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Plasma Levels of MicroRNA-146a-5p, MicroRNA-24-3p, and MicroRNA-125a-5p as Potential Diagnostic Biomarkers for Rheumatoid Arthritis

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ABSTRACT

Rheumatoid arthritis (RA) is an autoimmune disease that is characterized by inflammation of the articular tissue. This study aims to evaluate the expression of microRNA (miR)-146a-5p, miR-24-3p, and miR-125a-5p in the plasma of RA patients and compare them with those of healthy controls to obtain a specific expression profile for earlier diagnosis and assistance in treating patients.

This study was performed on 50 RA patients and 50 healthy controls. Five microliters of blood were taken from each patient/control. Plasma RNA was extracted using the Trisol solution. cDNAs were synthesized; using moloney murine leukemia virus (MMLV) and deoxynucleoside triphosphate (dNTP). Real-time PCR was performed using SYBR green kit.

The mean expression of miR-146a-5p, miR-24-3p, and miR-125a-5p in the RA group were 8.1±1.9, 6.5±1.2, and 6.8±2.2 and in the healthy group were 4.8±1.6, 3.6±2.2, and 3.4±1.7, respectively. Significant differences were also observed in the mean expression of these three miRNAs in four subgroups of RA patients with different disease activity based on disease activity score 28 (DAS28) (p<0.05). ROC curve analysis showed that miR-146a-5p (AUC=0.8, sensitivity=96%, specificity=86%), miR-24-3p (AUC=0.7, Sensitivity=95%, Specificity=75%) and miR-125a-5p (AUC=0.71, sensitivity=93%, specificity=84%) could be used as suitable biomarkers for RA diagnosis.

Increased expressions of miR-146a-5p, miR-24-3p, and miR-125a-5p in RA patients indicate that the miRNAs are involved in disease incidence and progression, and the measurement of their expression can play an essential role in the diagnosis and treatment of the disease.

Keywords: MiR-146a; MiR-24-3p; Rheumatoid arthritis

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INTRODUCTION

Rheumatoid arthritis (RA) is a polygenic autoimmune disease characterized by chronic inflammation of the synovial tissue and ultimately leading to irreversible joint destruction and lifelong disability,[1] and its prevalence is estimated to be 1% in the world.[2,3] Environmental parameters and specific genetic backgrounds are the contributing factors related to the disease. Approximately 50% of the causes of rheumatoid arthritis are related to human genetics.[4]

The diagnosis of RA is based on American College of Rheumatology (ACR) and European League Against Rheumatism (EULAR) criteria, which was revised in 2010.[5,6] The main goal of that revision was to improve the process of early diagnosis of RA disease by identifying the patients who benefit from the early administration of disease-modifying therapy. These criteria include the number of joints involved, serological tests (Rheumatoid Factor titer, Anti-CCP), C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) levels, and also the duration of symptoms.[5] Nowadays, diagnostic tests for RA are not sufficiently accurate, and leading to the late diagnosis of the patients.[7] Therefore, new biomarkers need to be identified to provide a rapid, simple, with high sensitivity and specificity for the diagnosis of rheumatoid arthritis.

New biomarkers called "microRNAs" were introduced in the discussions related to autoimmune diseases in recent years.[8,9] MicroRNAs are a class of non-coding RNAs of 21-25 nucleotides in length which act as one of the main epigenetic mechanisms in regulating the expression of genes involved in cell development and innate and acquired immune responses. They have the potential of being biomarkers with high sensitivity and specificity that can be identified at different stages of the disease.[10] Also, it is important to regulate microRNAs (miRNAs or miRs) expression to maintain immune homeostasis. As a result, it can be expected that impaired miRNA expression may cause autoimmune diseases.[11] Recent studies have suggested that circulating miRNAs can be suitable for clinical use. These molecules are stable in extracellular conditions (cell-free form) in body fluids such as plasma and serum and protected from endogenous RNase activity.[12,13] The circulating miRNAs are stable at room temperature for up to 24 hours and resistant to freezing and thawing from -80°C to room standard temperature.[12] Therefore, free circulating miRNAs play a crucial role as diagnostic markers and therapeutic targets.[14] The selection criteria of miRNAs were shaped based on the agreements and gaps existing in previous studies. Moreover, currently, there is insufficient information concerning plasma miRNAs in RA patients. Most investigations were implemented on the cellular miRNA alterations and their ability to affect RA's critical cellular functions.

