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ORIGINAL ARTICLE

Role of miRNA146a and miRNA155 as Biomarkers in Rheumatoid Arthritis Disease Activity

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ABSTRACT

Background: *Rheumatoid arthritis (RA) represents a worldwide health concern as it leads to joint deformities or even permanent disability. As epigenetic directors of biological signalling mechanisms, the erratic expression of various micro-RNAs (miRNAs) is strongly associated with RA.* ***Objectives:*** *The purpose of the current research was to assess the significance of miRNA-146a and miRNA-155 as predictors of the development of RA. We also assessed their promising application as biomarkers for RA disease activity.* ***Methodology:*** *This case-control study included 62 subjects (31 RA patients and 31 healthy controls). Participants sera were used for laboratory tests [anti-cyclic citrullinated peptide (anti-CCP), rheumatoid factor (RF), erythrocyte sedimentation rate (ESR), and C-reactive protein (CRP)] and for the measurement of miRNA-146a, and miRNA-155 gene expression.* ***Results:*** *In RA patients, levels of miRNA-146a and miRNA-155 were significantly higher than in controls (P<0.001). The receiver operating characteristic (ROC) curve analysis showed that miRNA-146a could predict RA with area under curve 0.857, sensitivity 77.4% and specificity 80.6% (P<0.001) at 95% confidence interval (CI) (0.765 – 0.95). MiRNA-155 could predict RA with area under curve 0.829, sensitivity 77.4% and specificity 67.7% (P<0.001) at 95% CI (0.727 – 0.932). Active RA patients expressed more miRNA-146a and miRNA-155 than the inactive group. There was a significant positive correlation (P<0.05) among miRNAs-146a and155 expression levels and DAS-28 score in RA patients. MiRNA -146a was found to increase the risk of active RA significantly independently by 2.023 folds. The ROC curve showed that miRNA -146a is useful for prediction of RA disease activity with area under curve 0.905, sensitivity 91.3% and specificity 87.5% (P<0.001) at 95% CI (0.791 – 1).* ***Conclusion:*** *MiRNA-146a and miRNA-155 expressions were remarkable in RA patients and could be recognized as potential biomarkers for early RA diagnosis. However, miRNA- 146a expression is an independent risk for active RA and could be a valuable biomarker to predict RA disease activity.*

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease described mainly by inflammation, erosions, and joint damage[1]. It also involves many extra articular organs such as skin, eyes, heart, lungs, and nervous system. The extra articular involvement in RA is more frequent with sever disease, long disease duration and uncontrolled disease activity[2].

Numerous genetic, epigenetic, immunological, and environmental factors interact in the pathophysiology of RA[3]. Among the epigenetic elements involved in the pathophysiology of RA are micro-RNAs , which are small, non-coding RNAs with a length of about 22 nucleotides, they play an important role in many biological process including the cell regulation mechanisms and immune responses[4]. In several malignancies and autoimmune illnesses like RA, altered microRNA expression has been linked to increased release of proinflammatory cytokines and the stimulation of numerous inflammatory signaling pathways[5]. Variable miRNAs, including miR-26, miR-146a, miR-146b, miR-150, and miR-155, have been implicated in the joint synovium's generation of proinflammatory cytokines in RA, leading to activation of the inflammatory cells, synovial tissue proliferation and osteoclast differentiation[6]. Moreover, miR-146, miR-150 and miR-155 have been found to be associated with the upregulation of the IL-17 producing cells and activation of Th17 in RA pathogenesis[7]. Additionally, when comparing RA patients to healthy controls, it has been demonstrated that miR-132, miR-16, miR-146a and miR-155 are linked to higher peripheral blood mononuclear cells (PBMNCs)[8].

Recently, many miRNAs, with a specific expression profile, have been introduced as new biomarkers

connected with the progression of many autoimmune diseases and cancers. Murata et al.[9] found that miR-24 and 125-5p might be used to predict early RA diagnosis; additionally, miR-125b values were correlated with RA disease activity and treatment response.

