Iraqi Journal of Biotechnology, 2026, Vol. 25, No. 1, 128-138

Study The Role of *miRNA-499* Gene Expression in Susceptibility of Rheumatoid Arthritis in a Sample of Patients from Baghdad Government

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Received: October 1, 2024 / Accepted: November 10, 2024 / Published: March 30, 2026

Abstract

Background. Rheumatoid arthritis (RA)is an inflammatory autoimmune disease that progressively deteriorates the joints.*miRNA-499* is associated with an increased risk of autoimmune disease and may influence susceptibility of rheumatoid arthritis .*MicroRNA-499* targets IL-17 and is a critical component in RA progression. **Aim**. To investigate the gene expression of *miRNA-499* in the susceptibility of Iraqi patients with Rheumatoid arthritis. **Methods.** The study involved two groups: one hundred RA patients identified clinically and confirmed by rheumatologists and laboratory testing and fifty healthy individuals form the control group. This study was carried out at the Institute of Genetic Engineering and Biotechnology. The time frame for this study has been extended from November 2023 to September 2024. **Results.** The expression level of *miRNA-499* was found to be significantly higher in rheumatoid arthritis patients than in the control group. The mean expression value in RA patients reached 6.276 ± 1.42, whereas the control individuals exhibited a mean expression level of 1.00 ± 0.00.This significant difference indicates a strong association between *miRNA-499* expression and RA. **Conclusion.** The high gene expression levels of *microRNA-499* could be Promising options for biomarker in diagnosis and treatment applications of RA.

Keywords: *miRNA-499*, gene expression, rheumatoid arthritis

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Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease characterized by continuous synovial inflammation and the degradation of bones and cartilage, eventually leading to joint loss. (1). Initially, this disease affects just one joint, but as the disease progresses, it is characteristic by extra-articular involvement (2). Therefore, it is very important to detect the disease at an early stage (3). Ages (40–60)years old are the main age group affected with RA (4). Inflammation of the synovium, the tissue that lines the joint is the most common cause of joint stiffness. the synovium has an extensive development called the pannus that passes over and under the articular cartilage and contains pro-inflammatory cytokines, which cause an overproduction of synovial fluid (5,6). The pain will be the final result of this inflammation and swelling in the joints,

which will lead to life-limiting consequences (7).

The etiology of RA remains unknown (8), even though significant progress made in recent years. Genetic changes in several genes/loci, which may cause functional and biological variances, have been associated with RA. *MiRNAs*, formed by longer nuclear transcripts, play a role in RNA interference by regulating gene expression after transcription (9). A single miRNA may bind to hundreds of mRNA targets, potentially regulating nearly every biological function. The *miRNA-499* gene has a location on chromosome 20 and regulates the expression of its target genes, which include IL-6, IL-2, IL-17RB, IL-21, and IL-23a. The liver produces CRP and fibrinogen in response to the IL-6. IL-17RB, IL-23a, IL-2Rb, IL-2, IL-6, and IL-18R are critical to the progression of rheumatoid arthritis (10).

Consequently, *mir-499* has the capacity to influence the synthesis of CRP and the occurrence of inflammation in RA. The PADI4 gene is a significant target of *miRNA-499* since it encodes the PADI4 enzyme, which promotes the synthesis of citrullinated peptides identified by anti-CCP. These peptides through post-translate, altering self-antigens and increasing the probability of self-tolerance breaking (11).

This research estimates the role of *miRNA-499* gene expression in susceptibility of RA and examines age, Body Mass Index (BMI), ESR, CDAI scores, disease duration, Rheumatic factor (RF), CRP in rheumatoid arthritis patients and its correlation with *miRNA-499* gene expression.

Materials and Methods

The work was carried out The Institute of Genetic Engineering and Biotechnology at the University of Baghdad. The study's samples were taken at Department of Chronic Arthritis Diseases, Baghdad Teaching Hospital. The time frame for this study has been extended from November 2023 to September 2024.