Recently, miR-146a-5p, miR-24-3p, and miR125a-5p were reported as potential biomarkers for RA diagnosis. MiR-146a, a popular miRNA, is one of the most thoroughly studied in RA. Increased expression levels of miR-146a have been documented in synovial fluid,[15-17] synovial tissue,[17-19] and RASFs.[20,21] Nevertheless, all of these methods are invasive resulting in conveying researchers looking for less invasive approaches to diagnose the diseases. However, few studies are available regarding the amount of this microRNA in serum and plasma. Moreover, recently it has been reported that miR-24-3p and miR-125a-5p have potential diagnostic value with high sensitivity and specificity in RA patients.[12] In addition, there is some conflicting evidence about the association between the level of these miRNAs and the disease period. For this reason, particular attention will be paid to the three miRNAs mentioned above in the additional studies in different populations. Regarding the genetic-related studies, the different races and ethnicities may have influential effects on gene expression results. In addition, the genetic of a specific population may vary from the others.

The purpose of this study was to evaluate the expression of miR-146a-5p, miR-24-3p, and miR-125a-5p in the plasma of Iranian patients with RA and compare it with healthy controls, and analyze the correlation between these selected miRNAs and disease activity indices to obtain a specific expression profile for earlier diagnosis and assistance in treating patients with RA.

MATERIALS AND METHODS

Patients and Controls

This case-control study was performed on 50 patients suffering from rheumatoid arthritis and 50 normal individuals of the same age and sex as the control group. The patient group's RA disease had previously been diagnosed based on ACR/EULAR

criteria [5,6] (clinical symptoms, physical examination, and laboratory tests) by expert rheumatologists.

The exclusion criteria included heart, kidney, liver disease, pregnancy, hematologic malignancies (leukemia, lymphoma, and cancers), and infection during the last three weeks of the beginning of this study.

All the necessary information related to this investigation was explained to the study population and informed written consent was obtained from all patients and controls. All the procedures, methods, experiments, and scientific investigations were approved by the ethics committee of the Tehran University of Medical Sciences (IR.TUMS.MEDICINE.REC.1397.126).

Based on the proposed method, 5 mL blood with EDTA anticoagulant was taken from each patient/control. To isolate the plasma, the blood samples were centrifuged at 4000 g for 10 minutes, and the plasma tubes were stored at -70°C until the beginning of necessary tests.

Plasma MicroRNA Expression

At the first step, a volume of 250 µL plasma was thawed on ice and then lysed with 750 µL TrizoLEX reagent (DNA biotech, I.R. Iran). The 25 fmol (5 µL) synthetic exogenous *C.elegans* miRNA (cel-miR-39: 5′-UCACCGGGUGUAAAUCAGCUUG-3′) was added to each denatured sample to normalize sample to sample variation caused by RNA isolation.[16] The mixture was vortexed and centrifuged at 12000g for 8 minutes at 4°C. The aqueous phase was separated, and 200 µL chloroform was added and mixed by inverting the tube for 15 seconds. After 3 minutes of incubation at room temperature, 15 minutes centrifugation at 12000g at 4°C was performed. The aqueous and organic phase containing the RNA was transferred to a new microtube. The 500 µL isopropanol was added and centrifuged at 12000 g for 15 minutes at 4°C. After 24 hours of incubation at -20°C, centrifugation at 12000 g for 15 minutes at 4°C was implemented. The supernatant was discarded, and the pellet was washed with 75% ethanol subsequently and then dried by exposure to ambient. Finally, RNA dissolved in 20 µL DEPC water. The quantity of extracted RNA was measured by using a NanoDrop spectrophotometer (Thermo 2000).

Primer Design

To design the primer, a new method called miR-Q was applied using the instructions presented in a research performed by Sharbati-Tehrani et al.[22] This novel and innovative method is used to identify and measure the small RNA molecules, especially miRNA. First, RNA is transcribed into cDNA; using RT6-miR-X primer (X is the unique name for each miRNA), and then cDNA is amplified and quantified using qPCR based on the use of 3 DNA-Oligonucleotide (Short-miR-X, MP-forward, MP-reverse) at different concentrations (Figure 1).[22] The sequences of primers required for target microRNAs are presented in Table 1.

