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Comparison of Plasma Levels of MicroRNA-155-5p, MicroRNA-210-3p, and MicroRNA-16-5p in Rheumatoid Arthritis Patients with Healthy Controls in a Case-control Study

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ABSTRACT

Rheumatoid arthritis is a chronic autoimmune disease that causes inflammation and destruction of the joints. The objective of the current study was to evaluate the expression of microRNA (miR)-155-5p, miR-210-3p, and miR-16-5p in the plasma of patients with rheumatoid arthritis in comparison with a healthy control group to attain an expression profile for earlier diagnosis and treatment.

To carry out this study, 100 individuals were chosen as two equally sized groups of rheumatoid arthritis patients and healthy controls. Five milliliters of blood were drawn from each individual, and plasma RNA was extracted using Trisol solution. Complementary DNAs were synthesized using the Moloney leukemia virus (MMLV) and deoxynucleoside triphosphate (dNTP). Finally, real-time polymerase chain reaction (PCR) was implemented using the SYBR Green kit.

The mean expression of miR-155-5p, miR-210-3p, and miR-16-5p were 2.46±2.79, 1.97±1.90, and 69.62±88.44 in the rheumatoid arthritis group, and 0.34±0.33, 9.82±9.34, and 7.94±7.09 in the healthy group, respectively. Additionally, significant differences were revealed in the relative expression of the selected microRNAs in 4 subgroups of rheumatoid arthritis patients with different

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disease activities based on the disease activity score 28 (DAS28). ROC curve analysis showed that miR-155-5p (area under the curve, AUC=0.90, sensitivity=80%, specificity=81%), miR-210-3p (AUC=0.75, sensitivity=66%, specificity=71%), and miR-16-5p (AUC=0.96, sensitivity=89%, specificity=82%) could be potential biomarkers for rheumatoid arthritis diagnosis.

Increased expressions of miR-16-5p and miR-155-5p and decreased expression of miR-210-3p in rheumatoid arthritis patients compared with healthy individuals demonstrate the effectiveness of these microRNAs in disease incidence and progression. Thus, the expression levels of these microRNAs can be used for diagnostic and therapeutic purposes.

Keywords: Biomarkers; miRNA-155-5p; miRNA-210-3p; miRNA-16-5p; Rheumatoid arthritis

INTRODUCTION

Rheumatoid arthritis (RA) is one of the most common autoimmune diseases, affecting 5 out of every 1000 adults worldwide.[1] RA primarily affects the synovial membrane and ultimately destroys the articular cartilage and juxta-articular bones.[2] Women are likely to be affected by RA 2 to 3 times more than men. This inflammatory disease could occur at any age, but people are more susceptible to RA in their sixth decade of life.[3]

Although the etiology of RA is unknown, studies show that both genetic and environmental factors contribute to this disease. The contribution of genetic factors to the incidence of the disease is estimated at 50%.[4] There are no definitive diagnostic criteria for RA despite its high prevalence. However, the 1987 American College of Rheumatology (ACR) classification criteria and European League Against Rheumatism (EULAR) criteria for RA diagnosis were revised in 2010. This revision improved the identification of patients with a fairly short duration of symptoms who may benefit from disease-modifying antirheumatic drugs.[5,6]

The 2010 criteria include the number of joints involved, based on physical examination or imaging by ultrasound or magnetic resonance imaging. They also include serological tests for elevated levels of rheumatoid factor (RF), anti-citrullinated protein antibodies (ACPAs), or both; an increase in the acute phase reactants response, such as C-Reactive Protein (CRP) level or erythrocyte sedimentation rate (ESR); and the duration of symptoms (≥6 weeks). The sensitivity and specificity of the 2010 criteria are 82% and 61%, respectively.[1] Since the current diagnostic tests for RA are neither accurate nor sensitive,[7] and an early diagnosis and treatment are beneficial in 90% of patients with early RA, new approaches and novel biomarkers with higher sensitivity and specificity are essential to identify patients with RA as early as possible.