The research studied 150 blood samples, including 100 samples from patients with rheumatoid arthritis and 50 samples from normal healthy controls without rheumatoid arthritis or any other medical disorders. All the patients were diagnosed by the consultant rheumatologists. Patients completed questionnaires on their sex, age, height, weight, CDAI scores, duration of disease and the correlation with other diseases. The ESR was detected by using a 9x120mm ESR vacuum tube to collect venous blood according to standard specifications. For direct detection of RF /CRP and it correlated with RA, a rapid agglutination procedure RA The RF/CRP Latex kits was used. RNA extraction was performed using the TransZol Up Plus RNA Kit. The concentration and purity of RNA were measured using a Nanodrop-2000 spectrophotometer (NanoDrop™ 2000 /2000c Spectrophotometers, USA). The RNA purity valuated by measuring the absorbance of the sample at 260 and 280 nm. Total RNA was reverse-transcribed to complementary DNA (cDNA) with the EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix Kit. particular primers were utilized each gene. Alpha DNA Company provided all primers as

lyophilized products in various picomole concentrations. The primer sequences are represented in Table 1. The levels of expression of the *miRNA-499* gene were performed by the qRT-PCR SYBR Green test using Q Real-time PCR System, to estimate the expression of the target gene.

he Endogenous control gene *U6* (Table 1) mRNA levels were amplified and used to normalize the *miRNA-499* gene expression. Use of the Statistical Analysis System-SAS (2018) program was done to determine the impact of both groups (control and patients) on study parameters. (12).

Table (1): Designed primers for the study

Primer	Sequence (5′→3′ direction)	primer size bp	Product size bp	Tm °C	References
(miRNA Gene Expression)					
miRNA-499	TTAAGACTTGCAGTGATGTTT	21	79	56	Alpha DNA company
miRU6 F.P.	AGAGAAGATTAGCATGGCCCCT	22	80		
miRNA-universe R.P.	GCGAGCACAGAATTAATACGAC	22	-		
Universal R. transcription p	GAGGTCCAGTTTTTTTTTTTTT TVN	26	-	-	

Results

Study samples distribution

The distribution was based on age, sex, BMI, CDAI, Duration, ESR, other diseases, RF, and CRP in patients and control groups. Table 2 shows the Sample distribution by sex in control and patient groups. In women, the percentage of RA has significantly increased. (82%) as compared with men (18%) with (P-value= 0.0001). Table 3 presents a comparison of the ages of RA patients and the control group. Table 4 showed the mean of BMI observations in patients was (30.99 ±0.84), while the control group's mean was (25.82 ±0.56). Results of the current study indicate a highly significant correlation between the two groups (P≤0.01). the distribution of patients groups according to CDAI the results showed that CDAI in RA patients was 18.75 ±0.84. While this study found that the mean duration of the disease was (10.16 ±0.79) years. The ESR values of the patient group were significantly higher (P≤0.01) than those of the control group. as shown in Table 5. Table 6 showed the correlation between RA patients and other diseases, none of the healthy controls in this research had diseases. RA patients had 25% hypertension and 16% diabetes. Additionally, 8% of RA patients had diabetes and hypertension. 3 %of RA patients had thyroid dysfunction. Significant results with a p-value (0.0001). Table 7 shows RF and CRP distribution in control and patient groups.

Table (2): Distribution of the sample study by sex in the control and patient groups

Factor		Patients	Control
Sex	men	18 (18.00%)	18 (36.00%)
	woman	82 (82.00%)	32 (64.00%)
	P-value	0.0001 **	0.0477
** (P≤0.01).			

Table (3): age distribution of the sample study in the control group and patient group

Factors		Patients No (%)	Control No (%)
Age (year)	Mean ±SE	47.62 ±1.17	37.88 ±1.43
** (P≤0.01).			

Table (4): Distribution the sample study according to BMI, CDAI, and Duration in patients and control groups

Factors		Patients	Control
BMI (kg/m^2)	Mean ±SE	30.99 ±0.84	25.82 ±0.56
	P-value	0.0001 **	
CDAI	Mean ±SE	18.75 ±0.84	-
Duration (year)	Mean ±SE	10.16 ±0.79	-
** (P≤0.01), CDAI: Clinical Disease Activity Index			

Table (5): Comparison between the patient and control groups based on the ESR values

Group	Mean ±SE of ESR (mm/hr.)
Patients	38.93 ±2.52
Control	13.00 ±0.83
T-test	7.152 **
P-value	0.0001
* (P≤0.05)	

Table (6): The correlation between RA patients and other diseases

Factors		No	%	P-value
Others disease	Hypertension	25	25.00%	0.0001 **
	hypertension + Diabetes	8	8.00%	
	Diabetes	16	16.00%	
	thyroid disease	3	3.00%	
** (P≤0.01).				