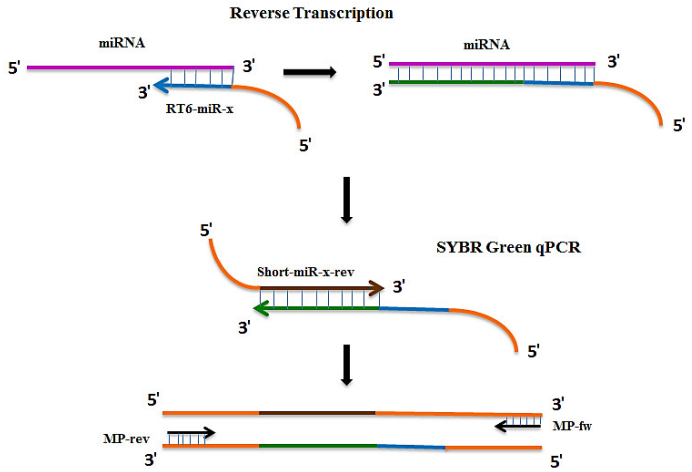

Figure 1. Schematic view of the desired primers activity (Figure Source[22])

Table 1. DNA-oligonucleotides were used for reverse transcription (RT) and quantitative polymerase chain reaction (qPCR in this study.

DNA-oligonucleotides	Sequence (5' to 3')
RT- 6- 24	tgtcaggcaaccgtattcaccgtgagtggtctgttc
Short- 24	cgtcagatgtccgagtagagggggaacggcgtggctcagttcagcag
RT- 6- 125	tgtcaggcaaccgtattcaccgtgagtggttcacag
Short- 125	cgtcagatgtccgagtagagggggaacggcgtccctgagacccctttaac
RT-6- 146	tgtcaggcaaccgtattcaccgtgagtggtaaccca
Short- 146	cgtcagatgtccgagtagagggggaacggcgtgagaactgaattcca
MP- fw	tgtcaggcaaccgtattcacc
MP- rev	cgtcagatgtccgagtagagg
RT- 6- cel- 39	tgtcaggcaaccgtattcaccgtgagtggtcaagct
Short- cel- 39	cgtcagatgtccgagtagagggggaacggcgtcaccgggtgtaaatc

Reverse Transcription

RNA was reverse- transcribed; using the Moloney murine leukemia virus (MMLV) enzyme (Yektatajhiz, Iran). Briefly, the mixture containing 2.5 µL RNA, 1 µL RT-primer, 10.5 µL DEPC water incubated at 70°C for 5 minutes. Then, 1 µL dNTP, 1 µL MMLV enzyme and 4 µL Buffer were added to each microtube, followed by incubation at 42°C for 60 minutes and 70°C for 5 minutes on the thermocycler (Applied Biosystems). Finally, the cDNA was stored at -20°C for additional tests.

Real-time Quantitative PCR

The real-time quantitative PCR assay was performed using a 2X SYBR Green qPCR Master Mix (Norgenbiotek, Canada) on the Corbett Rotor-Gene 6000 real-time PCR system (Qiagen). Five 10-fold serial dilutions (1, 1/10, 1/100, 1/1000, 1/10000) of cDNA samples were prepared and the efficiency of each primer was determined by analyzing a standard linear curve based on $E=10^{-1/slope}$. The results obtained by this analysis demonstrated an excellent amplification efficiency (0.98-1.03) for each primer set of experimental miRNA and reference genes. The 10µL of PCR mixture included 2 µL cDNA, 5 µL 2x SYBR Green Master Mix, 1 µL Short- primer, 1 µL MP-primer (MP-fw + MP-rev) and 1µL DEPC water. The PCR was executed in 95°C for 5 minutes, followed by 40 cycles of 95°C and 60°C for 15 and 30 seconds, respectively. For Non-template Negative Control (NTC) samples, the 2 µL of distilled water was added instead of a 2 µL cDNA template. The levels of plasma miRNA were calculated and assessed; using the ΔCT method. All the samples were tested in duplicate.

Moreover, the specificity of primers activity in the Real-time PCR reaction was confirmed by examining the size of the products on 3.5% agarose gel electrophoresis (Figure 2). The PCR product sizes of mir-146a-5p, miR-24-3p, miR-125a-5p, and cel-miR-39 were 69, 69, 73, and 67 bp, respectively. The real-time PCR products of miR-146a-5p (well 4), miR-24-3p (well 3), miR-125a-5p (well 2), cel-miR-39 (well 1) showed single band and expected size.

Statistical Analysis

Statistical analysis was performed using SPSS software version 25.0. Data were shown as mean±*SD* or counts (percentages). To compare the mean expression of miRNAs between the patient and control groups, a statistical t-test was used. Spearman's correlation coefficient was applied to measure the linear relationship between miRNAs expression and disease activity indices (ESR, CRP, TJC, SJC). It should be noted that the differences were considered statistically significant when P-value was less than 0.05. Also, the sensitivity and specificity of miRNAs for RA diagnosis were determined using the ROC (Receiver Operating Characteristic) curve.

RESULTS

Demographic, Clinical, and Laboratory Data

Demographic data and clinical findings of the study population are presented in Table 2.