The current research evaluated the impact of miRNA-146a and miRNA-155 as predictors of the development of rheumatoid arthritis. Also, their potential use as biomarkers of RA disease activity was assessed, which could open the field for new miRNAs targeted therapy with better disease prognosis and outcomes.

METHODOLOGY

A case-control study included 62 subjects categorized into 2 groups; A) Case group which involved 31 RA patients who were attending the Outpatient Clinics and the Inpatients of the Rheumatology and Rehabilitation Department, Zagazig University Hospitals. B) An age and sex-matched control group comprising thirty-one subjects who appeared to be in good health and had no clinical findings suggesting immunological diseases.

Rheumatoid arthritis patients fulfilling the 2010 American College of Rheumatology (ACR)/European League against Rheumatism (EULAR) criteria for the classification of RA were enrolled in the study[10]. Patients less than 16 years or more than 70 years old and those suffering from severe comorbidities such as malignancy, severe cardiovascular illness or mentally disorders were excluded from the study. Each participant was given a thorough explanation of the nature of the investigation and the study's objectives before providing written consent. All participants data were confidential. The Zagazig University Hospitals Institutional Review Board (IRB #11114-17/9-2023) authorized this research, and the work was done following the World Medical Association's (Declaration of Helsinki) Code of Ethics for Human Research. Every participant underwent a thorough history taking procedure, thorough general, systemic and musculoskeletal examination. The Disease Activity Score 28 (DAS28), pain visual analogue scale (VAS) (0-10), ESR and counting the number of tender and swollen joints (range 0-28) were utilized to measure the disease activity of RA patients; in addition to other RA clinical parameters such as morning stiffness, Patient and physician global health (0-10)[11]. Modified health assessment questionnaire (MHAQ) is a self-applied questionnaire for evaluating physical disability through asking about daily activities and perceived levels of difficulties[12].

Blood Sampling:

Venous blood samples (5ml) were collected from the participants into non-heparinized red topped blood collection tubes. Additionally, 2 ml of venous blood were collected into EDTA containing blood collection tubes for ESR test. The 5 ml blood samples were separated into 2 parts: one portion for performing laboratory tests such as anti-CCP, RF, and CRP. Prior to analysis, these samples underwent centrifugation and were kept at -20°C. The other portion of samples was used for measurement of miRNA-146a, and miRNA-155 expression. Serum was centrifuged and immediately removed using RNAse free tips into RNAse free Eppendorf and stored at −80°C for further analysis.

Total RNA Extraction:

Total RNA extraction was carried out using the miRNeasy Mini Kit (Qiagen, Germany, catalogue No 217004) based on the manufacturer's guidelines. Using a NanoDrop-2000 spectrophotometer (Thermo Scientific, USA), the concentration and purity of RNA were ascertained according to the ratio of absorbance (OD) at 260 and 280 nm. A ratio of ~2.0 is generally accepted as "pure" for RNA.

Reverse Transcription of RNA to complementary DNA (cDNA):

The cDNA was synthesized using miRCURY LNA RT Kit (Qiagen, Germany, catalogue No 339340) following the producer's instructions. A final volume of 10 µl of reverse transcription master mix was created through adding the following components: (2 µl 5x miRCURY RT Reaction Buffer, 1 µl 10x miRCURY RT Enzyme Mix, 2 µ Template RNA (5 ng/µl), and 5 µl RNase-free water). Reaction mixture was incubated for 60 min at 42ºC, then incubated for 5 min at 95ºC, to inactivate the reverse transcriptase, immediately cooled to 4°C and stored at -20ºC to be used within 5 weeks.