In recent years, novel biomarkers known as microRNAs (miRNAs or miRs) have been found to play a critical role in immune system regulation and are, therefore, related to autoimmune disorders. MicroRNAs are a class of small single-stranded noncoding RNA molecules of 21 to 25 nucleotides. They play essential roles in the regulation of innate and adaptive immune responses and immune cell development, and due to their high sensitivity and specificity, they can be recognized at different stages of the disease.[8-10]

Several studies have confirmed the role of microRNAs in RA and emphasized the clinical importance of circulating or extracellular microRNAs as promising biomarkers in RA. These molecules serve as potentially suitable biomarkers due to several essential characteristics. Firstly, they are stable in different biological fluids, including plasma, serum, and cell culture. Secondly, they are resistant to RNase digestion and other severe conditions such as extreme pH, boiling, and multiple cycles of freezing and thawing.[11,12]

Studies on the plasma level of microRNAs in individuals suffering from RA are insufficient. So far, studies have been conducted on changes in microRNA expression levels in cells and their effect on cell function in RA patients. Due to the potential role of free circulating microRNAs as diagnostic markers and therapeutic targets,[13] this study attempts to evaluate the expression of miR-155-5p, miR-210-3p, and miR-16-5p in the plasma of Iranian RA patients and compare it with healthy individuals, and identify the relationship between these designated microRNAs and disease activity indices to obtain a specific expression profile to diagnose and treat RA patients earlier.

MATERIALS AND METHODS

Patients and Controls

This case-control study was carried out in patients attending the Department of Rheumatology, Imam Khomeini Hospital, Tehran, Iran. Individuals with RA had previously been diagnosed according to the ACR/EULAR criteria[5,6] by professional rheumatologists based on clinical symptoms, physical examination, and laboratory tests. After obtaining written informed consent from all participants, 5 mL of blood was collected in EDTA-containing tubes after 10 to 12 hours of fasting from 50 patients with RA and 50 healthy individuals of the same age and sex as the control group. Patients with heart, kidney, and liver diseases, pregnant women, those suffering from hematologic malignancies (leukemia, lymphoma, and other cancers), or those infected during the past 3 weeks were excluded from the study. After the blood draw, plasma was isolated from the samples. For this purpose, blood samples were centrifuged at 4000*g* for 10 minutes, and tubes containing plasma were stored at −70°C for further analysis.

RNA Extraction from Plasma

In the beginning, 250 µL of human plasma was thawed on ice, and then 750 µL of the TrizoLEX reagent (DNA Biotech, Iran) was used for lysis. For normalization of the sample-to-sample variation caused by RNA isolation, 25 fmol (5 µL) of synthetic *Caenorhabditis elegans* microRNA cel-miR-39, as an external control, was added to each denatured sample.[14] The resulting mixture was vortexed and centrifuged at 1200*g* for 8 minutes at 4°C.

After aqueous phase separation, 200 µL chloroform was added and mixed by inverting the tube gently for 15 seconds. Following a 3-minute incubation of the tubes at room temperature, centrifugation at 12000*g* at 4°C was performed. Having transferred the aqueous and organic phases containing the RNA to new microtubes, 500 µL isopropanol was added and centrifuged at 12000*g* for 15 minutes at 4°C. After incubating the tubes for 24 hours at −20°C, centrifugation was carried out at 12000*g* for 15 minutes at 4°C. Finally, by discarding the supernatant, the pellet was washed with 75% ethanol and left to dry at ambient temperature. After drying, RNA was dissolved in 20 µL diethylpyrocarbonate (DEPC) water, and the purity of the RNA molecules extracted was evaluated by a NanoDrop spectrophotometer (Thermo Fisher Scientific Co. USA).

Primer Design

A novel method called miR-Q was performed to design the primers, according to the instructions presented by Sharbati-Tehrani et al.[15] This new method has made it possible to identify and measure small RNA molecules, particularly microRNAs. Initially, RNA is converted to complementary DNA (cDNA) using the reverse transcription polymerase chain reaction (RT-PCR) 6-miR-X primer (X is the specific name of each miR). Then, the cDNA is amplified and quantified by the quantitative polymerase chain reaction **(**qPCR) method based on three different types of DNA oligonucleotide, short-miR-X, multiplex PCR (MP) forward primer, and MP reverse primer at different concentrations. The sequence of primers used for each microRNA is listed in Supplementary Table.