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Table 2. Demographic data and clinical findings of the study population

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
Medication, n (%)		
Methotrexate	31 (62%)	NA
sulfasalazine	(54%)27	NA
Prednisolone	23 (46%)	NA
Hydroxychloroquine	20 (40%)	NA
Alendronate	8 (4%)	NA

*Data shown in the above table are expressed as mean±SD or counts (percentages).

N.A., 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 on 28 joints; TJC28, Tender joint count on 28 joints.

Altered Expression of MiR-146a-5p, MiR-24-3p, and MiR-125a-5p in Plasma of Patients with RA Compared to Healthy Controls

To identify the changes that happened in the expression levels of the selected miRNAs in plasma from patients with RA compared to healthy controls, real-time qPCR was implemented. To show the mean expression, the formula ΔCt=Ct (Reference gene) - Ct (miRNA of interest) was used. In this case, an increase in ΔCt indicates an increase in expression.[23] The overall analysis of the results showed a significant increase in the expression levels of miR-146a-5p, miR-24-3p, and miR-125a-5p in patients compared to healthy controls (p<0.05) (Figure 3).

Analysis Based on DAS28 Index

In this research, patients suffering from RA were divided into four groups according to the DAS28 index of disease activity. As shown in Table 3, if the DAS28 resulted in less than 2.6, it would be categorized in the remission group. The DAS28 in the 2.6-3.2 intervals were classified as low disease activity. In addition, the DAS28 resulted in 3.2-5.1 intervals were recorded as the moderate disease activity, and finally, the DAS28 resulted in more than 5.1 were classified in the high disease activity category. The Kruskal-Wallis statistical test results showed that the average expression of miR-146a-5p, miR-24-3p, miR-125a-5p, CRP, and ESR levels statistically varied between the four different subgroups in terms of disease activity. The expression levels of miR-146a-5p, miR-24-3p, and miR-125a-5p were increasing by elevating the DAS28 index (Table 3).

Comparison of the Average Expression of the Selected MiRNAs between Different Age Groups

Implementing the Kruskal-Wallis statistical test, the average expression of miR-146a-5p, miR-24-3p, miR-125a-5p, CRP, and ESR levels in three different age groups of 20-39, 40-59, and ≥60 years old were studied and compared. There were no significant differences between these three subgroups in the expression levels of these miRNAs and ESR levels. While CRP levels were significantly different between the three groups, they were increased dramatically in≥60 years old group (p <0.05) (Figure 4).

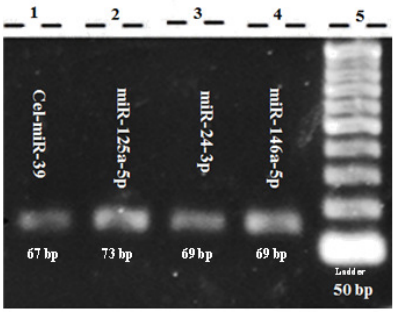

Figure 2. Real-time polymerase chain reaction (PCR) products on 3.5% agarose gel; 1: Internal Control Cel-miR-39, 2: miR-125a-5p, 3: miR-24-3p, 4: miR-146a-5p, 5: 50bp Ladder.

miRNA	A Relative expression	*P-value*
miR-146a-5p		
RA patients	8.1 ± 1.9	**0.01**
Healthy controls	4.8 ± 1.6	
miR-24-3p		
RA patients	6.5 ± 1.2	**0.02**
Healthy controls	3.6 ± 2.2	
miR-125a-5p		
RA patients	6.8 ± 2.2	**0.02**
Healthy controls	3.4 ± 1.7	

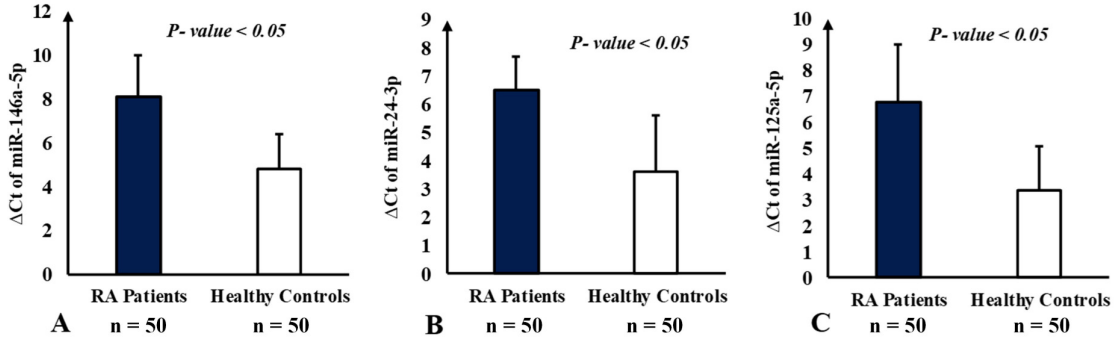

Figure 3. Comparison of levels of circulating miR-146a-5p (A), miR-24-3p (B), and miR-125a-5p (C) between rheumatoid arthritis (RA) patients and healthy controls. The formula ΔCt=Ct (Reference gene) - Ct (miRNA of interest) was used. In this case, an increase in ΔCt indicates an increase in expression.