Amplification and detection of cDNA:

This was done utilizing miRCURY LNA SYBR® Green PCR Kit (Qiagen, Germany, catalogue No 339345) and miRCURY LNA miRNA Primer Assay (Qiagen, Germany,catalogue No 339306) based on manufacturers' protocols. For dilution of the cDNA to 1:60, add 1180 µl of RNase-free water to the 20 µl RT reactions immediately before use. Human RNU6B was used as a normalization gene for later assessment of miRNA expression. Reaction mixture was prepared as follow to reach a total volume of 10 µl: (5 µl 2x miRCURY SYBR Green Master Mix, 3 µl cDNA template, 1µl 10x PCR primer mix (miR-146a, miR-155 or RNU-6), 0.5 µl RNase-free water, and 0.5 µl ROX Reference Dye. Real time PCR was done on "Quant studio 5 DX" platform (ThermoFisher Scientific, Singapore) using the following cycling conditions: 2 min at 95c to activate HotStar Taq DNA Polymerase followed by 40 cycles of (denaturation at 95 ºC for 10 sec, combined annealing/ extension at 56ºC for 60 sec). After that, melting curves were plotted displaying data gathered at a melting curve stage. Peaks on the melting curve can reveal a target's melting temperature (Tm) or nonspecific PCR amplification. Fluorescence data

collection was completed throughout the annealing/extension step.

Data Acquisition and Processing:

Threshold cycle (CT) values were recorded for both RA cases and controls, and then normalized against RNU-6 to determine ΔCT. Difference between ΔCT of cases and controls were calculated using ΔΔCT method. Relative expression of miRNA was measured using 2^-ΔΔCT method[13], where:

ΔCT = CT target gene – CT reference gene.

ΔΔCT = ΔCT test sample – ΔCT control sample.

Relative expression of miRNA= 2-ΔΔCT (normalized expression ratio)

Statistical analysis:

To analyze the data, we used the software SPSS (Statistical Package for the Social Sciences) version 26. When comparing categorical variables, the chi square test and, when necessary, the Monte Carlo tests were used to characterize the data using their absolute frequencies. Using chi square for trend test, ordinal data between two groups was compared. To validate assumptions for use in parametric tests, the Shapiro-Wilk test was employed. Depending on the type of data, the means and standard deviations or the median and interquartile range (IQR) were used to characterize quantitative variables. The independents sample t test was utilized (for normally distributed data) to compare quantitative data between two groups. For data that was not normally distributed, Spearman rank correlation coefficients were used to evaluate the direction and strength of the correlation between two continuous variables. In order to diagnose a specific health issue, the ROC curve was utilized to determine the best cutoff of a particular quantitative parameter. Binary logistic regression was applied to discover independent risk variables for certain medical conditions. The level statistical significance was established at P<0.05. A highly significant difference was noticed if p≤0.001.

RESULTS

This work involved thirty-one RA patients and thirty-one healthy individuals as a control group; their mean age was 44.52 ± 11.08 and 38.91 ± 11.67 years respectively, and most of them were females. Regarding RA disease activity measures, DAS 28 showed a mean of 4.4± 1.45 indicating moderate disease activities; however, the patient and the physician global health medians (IQR) were 5(4-7) and 3(3-4) respectively. Additionally, there was highly significant difference in the levels of miRNA-146a and miRNA-155 in RA patients compared to controls (P< 0.001) with higher levels in RA patients than healthy controls as shown in table 1.

Table 1 Demographic data of the studied groups:

	Case group (n=31)	Control group (n=31)	Test	p
Female gender n (%)	28 (90.3%)	25 (80.7%)	Fisher[¥]	0.473
Age (years) [mean ± SD]	44.52 ± 11.08	38.91 ± 11.67	1.941[∞]	0.057
BMI (kg/m^2) [mean ± SD]	31.6 ± 6.79	29.25 ± 7.72	1.273[∞]	0.208
Morning stiffness (min) [median (IQR)]	15(8 – 30)			
Number of tender joints NTJ [median (IQR)] **Number of swollen joints NSJ** [median (IQR)]	6(2 – 18) 0(0 – 2)			
DAS-28 [mean ± SD] Active > 2.6 Inactive ≤ 2.6	4.4 ± 1.45 23 (74.2%) 8 (25.8%)			
MHAQ [median (IQR)]	0.86(0.64 – 1.5)			
VAS pain [median (IQR)]	60(40 – 65)			
Patient global health (0-10) [median (IQR)]	5(4 – 7)			
Physician global health (0-10) [median (IQR)]	3(3 – 4)			
miRNA 146a (folds) [median (IQR)]	5.71(2.71 – 9.71)	1.57(1.02 – 2.68)	-4.873[§]	<0.001**
miRNA 155 (folds) [median (IQR)]	3.03(2.16 – 4.16)	1.2(0.16 – 2.46)	-4.459[§]	<0.001**