Reverse Transcription

In reverse transcriptase (RT)-PCR, the first step is to convert RNA templates into cDNAs using reverse transcriptase. For this purpose, RNA templates were reverse-transcribed by the Moloney murine leukemia virus (MMLV) enzyme (Yektatajhiz, Iran). Briefly, the reaction mixture containing 2.5 µL extracted RNA, 1 µL RT-primer, and 10.5 µL DEPC water was incubated at 70°C for 5 minutes. After 5 minutes of incubation, the samples were immediately placed on ice, and the following components were added to each microtube and incubated at 42°C for 60 minutes and 70°C for 5 minutes on the thermocycler (Applied Biosystems 4375786): 1 µL dNTP, 1 µL MMLV enzyme, and 4 µL buffer. Finally, cDNA was stored at −20°C for further experiments.

Real-time Quantitative PCR

To evaluate the expression of selected microRNAs in plasma samples, the real-time qPCR technique was performed using a 2X SYBR Green qPCR Master Mix (Norgenbiotek, Canada) on the Corbett Rotor-Gene 6000 real-time PCR system (Qiagen, Germany). Several 10-fold serial dilutions of cDNA samples were prepared, and to determine the efficiency of the primers, which in turn indicates PCR efficiency, a standard linear curve was analyzed based on $E=10^{-1/slope}$. For all the dilutions, the real-time PCR reaction was performed in duplicate. The results demonstrated an amplification efficiency of 0.98 to 1.03 for each primer set of experimental microRNA and reference genes, which indicates minimal

inhibition or variation in the reactions. The 10-µL PCR reaction mixture contained 2 µL cDNA, 5 µL 2x SYBR Green Master Mix, 1 µL short-primer, 1 µL MP-primer (MP-forward + MP-reverse), and 1 µL DEPC water. PCR was implemented at 95°C for 2 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds and extension at 60°C for 30 seconds. For the no template control (NTC) samples, 2 µL of distilled water was added instead of the 2 µL of cDNA. It should be noted that for these samples, no signal should be observed. The ΔΔCt method was used to measure and calculate plasma microRNA levels. To examine and confirm the specificity of primer activity in the real-time PCR reaction, PCR products were run on 3.5% agarose gel electrophoresis (Figure 1). The PCR product sizes of miR-155-5p, miR-210-3p, miR-16-5p, and cel-miR-39 were 73 bp, 69 bp, 69 bp, and 67 bp, respectively. The real-time PCR products of miR-155-5p (well 4), miR-210-3p (well 3), miR-16-5p (well 2), and cel-miR-39 (well 1) were displayed as single bands and expected sizes.

Statistical Analysis

Data were analyzed using SPSS statistical software version 18.0. Data are shown as mean±SD or counts (percentages). A statistical *t*-test was used to compare microRNA mean expression between the case and control groups. The linear relationship between microRNA expression and the disease activity indices ESR, CRP, tender joint count (TJC), and swollen joint count (SJC) was measured by Spearman correlation coefficient. $p<0.05$ was considered statistically significant. Additionally, the receiver operating characteristic (ROC) curve was used to evaluate the sensitivity and specificity of microRNAs for RA diagnosis.

RESULTS

Demographic, Clinical, and Laboratory Data

Demographic data and clinical findings of patients and controls who participated in the study are shown in Table 1.

Altered Expression of miR-155-5p, miR-210-3p, and miR-16-5p in Plasma of Patients with RA Compared to Healthy Controls

The real-time qPCR technique was applied to demonstrate the changes that happened in the expression levels of the selected microRNAs in plasma from patients with RA in comparison with healthy controls. The following formula was used to show the mean expression levels:

$$\Delta\Delta Ct = \Delta Ct_{\text{reference gene}} - \Delta Ct_{\text{miRNA of interest}}$$

An increase in ΔΔCt represents an increase in expression. Overall, in this study, a significant increase in the expression levels of miR-155-5p and miR-16-5p and a decrease in the expression level of miR-210-3p was observed in patients compared to healthy controls ($p<0.001$; Figure 2).

Comparison of Selected MicroRNAs Average Expression between Genders

The average expression levels of miR-155-5p, miR-210-3p, miR-16-5p, CRP, and ESR in male and female patients included in this study were evaluated and compared with the Kruskal-Wallis statistical test. The results showed no significant differences between these groups in the expression levels of selected microRNAs and also the ESR and CRP levels, as represented in Table 2.

