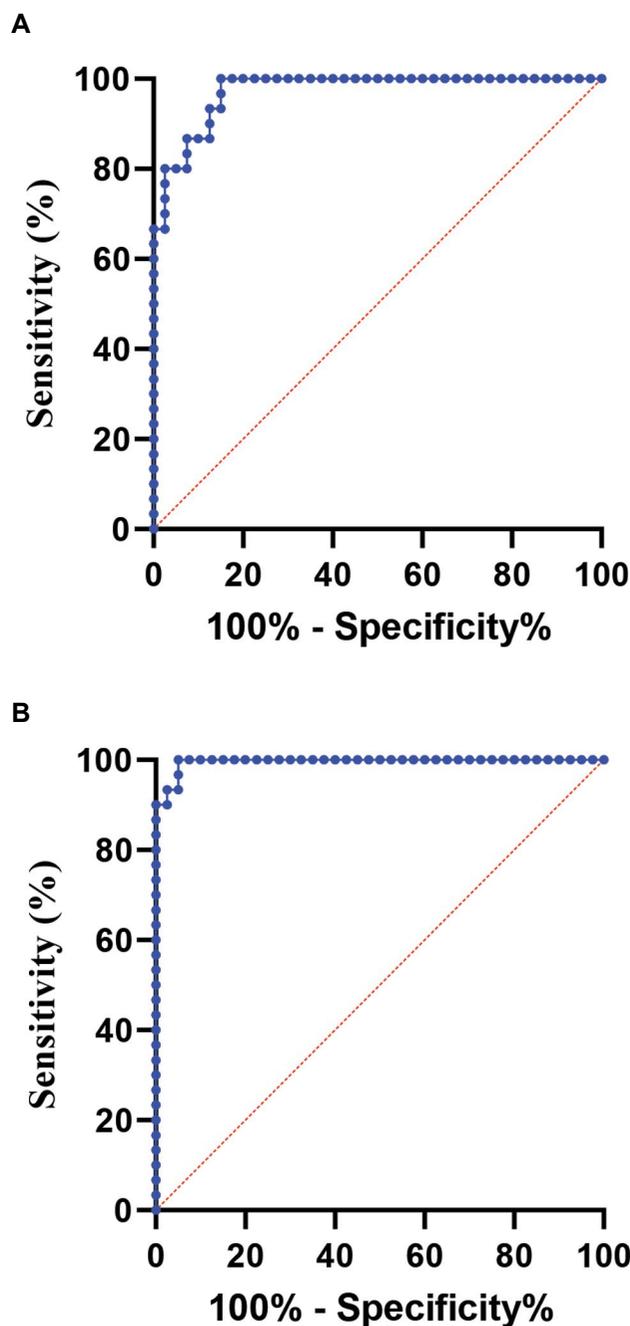


Fig.5: The Sensitivity and specificity of anti-CCP and miRNA-146a. **A.** The sensitivity and specificity of anti-CCP were 76 and 96% respectively whereas, **B.** Shows a high sensitivity (85%) and specificity (100%) of miRNA-146a in patients with RA. anti-CCP; Anti-citrullinated protein antibody and RA; Rheumatoid arthritis.

Discussion

In this study, the expression levels of TNF- α and miRNA-146a were evaluated in patients with RA compared with HCs. The potential role of miRNA-146a in the regulation of immune responses and different biological activities has been reported by multiple studies (14, 16, 28). miRNA-146a is involved in controlling inflammatory responses through different mechanisms such as the regulation of anti-inflammatory cytokine IL-1 β (28, 29). This miRNA, together with miRNA-155, has a critical role in the gene expression profiling of Th1/Th2 lymphocytes through various mechanisms such as targeting critical inflammatory cytokines and different signaling pathway proteins (30, 31). In addition, miRNA-146a modulates the regulation of signal transducer and activator transcription 1 activator and promotes lymphocyte responses to Th1 cells (32). A positive association was also reported between the increased expression of miRNA-146a with various inflammatory disorders of the central nervous system, such as Alzheimer disease (33). Kriegsmann et al. (34) detected higher levels of miRNA-146a in synovial fluid of RA patients compared with patients with osteoarthritis and introducing miRNA-146a as a beneficial biomarker for diagnosis of Sjögren syndrome.

A potential correlation between the relative expression levels of this miRNA-146a and various concentrations of CRP, RF, and anti-CCP was also examined in reference to studies that have shown a substantial correlation between the levels of expression of this miRNA and the severity of the RA.

In the present study, data analysis indicated a significant positive correlation between the relative expression levels of miRNA-146a with RF and CRP. However, the results showed that miRNA-146a is inversely associated with anti-CCP concentration, which may be related to an increase in the frequency of T follicular regulatory cells in inactive or stable-remission patients (35). T follicular regulatory cells are a subset of T cells that control immunological responses in the germinal center (GC) by interacting with T follicular helper and preventing the generation of antibodies such as anti-CCP (36). These data could be suggested a positive association between miRNA-146a and the pathogenesis of RA. In contrast to these findings, other studies have revealed contradictory data on the relationship between miRNA-146a and the pathophysiology of several autoimmune disorders, including RA. Churov et al. (37) reported a limited association between the levels of miRNA-146a expression and RA disease severity in a systematic review study. Fan et al. (38) have reported a decreased expression of miRNA-146a in the serum of patients with systemic lupus erythematosus (SLE), which was associated with a reduction in CRP in these patients. However, Sun et al. (39) showed no association between the levels of miRNA-146a and the risk of SLE.

Another meta-analysis study conducted by Fan et al. (38) on the association between miRNA-146a and SLE showed

a significant association between the expression levels of miRNA-146 with susceptibility to SLE in European and Asian populations. This discrepancy may be due to the use of a limited sample size in previous research.

On the other hand, we evaluated the potential effects of OLEU, as a natural anti-inflammatory and anti-oxidative product, on the expression levels of TNF- α and miRNA-146a. Data showed that OLEU significantly decreased the production of TNF- α in the culture media of treated PBMCs compared with untreated cells, which is inconsistent with prior findings. It also has the ability to reduce the level of miRNA-146a in treated PBMCs. Several studies have revealed the potential effects of OLEU on the expression pattern of different miRNAs in various disorders. Besides, they proposed OLEU as an important anti-inflammatory product (21, 40). However, the small sample size is the main limitation of the present study. In addition, the potential effects of OLEU on other RA pathogenesis-related parameters have not been studied. Future research is necessary to introduce OLEU as a beneficial natural medicine for RA treatment.

Conclusion

The present study revealed a significant correlation between miRNA-146a expression levels and key RA pathogenesis-related parameters, including RF and anti-CCP. This suggested that miRNA-146a might play an important role in RA susceptibility as well as other RA-related autoimmune diseases including SLE. Consequently, miRNA-146a may be a useful and valuable biomarker for the diagnosis or early detection of RA. In addition, it seems that OLEU could modulate immune responses by decreasing the levels of main proinflammatory factors such as TNF- α . However, future studies with larger sample sizes are necessary to introduce OLEU as a beneficial natural medicine for RA treatment.

Acknowledgments

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Authors' Contributions

H.M., Z.Y., F.T., M.Y., M.J.S., R.J.; Conceptualization, Investigation, Writing - Original Draft, and Methodology. Z.Y., R.J.; Review and Editing. M-H.S., N.S; Data analysis. The authors read and approved the final manuscript.

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