[¥]Chi square test, [∞]independent sample t test, [§]Mann Whitney test , **p≤0.001 is statistically highly significant, n: number, IQR: interquartile range, SD: standard deviation, MBI: Body mass index, NTJ: Number of tender joints, NSJ: Number of swollen joints, DAS: disease activity scale, MHAQ; Modified health assessment questionnaire, VAS: visual Analogue scale.

The ROC curve in figure 1 showed that the best cut-off of miRNA 146a in prediction of RA was ≥ 2.694 folds with area under curve 0.857, sensitivity 77.4% and specificity 80.6% (P<0.001) at 95% CI (0.765 – 0.95). Also, best cut-off of miRNA 155 in prediction of RA was ≥2.1475 folds with area under curve 0.829, sensitivity 77.4% and specificity 67.7% (P<0.001) at 95% CI (0.727 – 0.932).

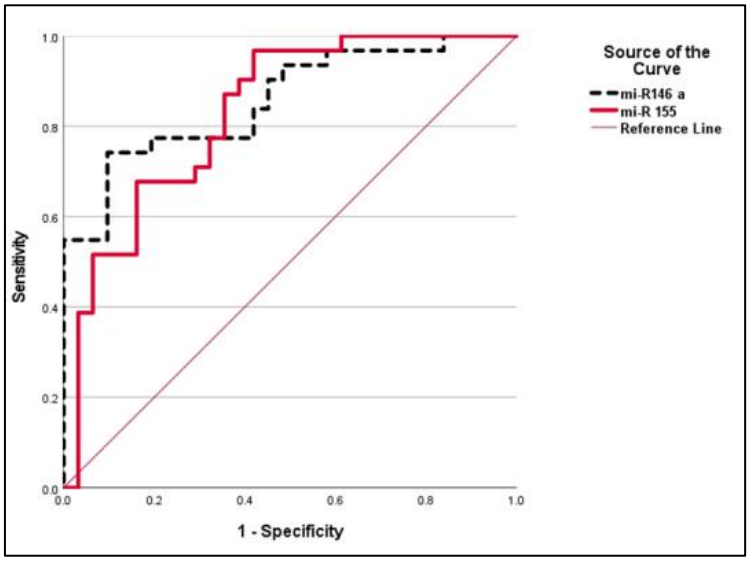

Fig. 1: ROC curve showing the performance of miRNA-146a and miRNA-155 in prediction of RA.

Studying the relationship between RA disease activity and different clinical and laboratory parameters in RA patients revealed statistically significant relations among active RA and the number of tender joints (P =0.002), DAS-28(P<0.001), ESR (P=0.03), CRP (P=0.02), and physician global health (P=0.002), miRNA-146a (P<0.001) and miRNA-155(P=0.009) as illustrated in table 2.

Table 2: The association between RA disease activity and different clinical and laboratory parameters in RA group:

	Inactive	Active	Z	p
	Median (IQR)	Median (IQR)		
Female gender n(%)	8(100%)	20(87%)	Fisher¥	0.55
Smoking n(%)	0 (0%)	3(13%)	Fisher¥	0.55
Comorbidity n(%)	3(37.5%)	16(69.6%)	Fisher¥	0.206
Morning stiffness [median(IQR]	15(0 – 26.5)	10(8 – 30)	0.939 §	0.348
NSG [median(IQR]	0(0 – 0)	0(0 – 2)	1.443 §	0.149
NTG [median(IQR]	1(0 – 2.5)	8(3 – 18)	3.107 §	**0.002***
DAS [Mean ± SD]	3.02 ± 1.28	4.88 ± 1.19	-3.674∞	**<0.001****
miRNA 146a [median(IQR]	2.09(1.75 – 3.42)	6.51(4.22 – 10.02)	3.367 §	**<0.001****
miRNA 155 [median(IQR]	2.04(2.0 – 2.66)	3.65(2.65 – 5.45)	2.578 §	**0.009***
CRP (mg/L) [median(IQR]	7.55(2.3–13.68)	16(12.1 – 28.7)	2.309 §	**0.02***
ESR (mm/hour) [median(IQR]	16(10.25 – 24)	27(19 – 43)	2.149 §	**0.03***
VAS pain [median(IQR]	60(42.5 – 60)	60(30 – 70)	-0.208 §	0.842
Patient global health [median(IQR)]	4.5(4 – 5)	6(3 – 7)	-1.084 §	0.278
Physician global health [median(IQR]	2(2 – 2.75)	4(3 – 4)	-3.139 §	**0.002***
Disease Duration [median(IQR]	8(5.75 – 11)	5(3 – 10)	-1.455 §	0.146
MHAQ [median(IQR]	0.75(0.36 – 0.86)	1(0.64 – 1.5)	1.822 §	0.068
RF (IU/mL) [median(IQR]	86(46.25–128)	34(9 – 239)	-0.975 §	0.329
Anti-CCP (EU/mL) [median(IQR]	31.5(20– 401)	11(6 – 373)	-1.655 §	0.098

¥Chi square test, ∞independent sample t test, §Mann Whitney test, **p≤0.001 is statistically highly significant, IQR: interquartile range, IU: international unit, EU: enzyme units

Serum levels of miRNA-146a and miRNA-155 expression were higher in active RA patients with a median (IQR) of 6.51 (4.22 – 10.02) folds and 3.65(2.65 – 5.45) folds respectively; versus 2.09 (1.75 – 3.42) folds and 2.04 (2.0 – 2.66) folds in the inactive group as represented in figure 2. Additionally, there was a significant positive correlation (P<0.05) among mi-RNAs 146a and155 expression levels and DAS-28 in RA patients as demonstrated in figure 3.