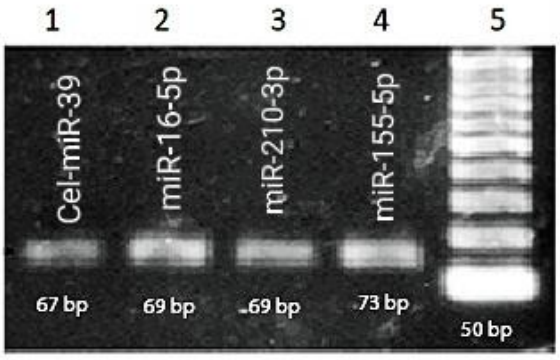

Figure 1. Real-time polymerase chain reaction products on 3.5% agarose gel; 1: cel-miR-39 (internal control), 2: miR-16-5p, 3: miR-210-3p, 4: miR-155-5p, 5: 50basepair ladder.

Table 1. Comparison of demographic and clinical characteristics between RA patients and healthy controls

Characteristics	RA patients (N=50)	Healthy Controls (N=50)
Age, years	48.6±11.1	47.9±10.4
Female/male (n)	35/15	35/15
Disease duration, years	8.8±7.4	NA
RF positive, n (%)	35 (70%)	NA
Anti-CCP positive, n (%)	28 (56%)	NA
ESR, mm/h	34.2±14.7	NA
CRP, mg/dL	32.8±30.7	NA
DAS28	4.1±1.4	NA
High Disease Activity (>5.1), n (%)	16 (32%)	NA
Moderate Disease Activity (3.2–5.1), n (%)	21 (42%)	NA
Low Disease Activity (2.6–3.2), n (%)	4 (8%)	NA
Remission (<2.6), n (%)	9 (18%)	NA
SJC28, (count)	7.4±6.6	NA
TJC28, (count)	9.3±8.1	NA
Medications, N (%)		
Methotrexate	31 (62%)	NA
Sulfasalazine	27 (54%)	NA
Prednisolone	23 (46%)	NA
Hydroxychloroquine	20 (40%)	NA
Alendronate	8 (4%)	NA

Data is shown as mean±SD or counts (percentages).

NA: not applicable; RA: rheumatoid arthritis; RF: rheumatoid factor; anti-CCP: anti-cyclic citrullinated peptide; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; DAS28: disease activity score in 28 joints; SJC28: swollen joint count in 28 joints; TJC28: tender joint count in 28 joints

Table 2. Average expression levels of selected microRNAs, CRP, and ESR in different sex groups of patients.

Parameters	Female	Male	p-value
miR-16-5p	88.28±46.57	88.36±77.78	0.998
miR-155-5p	2.16±2.46	3.11±2.52	0.318
miR-210-3p	1.27±1.66	2.15±2.11	0.221
CRP, mg/dL	33.3±3.4	31.8±2.1	0.5
ESR, mm/h	31.6±13.2	40.2±16.6	0.1