Correlation of Selected MiRNAs with Markers of Disease Activity in RA Patients

To determine the correlation between miR-146a-5p, miR-24-3p, and miR-125a-5p expression with CRP, ESR, TJC, and SJC indices, the Spearman correlation test was conducted. Results showed that SJC was positively correlated with miR-24-3p and miR-125a-5p. The results showed a significant correlation between levels of miR-146a-5p, miR-24-3p, and miR-125a-5p and ESR levels in patients with RA. Levels of miR-24-3p and miR-125a-5p correlated with a change in CRP levels. None of these miRNAs significantly correlated with the TJC index. However, the levels of miR-146a-5p did not correlate with CRP, SJC, and TJC measures (Table 4).

Table 3. Comparison of the average expression of miR-146a-5p, miR-24-3p, miR-125a-5p, CRP, and ESR levels between the four different subgroups in terms of disease activity

Parameters	DAS28				
	High N=16	Moderate N=21	Low N=4	Remission N=9	*p*
miR-146a-5p	4.9 ±2.9	2.9 ±1.9	1.7 ±0.1	1.4 ±1.8	**0.004**
miR-24-3p	4.4 ±3.04	3.6 ±2.5	2.06 ±1.1	0.03 ±4.1	**0.03**
miR-125a-5p	5.3 ±3.8	3.2 ±2.3	2.9 ±0.3	1.3 ±1.9	**0.01**
CRP, mg/dL	58 ±33.8	29 ±22.5	13.2 ±11.5	5.8 ±4.1	**<0.001**
ESR, mm/h	42.8 ±14.4	33.5 ±14.1	22 ±11.1	26 ±1.01	**0.01**

*Data shown in the above table are expressed as mean±SD or counts. ESR, Erythrocyte sedimentation rate; CRP, C-reactive protein

Analysis Based on RF and Anti-CCP Results

In the present study, the mean expression of miR-146a-5p, miR-24-3p, miR-125a-5p were compared between two groups of Rheumatoid Factor (RF) positive and negative patients. The results of this analysis showed a significant difference at the levels of miR-24-3p (p=0.02), miR-125a-5p (p=0.01), and miR-146a-5p (p=0.01) between the RF positive and negative groups (Figure 4). Furthermore, the relative expression of miR-146a-5p (p=0.03), miR-24-3p (p=0.03), miR-125a-5p (p=0.02) were statistically different between patients with Anti-CCP positive and Anti-CCP negative results (Figure 4).

Moreover, the expression levels of miR-146a-5p, miR-24-3p, and miR-125a-5p were investigated in subjects with RA, whose RF test was negative or positive with a healthy control group. The results showed that the miRNAs expression levels were considerably increased in RA patients with negative/positive RF and Anti-CCP results compared to healthy controls (Figure 5).

The MiR-146a-5p, MiR-24-3p, and MiR-125a-5p as Potential Diagnostic Markers for RA

The Receiver Operating Characteristic (ROC) curve was applied to assess the biomarker's ability to distinguish disease status. The best cutoff values were calculated based on Youden's index, the highest possible effectiveness of a biomarker for all three miRNAs. As shown in Figures 6, the ROC curve analysis for miR-146a-5p, miR-24-3p, and miR-125a-5p indicates that the plasma levels of these miRNAs can be used as potential diagnostic markers to differentiate RA patients from healthy individuals with high sensitivity and specificity (p<0.0001).