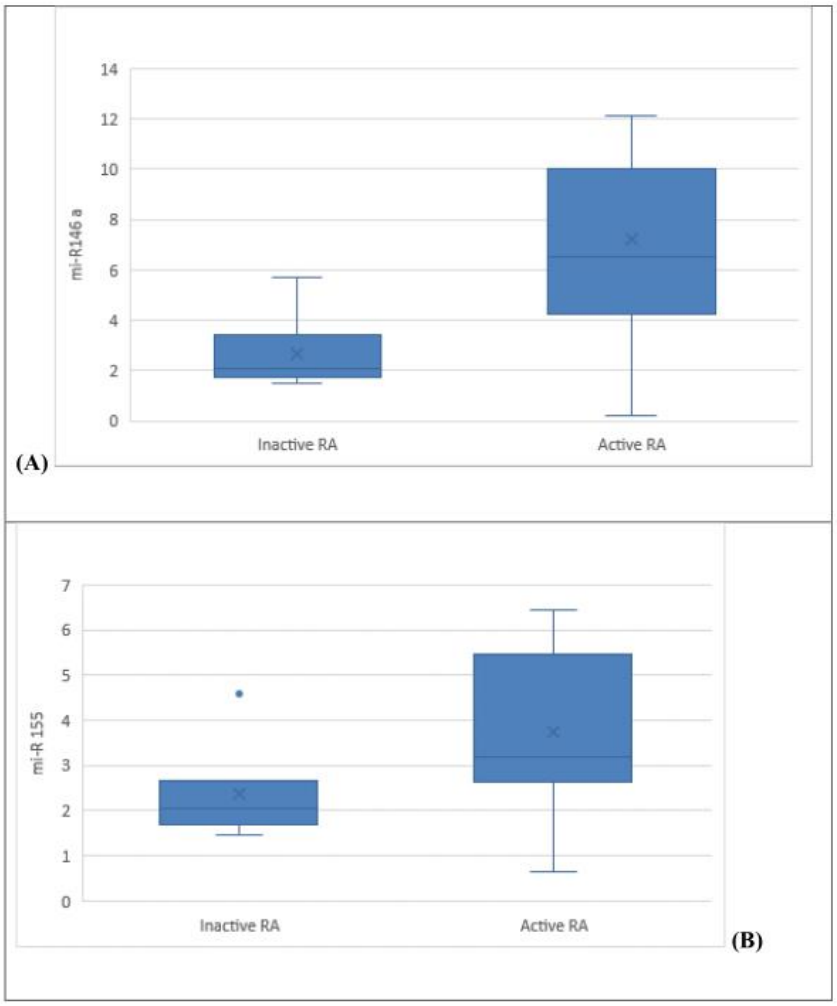

Fig. 2: levels of miRNA-146a (A) and miRNA-155 (B) in RA patients.

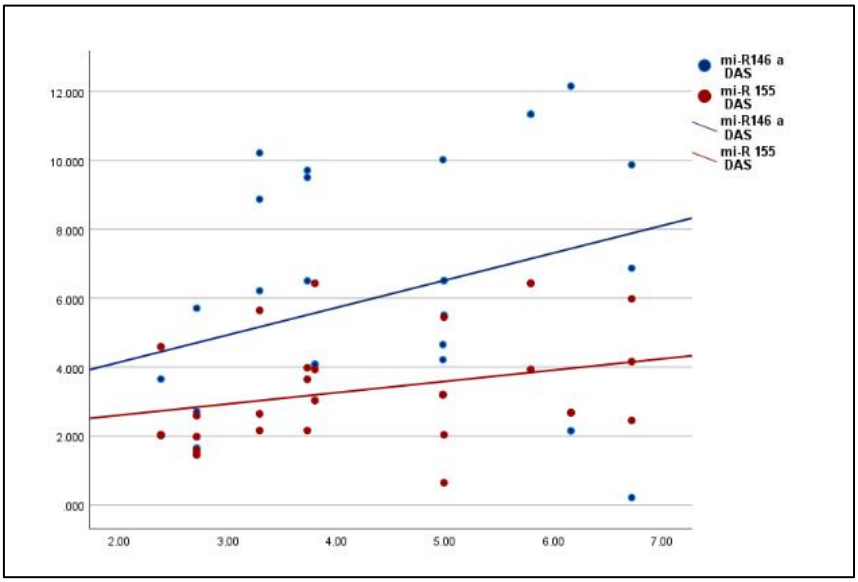

Fig. 3: Scatter dot showing significant positive correlation between DAS-28 and both miRNA-146a and miRNA-155.

On doing multivariate backward regression analysis of the factors associated with RA disease activity, micro-RNA 146 a was found to increase the risk of active RA significantly independently by 2.023 folds as shown in table (3). Furthermore, the ROC curve in figure 4 showed that the best cutoff of miRNA 146a in prediction of disease activity in RA patients was ≥3.8695 folds with area under curve 0.905, sensitivity 91.3% and specificity 87.5% (P<0.001) at 95% CI (0.791 – 1).