miR: MicroRNA; CRP: C-reactive protein; ESR: erythrocyte Sedimentation Rate

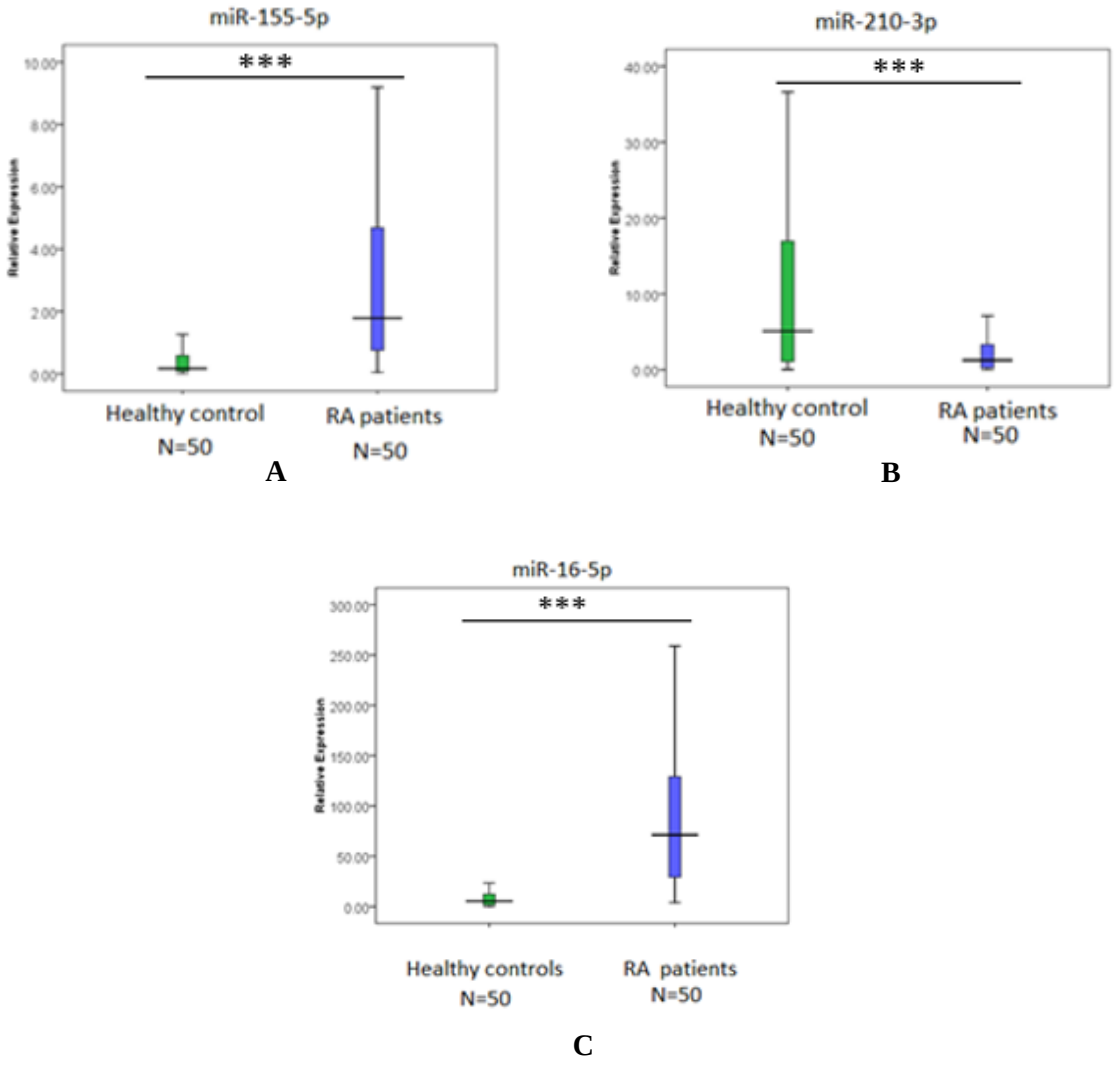

Figure 2. Comparison of levels of circulating miR-155-5p (A), miR-210-3p (B), and miR-16-5p (C) between RA patients and healthy controls. RA: rheumatoid arthritis; ***p<0.001

Analysis Based on RF and Anti-CCP Results

The mean expression levels of miR-155-5p, miR-210-3p, miR-16-5p, CRP, and ESR were compared between patients with positive and negative RF. There was a significant difference in the expression of miR-155-5p between the positive and negative RF groups (p<0.05), while the levels of miR-16-5p and miR-210-3p showed no significant difference between the two groups. A significant difference was observed between the two groups (p<0.002; Table 3) regarding the CRP and ESR levels.

Additionally, the expression levels of the selected microRNAs, CRP, and ESR were investigated between patients with positive and negative anti-CCP results. Analysis showed a significant difference in the mean expression levels of miR-155-5p, miR-210-3p, CRP, and ESR in RA patients with positive and negative anti-CPP results (p<0.05; Table 4). However, for the mean expression level of miR-16-5p, there was no significant difference between the two groups.

In addition, the expression levels of the selected microRNAs were assessed and compared in patients with RA, whose RF test and anti-CCP test were negative or positive, with the healthy control group. The results demonstrated that the expression levels of miR-155-5p and miR-16-5p were significantly

increased in RA patients with both positive and negative RF and anti-CCP, compared to healthy controls. Conversely, for the expression of miR-210-3p, a significant decrease was observed compared to healthy individuals.

Analysis Based on DAS28 Index

In this study, RA patients were classified into 4 groups based on the DAS28 index of disease activity. The 4 groups were as follows: 1) remission group (patients with DAS28<2.6), 2) low disease activity group (patients with DAS28 in the 2.6 to 3.2 interval), 3) moderate disease activity (patients with DAS28 in the 3.2 to 5.1 intervals), and 4) high disease activity (patients with DAS28 >5.1). The average expression of miR-155-5p, miR-210-3p, miR-16-5p, CRP, and ESR levels were assessed with the Kruskal-Wallis statistical test. The results demonstrated that the average expression levels of selected microRNAs, ESR, and CRP varied significantly between the 4 groups in terms of disease activity (Table 5).