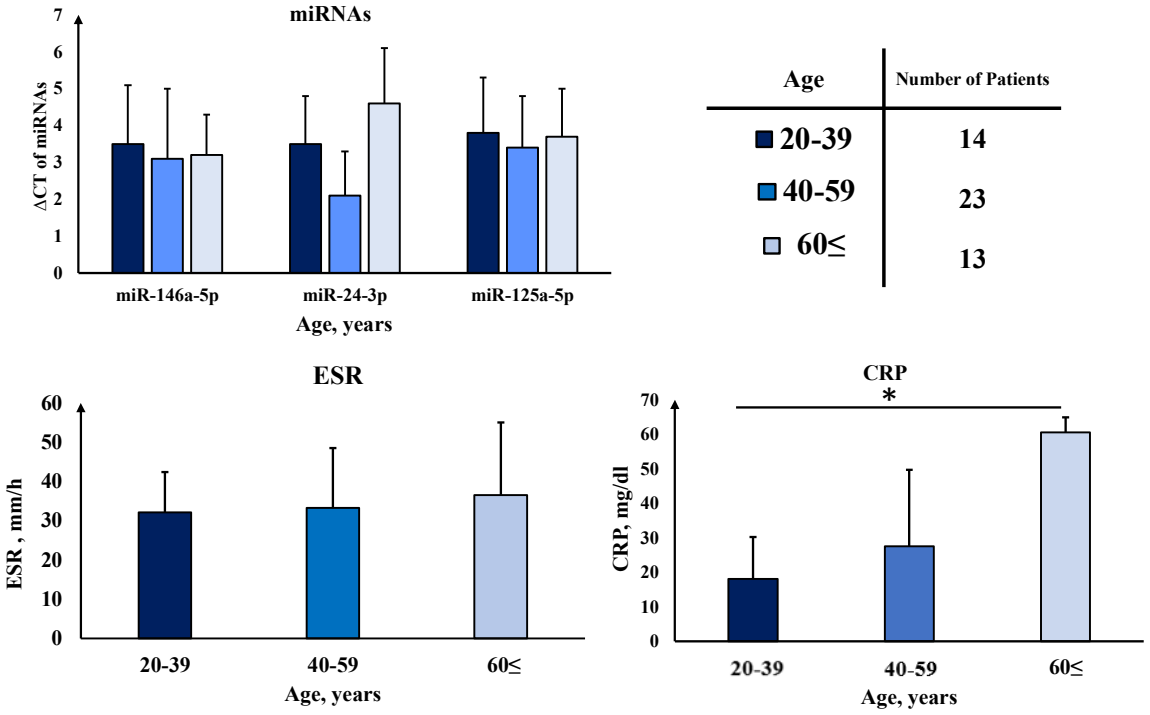

Figure 4. Comparison of levels of circulating miR-146a-5p, miR-24-3p, miR-125a-5p, C-reactive protein (CRP), and erythrocyte sedimentation rate (ESR) between three different age groups. *p<0.05. To show the mean miRNAs expression, the formula ΔCt=Ct (Reference gene) - Ct (miRNA of interest) was used. In this case, the more ΔCt indicates, the more expression.

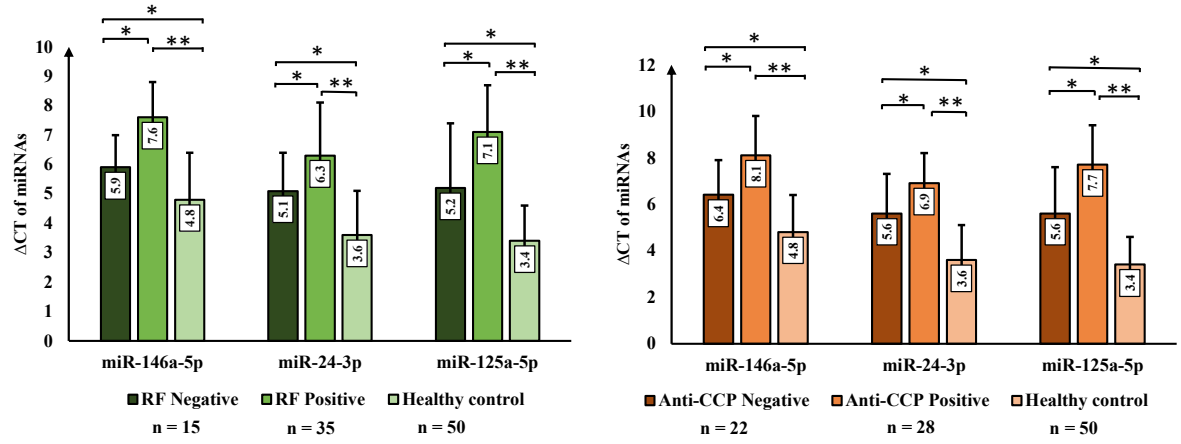

Figure 5. Comparison of levels of circulating miR-146a-5p, miR-24-3p, miR-125a-5pbetween positive/negative rheumatoid factor (RF) or anti-cyclic citrullinated peptide (Anti-CCP) with the healthy control group. *p<0.05, **p<0.01.