Table 3: Multivariate regression analysis to predict activity in RA patients:

	β	P	AOR	95% C.I.	
				Lower	Upper
miRNA146 a	0.705	0.015*	2.023	1.148	3.565

*p<0.05 is statistically significant, AOR: adjusted odds ratio, CI: confidence interval

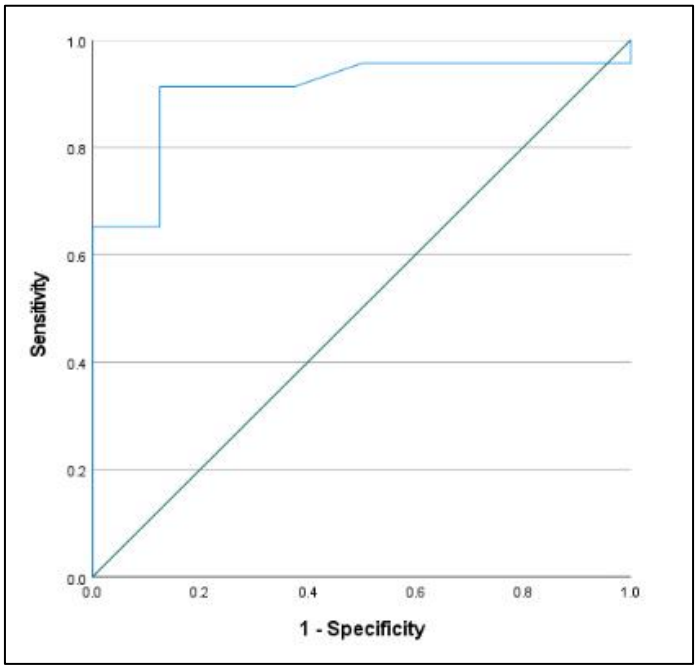

Fig. 4: ROC curve showing the performance of miRNA-146a in the prediction of disease activity in RA patients.

DISCUSSION

Rheumatoid arthritis represents a worldwide health problem that is characterized by chronic synovitis of variable clinical progression with subsequent joint deformities and permanent disability[14]. In the current work, we determined two distinct circulatory miRNAs (miRNA-155 and miRNA-146a), linked to immunological function, as putative biomarkers of RA. Previous research revealed that RA synovium and synovial fibroblasts had altered expression of miRNA-155 and miRNA-146a for the first time on 2008[15]. So far, miRNA-146 and miRNA-155 are the most well-known miRNAs linked to RA; they are both involved in controlling the NF-kB pathway, a critical mediator of inflammation[16]. Among the miRNAs in RA, miRNA-155 is the most adaptable. It can function as a biomarker and exert strong regulatory control over distinct miRNAs in various cells[17]. Furthermore, contemporary methods such as microarray, small RNA sequencing, in situ hybridization, quantitative reverse transcriptase PCR (qRT-PCR), and in situ hybridization facilitate the straightforward detection and precise quantification of miRNA expression. Thus, circulating miRNAs are appealing and encouraging biomarkers for clinical uses in RA, including support for prompt diagnosis, tracking the progression of the illness and forecasting treatment outcome[18].

In this study, when compared with controls, we discovered that the expressions of miRNA 146a and miRNA 155 were significantly higher in the serum of RA patients. This finding was in agreement with numerous research that estimate the expression of both miRNAs in synovial fluid, PBMC, synovium, synovioblast and specific cells in RA patients[19-24]. Filková and colleagues[25] discovered decreased levels of

miR-146a and miR-155 in the sera of individuals with early RA compared to those with chronic RA. Additionally, patients with RA and controls exhibited comparable values of these miRNAs, consistent with the findings published by Murata et al.[26]. Furthermore, Cunningham and his colleagues[16] demonstrated a significant increment in miRNA-146 in RA and arthralgia compared with controls and remarkably, there was no change in miRNA-155 levels, despite the fact that these levels were elevated in a variety of RA. Nevertheless, the contradictory hypothesis that serum miRNA-155 expression is similar in RA patients and controls was later put forth by Taha et al.[27]. The factors that influence the levels of circulating miRNA, both intrinsic and extrinsic, have not yet been thoroughly characterized. Disparities in circulating miRNA signatures may be caused by the study design, the sex, age, race, and lifestyle of the participants, as well as by the methods used for miRNA analysis[18].