Table 3. Mean expression levels of selected microRNAs, CRP, and ESR among positive RF and negative RF patients

Parameters	Patients with Positive RF	Patients with Negative RF	*p* value
miR-16-5p	92.77±68.61	76.76±74.72	0.542
miR-155-5p	2.37±2.41	4.20±2.15	0.048
miR-210-3p	2.03±2.02	1.55±1.87	0.496
CRP, mg/dl	38.1±28.6	20.8±3.2	0.002
ESR, mm/h	38.5±13.8	24.2±11.8	0.002

miR: microRNA; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; RF: rheumatoid factor

Table 4. Mean expression levels of selected microRNAs, CRP, and ESR in positive and negative Anti-CCP patients

Parameters	Patients with Positive Anti-CCP	Patients with Negative Anti-CCP	p-value
miR-16-5p	63.43±81.43	92.24±73.79	0.659
miR-155-5p	1.43±1.70	2.73±3.47	0.027
miR-210-3p	2.17±2.35	1.27±1.09	0.046
CRP, mg/dl	44.7±30.4	15.1±21.8	0.0001
ESR, mm/h	38.2±15.5	28.5±11.9	0.03

miR: MicroRNA; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate, Anti-CCP: Anti-cyclic citrullinated peptide

Table 5. Average expression levels of miR-16-5p, miR-210-3p, miR-155-5p, CRP, and ESR in the 4 different subgroups in terms of disease activity.

Parameters	DAS28				
	High n=16	Moderate n=21	Low n=4	Remission n=9	*p* value
miR-16-5p	124.4±68.4	76.4±74.1	38.6±25.5	34.3±22.5	0.005
miR-210-3p	0.78±0.95	1.1±1.5	1.2±1.4	2.8±2.1	0.048
miR-155-5p	4.1±1.9	2.8±2.8	2.0±1.8	0.8±0.9	0.037
CRP, mg/dl	58±33.8	29±22.5	13.2±11.5	5.8±4.1	0.000
ESR, mm/h	42.8±14.4	33.5±14.07	22±11.1	26±1.01	0.01

Data are expressed as mean±SD or counts. ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; miR: MicroRNA

Table 6. The correlation between expression levels of selected microRNAs and disease activity indices.

Parameters	ESR	CRP	TJC	SJC
miR-16-5p	r=0.385	r=0.409	r=0.345	r=0.381
	p=0.027	p=0.018	p=0.046	p=0.022
miR-155-5p	r=−0.18	r=0.351	r=0.345	r=0.348
	p=0.305	p=0.039	p=0.039	p=0.032
miR-210-3p	r=0.10	r=0.06	r=−0.330	r=0.07
	p=0.537	p=0.704	p=0.037	p=0.687

$p<0.05$ was considered significant. The number of patients is 50.

Comparison of Selected MicroRNAs Average Expression between Different Age Groups

Using the one-way analysis of variance (ANOVA) statistical test, the average expression levels of miR-155-5p, miR-210-3p, miR-16-5p, CRP, and ESR were investigated and compared between the 3 different age groups of 20–39, 40–59, and ≥60 years old. The results showed no significant difference between these 3 groups in the expression levels of selected microRNAs or ESR levels ($p>0.05$). However, applying Tukey's statistical test, CRP levels differed significantly between the 3 groups, as they were increased in the ≥60-year-old group ($p<0.001$).

Correlation of Selected MicroRNAs with Markers of Disease Activity

The correlation between miR-155-5p, miR-210-3p, and miR-16-5p expression and disease activity indices such as CRP, ESR, TJC, and SJC was investigated using the Spearman correlation test. The results indicated that miR-16-5p positively correlated with all disease activity indices, while miR-155-5p had a significantly positive correlation with TJC, SJC, and CRP. For miR-210-3p, a significant inverse correlation was observed only with the TJC index (Table 6).