Table 4. The correlation between expression levels of selected miRNAs and disease activity markers

Parameters	ESR	CRP	TJC	SJC
miR-146a-5p	r=0.46	r=0.07	r=0.16	r=0.18
	***p*=0.001**	*p*=0.6	*p*=0.13	***p***=0.11
miR-24-3p	r=0.43	r=0.4	r=0.22	r=0.38
	***p*=0.003**	***p*=0.01**	*p*=0.07	***p*=0.004**
miR-125a-5p	r=0.49	r=0.3	r=0.11	r=0.26
	***p*=0.001**	***p*=0.01**	*p*=0.23	***p*=0.04**

Spearman's correlation coefficient test was conducted and $p < 0.05$ was considered significant. The number of patients=50.

miRNA	Cutoff value	AUC	Sensitivity (%)	Specificity (%)
miR-146a-5p	1.53	0.8	96	86
miR-24-3p	1.51	0.7	95	75
miR-125a-5p	1.49	0.71	93	84

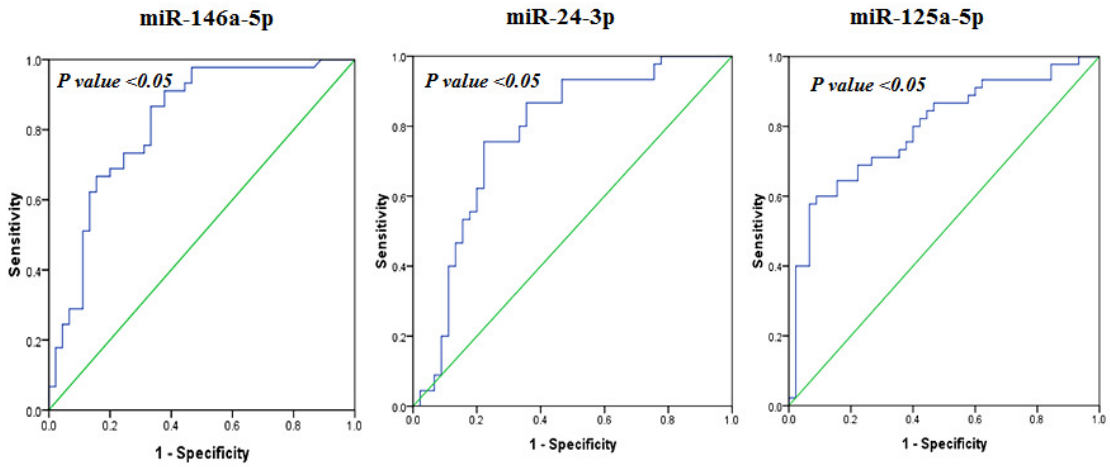

Figure 6. Receiver operating characteristic (ROC) curve analysis of plasma miR-146a-5p, miR-24-3p, and miR-125a-5p to differentiate rheumatoid arthritis (RA) patients (N = 50) from healthy subjects (N = 50).

DISCUSSION

Rheumatoid arthritis is a chronic autoimmune disease that influences approximately 1% of the world's population. This systemic inflammatory disease is characterized by inflammation of the joints, cartilage involvement, and bone erosion.[24] Early diagnosis and treatment have been emphasized to prevent and reduce RA disease complications.[25,26] Therefore, scientists have focused their research on introducing diagnostic and prognostic biomarkers to identify patients, as well as predicting disease progression and adopting appropriate therapeutic approaches. Suitable biomarkers should have these features: 1) stable expression, 2) high sensitivity and specificity, 3) detectable by conventional and inexpensive laboratory methods, 4) detectable in the early stages of the disease, 5) easy and non-invasive sampling.[27] In recent

years, new biomarkers called microRNAs have emerged in the discussions related to autoimmune diseases. The miRNAs, which are free in the blood circulation, are essential to be used as diagnostic markers and therapeutic targets. Previous studies firstly investigated the miRNAs' profile, and then, miR-24-3p, miR-125a-5p, and miR-146a-5p were selected for further investigations. Their results showed a high diagnostic value of the profile mentioned above in their study populations.[12,28] Based on the genetic-related studies, the different races and ethnicities may have influential effects on gene expression results. In addition, the genetic of a specific population may vary from the others. Therefore, in this study, the expression patterns of miR-146a-5p, miR-24-3p, and miR-125a-5p in the plasma of patients suffering from RA were investigated to obtain a specific expression profile for earlier diagnosis and assist in treating patients from the final results.