Since aberrant miRNA expression manifests earlier than protein markers, it serves as a prognostic biomarker during the early stages of the progression of RA[24]. Our research revealed that the best cutoff of miRNA 146a in prediction of RA is ≥2.694 with area under curve 0.857, sensitivity 77.4% and specificity 80.6% (P<0.001). In support of this finding, Erfan et al[21] demonstrated that miRNA-146a can be accurate in the diagnosis of RA with an AUC of 0.836 at a cut-off value of ≥1.433 folds with a sensitivity and specificity of 82.5% and 100%, respectively. Consequently, it is able to differentiate between healthy controls and patients with early RA. Additionally, we determined that the best cutoff of miRNA 155 in prediction of RA is ≥2.1475 folds with area under curve 0.829, sensitivity 77.4% and specificity 67.7% (P<0.001). Given the high prevalence of proinflammatory stimuli in RA, it has been proposed that miRNA-155 plays a counter-regulatory role in regulating excessive inflammation by cooperatively downregulating multiple signal-transducing molecules[28].

In this work, we observed that there was a statistically significant relation between the disease activity and the number of tender joints, DAS, ESR, CRP, miRNA 146a, miRNA 155 and physician global health. Moreover, a statistically significant positive correlation between DAS28 and both miRNA 146a and miRNA 155 was recorded in the present study.

Interestingly, on doing multivariate backward regression analysis, miRNA 146a was found to significantly independently increase risk of active RA by 2.023 folds. We also recognized that the best cutoff of miRNA 146a in prediction of RA activity is ≥3.8695 folds with area under curve 0.905, sensitivity 91.3% and specificity 87.5% (P<0.001). Hence, we anticipated that miRNA 146a could be a suitable marker for disease activity in RA patients. In concordance with our results, there is a documented positive correlation between the expression of miR-146a and disease activity in RA[26, 7]. Consistent with this finding, Abou-Zeid and coworkers[29] reported that the level of miR-146a was positively correlated with the levels of ESR and DAS28.

However, some researches determined that the expression levels of both miRNA-146a and miRNA-155 were positively correlated with the clinicopathologic characteristics of RA activity[22, 30]. These miRNAs have an impact on bone homeostasis and cell differentiation in the synovial region through their involvement in inflammatory signaling pathways[31-32] , thus highlighting the severity and activity of the disease in RA patients[32-33].

On the other hand, Erfan et al[21] showed that the expression levels of miR-146a were not correlated with DAS28, ESR, or RF. Likewise, in both synovial fluid and peripheral blood, Li et al.[34] could not discover a correlation between the levels of miR-146a expression and disease activity indices. Furthermore, Inflammation and miR-146a did not significantly correlate, according to Andonian et al.[23] They speculate that when comparing the inflammatory signatures of the long-term RA patients in their cohort with those with early-stage acute inflammatory disease, their findings will reveal differences.

The advantage of utilizing a serum miRNA signature as a biomarker is its simplicity, reproducibility, and minimal invasiveness. A minimal amount of serum is needed to concurrently identify a panel of chosen miRNAs[16]. Serum miRNAs have been reported as potential biomarkers for a wide range of diseases because they are stable and may be elevated in the circulation as a result of their shedding from a disease site or, alternatively, they function as a means of communication between distal cells and tissues via the circulation[35-36]. One of the strengths of our study is evaluating the predictive value of miRNA 146a and miRNA 155 in both diagnosis and early detection of RA disease activity for better disease control and outcomes.

One of the limitations of this work is that we could not include the other miRNAs that are also associated with RA in our study. Furthermore, the relatively small sample size and the same ethnicity of the studied groups could be another limitation. It is highly advised to conduct further research on multicentric and multiethnic cohorts.

CONCLUSION

MiRNA-146a and miRNA-155 expressions were remarkable in RA patients and could be recognized as potential biomarkers for early RA diagnosis. However, miRNA- 146a expression is an independent risk for active RA and could be a valuable biomarker to predict RA disease activity.

Declarations:
Consent for publication: Not applicable
Availability of data and material: Data are available upon request.
Competing interests: The author(s) declare no potential conflicts of interest with respect to the research, authorship and/or publication of this article.
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