The miR-155-5p, miR-210-3p, and miR-16-5p as Potential Diagnostic Markers for RA

The ROC curve was applied to determine the sensitivity and specificity of each selected miRNA to evaluate its diagnostic value as a noninvasive biomarker. The best cutoff values were calculated based on Youden's index, the maximum potential effectiveness of a biomarker for all the selected microRNAs. The ROC curve analyses for all the 3 microRNAs are shown in Table 7. The analysis indicates that the plasma levels of the selected microRNAs can be used as potential diagnostic biomarkers with high sensitivity and specificity to distinguish patients suffering from RA from healthy subjects. In addition, the sensitivity and specificity of microRNAs in combination with each other were evaluated by the ROC curve. The results are shown in Table 8.

Table 7. ROC curve analysis of plasma levels of miR-155-5p, miR-210-3p, and miR-16-5p to distinguish RA patients (N=50) between healthy individuals (N=50).

miRNA	Cutoff value	AUC	Sensitivity (%)	Specificity (%)
miR-155-5p	0.61	0.90	80	81
miR-210-3p	2.75	0.75	66	71
miR-16-5p	16.51	0.96	89	82

Table 8. The sensitivity and specificity of combined microRNAs as diagnostic markers

microRNAs	Cutoff Value	Area Under the Curve	Sensitivity (%)	Specificity (%)
miR-16-5p + miR-155-5p	0.72	0.99	94	100
miR-16-5p + miR-210-3p	0.46	0.99	94	92
miR-155-5p + miR-210-3p	0.43	0.95	89	85
miR-16-5p + miR-155-5p + miR-210-3p	0.41	0.99	97	97

DISCUSSION

Approximately 1% of the world's population is affected by RA.[16] RA causes polyarticular synovitis, cartilage, and bone destruction and frequently affects internal organs [17]. The available clinical tests to diagnose RA are not accurate enough. The late diagnosis of this disease has increased its mortality rate. Hence, early diagnosis and treatment are essential to thwart and decrease RA disease problems.[18,19] To find a simple and quick method that is also highly sensitive and specific for an early diagnosis, researchers have been focusing on the identification of new diagnostic and prognostic biomarkers. Ideal biomarkers should have constant expression, high sensitivity and specificity, safe, easy, and noninvasive sampling, be measurable by cost-efficient laboratory methods, and be able to detect the disease at its early stages.[20] Recently, studies have introduced new circulating microRNAs as noninvasive biomarkers for several diseases, including autoimmune disorders. Their first emergence as potential diagnostic markers was in B-cell lymphoma.[4] Having these characteristics, microRNAs are ideal diagnostic markers and therapeutic targets. Previous miRNA expression profiling studies in many diseases, such as cancer and RA, gave rise to common drawbacks in their expression profiling. These were due to different sample sizes, different ethnic groups, and the application of different methods in miRNA analysis. A population of Iranian patients with RA participated in this study to evaluate the expression patterns of miR-155-5p, miR-210-3p, and miR-16-5p in the plasma. The aim was to attain a clear expression profile to help patients with earlier diagnosis and treatment.

The nuclear factor kappa light chain enhancer of activated B cells (NF-κB) pathway is an important regulator of the immune response, inflammation, and cell survival. On the other hand, miR-20 hinders the activation of NF-κB pathway.[21,22] The expression and function of microRNAs are regulated by different environmental stresses, including hypoxia.[23] One of the most important microRNAs overexpressed in hypoxic conditions is miR-210.[24,25] Furthermore, excessive expression of miR-210 inhibits the secretion of TLR4-induced proinflammatory cytokines such as IL-6 and TNF-α.[26] Previous studies suggest that miR-210 plays a crucial role in RA and other autoimmune disorders. In the current study, the expression of miR-210-3p in the plasma of RA patients was analyzed using a real-time PCR technique. The results align with the findings of other studies showing a significant decrease in the relative expression of miR-210-3p in RA patients in comparison with the healthy controls.[27,28] The DAS28 index was used in this study to examine RA disease activity. The findings demonstrate that 32% of patients had high disease activity, and 18% were in remission. Based on the statistical analysis, there was a considerable difference in the expression level of miR-210-3p in the high disease activity stage compared to the remission phase. Also, the relative expression of miR-210-3p is reduced by increasing disease activity. On the other hand, it was observed that the relative expression of miR-210-3p had a significant inverse correlation with the TJC disease activity index, while with other disease activity indices such as CRP, SJC, and ESR, no significant correlation was seen. A study by Huang et al,[27] showed no significant correlation between the relative expression of miR-210-3p and the disease activity indices. The study of Abdul-Maksoud et al,[28] also shows that miR-210-3p was negatively correlated with ESR, CRP, SJC, TJC, DAS28, RF, anti-CCP, and elevated levels of TNF-α and Interleukin-1 beta (IL-1β). All in all, the results of these studies show that miR-210-3p may lead to RA disease occurrence and progression. Thus, this biomarker can

potentially be used for the assessment of patients in terms of disease activity.