The miR-146a-5p acts as an essential regulator in the differentiation and function of innate and adaptive immune cells.[29,30] The expression levels of miR-146a have been studied in different samples of rheumatoid arthritis patients, including synovial fluid, articular tissue, and fibroblasts.[12,18,20,31] Increased levels of miR-146a have been reported in all these studies, suggesting the miR-146a as a suitable biomarker for RA diagnosis. In the present study, real-time PCR analysis was performed to examine the expression of miR-146a-5p in the plasma of patients with RA. The results of this study showed that the relative expression of miR-146a-5p in the RA patients was significantly increased compared to the healthy control group, which was similar to the results of other studies.[15,32,33] The conflicting results regarding the relationship between the expression level of miR-146a and different courses of the disease were observed in various studies. Elsayed et al[34] found a significant positive correlation between miR-146a expression and disease activity. Also, a positive correlation between the expression of miR-146a, ESR, CRP, and Anti-CCP was reported. In contrast, Murata et al[12] showed that plasma miR-146a level was not significantly correlated with disease activity and indices such as ESR and CRP. In the present study, the RA disease activity was assessed by the DAS28 index, which showed 32% of all patients had High Disease Activity, and 18% were in the Remission Phase. Furthermore, statistical analysis showed that the level of plasma miR-146a-5p was remarkably different between High Disease Activity and Remission Phase. However, the relative expression of miR-146a-5p resulted in no considerable difference between the two modes of High Disease Activity and Moderate Disease Activity. The results of this study showed that the relative expression of miR-146a-5p rises by increasing disease activity. Moreover, the level of miR-146a-5p is positively correlated with ESR levels in RA patients. Therefore, these findings suggest that this miRNA may be involved in the occurrence and progression of RA disease. Consequently, it can be used as a potential biomarker to evaluate patients in terms of disease activity.

Recently, it has been reported that miR-24-3p and miR-125a-5p have potential diagnostic value in RA patients. One of the possible roles of miR-24-3p and miR-125a-5p is to increase the inflammatory process. Murata et al.[12] reported that the levels of plasma of the miR-24-3p and miR-125a-5p expression were significantly increased in patients with RA in comparison to healthy controls. The highest AUC was observed for miR-125a-5p (AUC=0.83) with miR-24-3p (AUC=0.8). The results of this study showed that the relative expression of miR-24-3p and miR-125a-5p were significantly increased in RA patients in comparison to healthy controls. In addition, the patients with high disease activity showed the increased expression of miR-24-3p and miR-125a-5p compared to patients who were in the Remission Phase. Moreover, the ROC analysis was implemented to investigate further whether these miRNAs can be used as diagnostic markers for rheumatoid arthritis or not. In this research, the highest AUC=0.8 was obtained for miR-146a-5p with 96% sensitivity and 86% specificity and then, the miR-125a-5p (AUC=0.71) and miR-24-3p (AUC=0.7). These three miRNAs examined in this study had high sensitivity and specificity for the RA diagnosis. Thus, it can be concluded that these miRNAs can be used as potential biomarkers for RA diagnosis.

In addition, CRP is a globin that is produced in the liver in response to inflammation or damage to the body. When inflammation or infection occurs, the CRP synthesis may reach maximum levels within 50 hours.[35] Both CRP and ESR are two suitable biomarkers for determining inflammation status in RA. The results of this study indicated that the levels of miR-24-3p and miR-125a-5p are positively correlated with CRP and ESR indices in RA patients, proposing that the increased expression of these miRNAs is positively

associated with the disease activity. However, in a study conducted by Murata et al., the level of miR-125a-5p did not associate with ESR and CRP levels, which was in contrast to our results.

Finally, the relative expression of miR-146a-5p, miR-24-3P, and miR-125a-5p was compared between RA patients who had negative Anti-CCP or RF test results and healthy controls. The results showed that the expression of all three miRNAs in RF negative or Anti-CCP negative patients was substantially higher than in healthy controls. Regarding the high diagnostic value of the Anti-CCP in RA patients, the patients with the negative test results may not be diagnosed at an early stage of the disease. Therefore, the miRNAs that result higher in seronegative (Anti-CCP- / RF-) patients than healthy controls should be more considered.

One of the limitations of this study was the restriction of the control group to healthy individuals. Consequently, it is suggested to consider additional control groups such as patients with lupus erythematosus, infection, osteoarthritis, and other inflammatory diseases to determine the specificity of these biomarkers for RA diagnosis.

In conclusion, increased expression of miR-146a-5p, miR-24-3p, and miR-125a-5p in the plasma of RA patients compared to healthy individuals indicates that these miRNAs may be involved in disease incidence and progression and the measurement of their expression can play a crucial role in the diagnosis and treatment of the disease.

CONFLICT OF INTEREST

The authors report no conflict of interest.

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