One of the most important microRNAs playing a significant role in autoimmune diseases like RA is miR-155-5p. It regulates various signaling pathways associated with RA pathogenesis.[29-31] Altered expression of miR-155-5p is involved in tissue destruction in inflamed joints, proinflammatory activation of myeloid cells, and the development of inflammatory arthritis.[29-31] Thus far, various studies have examined the expression level of miR-155-5p in different body samples of RA patients, including synovial tissue, $CD14^+$ and $CD68^+$ cells in synovial fluid, and synovial fibroblasts.[32-35] They all concluded that there were elevated levels of miR-155-5p in the above samples. However, this study compared the plasma of RA patients to that of a healthy group to examine the relative expression of miR-155-5p. According to the findings, the expression of miR-155-5p significantly increased in the plasma of RA patients. The results showed a significant increase in the expression of miR-155-5p in the plasma of RA patients. Additionally, there was a significant direct correlation between the relative expression of miR-155-5p and disease activity indices, except for ESR. Our results confirm the findings of previous studies showing that the expression of miR-155-5p significantly rises in RA patients compared to the healthy group.[11,28,36] One study, however, has shown a significant reduction in the circulating level of miR-155 in RA patients compared to controls, and another study has demonstrated significant upregulation of miR-155 levels in the plasma of RA patients.[37,38] Finally, the expression level of miR-16-5p was investigated in this study as the last miRNA. It has been stated that miR-16 is involved in RA, and it is suggested as a suitable candidate marker of RA disease activity. This microRNA is downregulated in the sera of early RA patients compared with healthy controls.[39] Also, the abundance of miR-16 and miR-155 changed in plasma during treatment with olokizumab.[40]

Consistent with the results of other studies, the expression of miR-16-5p significantly increased in the plasma of RA patients.[36]

The production of CRP, an acute-phase protein, reflects inflammation or damage to the body [41]. CRP and ESR are suitable biomarkers to detect the inflammatory status in RA. The present study also revealed a positive correlation between all the disease indices, such as CRP and ESR, and the level of miR-16-5p. It is concluded that increased expression of miR-16-5p is positively associated with disease activity.

Anti-CCP is a good indicator of RA, and the early diagnosis of patients with a negative anti-CCP is difficult. Thus, RA patients with negative anti-CCP or RF test results were compared with healthy controls based on the relative expression of the selected microRNAs. The results showed that the expression of miR-155-5p and miR-16-5p was significantly higher in RA patients with either negative anti-CCP or negative RF results compared to healthy individuals, while the expression of miR-210-3p was lower in these patients. These findings emphasized the diagnostic value of the selected microRNAs.

Since a suitable diagnostic biomarker must have high sensitivity and specificity, in this study, we examined the sensitivity and specificity of each of the microRNAs separately and also in combination. For this purpose, ROC curves were drawn, and the AUC was measured. The results represented high sensitivity and specificity for each microRNA, among which miR-16-5p had the highest sensitivity and specificity, 89% and 82%, respectively (AUC=0.96). Moreover, a 97% sensitivity and specificity were observed when using the combination of the 3 microRNAs. Hence, it can be concluded that these microRNAs have a high potential to be used as diagnostic biomarkers for RA diagnosis.

Increased expression of miR-155-5p and miR-16-5p and decreased expression of miR-210-3p in the plasma of RA patients compared to healthy individuals indicate the involvement of these microRNAs in disease incidence and progression. Therefore, they can be used as appropriate biomarkers in the diagnosis and treatment of RA patients.

STATEMENT OF ETHICS

The present study was approved by the Ethics Committee of Tehran University of Medical Sciences (Ethics Code: IR.TUMS.MEDICINE.REC.1397.305). Written informed consent was obtained from all participants.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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