Table (7): Study sample distribution by RF and CRP in both the Patients and control groups

Factors		Patients No (%)	Control No (%)
RF	Positive (+ve)	53 (53.00%)	2 (4.00%)
	Negative (-ve)	47 (47.00%)	48 (96.00%)
	P-value	0.548 NS	0.0001 **
CRP	Positive (+ve)	47 (47.00%)	0 (0.00%)
	Negative (-ve)	53 (53.00%)	50 (100%)
	P-value	0.548 NS	0.0001 **
** (P≤0.01), NS: Non-Significant.			

Molecular study

Comparison *miRNA-499 gene* between study groups

As a result, in Table 8, *miRNA-499* showed higher expression in patients compared to the control group with great statistical significance (6.276±1.42 *vs.* 1.00±0.00; p ≤0.01).

The association of *miRNA-499* gene expression with some parameters

Table 9 found the association expression of miRNA-499 with the parameters study of the patients group. Based on statistical analysis, there is no correlation between of ESR, CDAI, BMI, Age, and Duration of Disease with *miRNA-499* gene expression.

Table (8): Comparison *miRNA-499 gene* between study groups

Group	CT (miR499)	HKG (U6)	ΔCt	$2^{-}\Delta Ct$	Experimental group/ Control group	Fold change
Patients	32.34	20.23	12.110	0.00284	0.00284/0.000454	6.276 ±1.42
Control	31.53	20.42	11.106	0.000454	0.000454/0.000454	1.00 ±0.00
T-test (P-value)	--		--	--	--	1.272 ** (0.0001)
** (P≤ 0.01).						

Table (9): Correlation coefficient between fold change of miRNA-499 with parameters study

Parameters	Correlation coefficient-r with Fold change	P-value
ESR	-0.07 NS	0.507
CDAI	-0.09 NS	0.351
BMI	-0.02 NS	0.853
Age	0.11 NS	0.258
Duration	-0.13 NS	0.207
NS: Non-Significant.		

Discussion

This result about sex agreed with (13). that shows Women are impacted by rheumatoid arthritis (2-4) times more frequently than men. Sex hormones contribute significantly to gender differences. Androgen levels inversely correspond the severity of RA, which also might explain why the disease is less severe in men (14). Lesuis *et al*., (15), found that women have a greater value of chronic pain, disabilities, disease activity, and pain than males. The study is supported by the study of Gorial *et al*. (16), which found that the mean age of the patients was (47.88±10.68 years).

Stress, long-term environmental antigen exposure, and other factors suppress the immune system (17). Cells are subjected to injury by an act of life. The incidence of RA increases with age, despite the fact that it may develop at a young age. It has been proposed that RA may arise because of the immune system's premature aging (immunosenescence). The result about BMI is in agreement with the Iraqi study done by

Ismail and Alaaraji (18), which showed the mean BMI of RA patients was significantly greater (P= 0.0079) in comparison to healthy controls. People often view obesity as a systemic inflammatory condition, marked by elevated levels of inflammatory cytokines like IL-6 and tumor necrosis factor-alpha (19). These cytokines cause inflammation have the potential to enhance individuals' inflammatory responses. The distribution of the patient group according to CDAI, was 18.75 ±0.84. The CDAI is useful for assessing the disease activity in patients with RA since it demonstrates high sensitivity, high specificity, and a favorable predictive likelihood ratio. and response to treatment (20). The mean duration of the disease is comparable to the study by Younis *et al.* (21); the mean showed (11.19±7.55) years. In research conducted by Hassan *et al*. (22), the mean value was (6.31 ± 0.48) years, which may be due to the nature of life and the associated chronic disease. Similar results were found by Khadim and Al-Fartusie (23). ESR is an essential part of several rheumatic criteria for disease activity and progression assessment and laboratory activity indicator for therapy (24). According to Tishkowski and Gupt (25), an increase in fibrinogen levels may be the cause of a higher ESR in RA, resulting in red blood cell stickiness and an acceleration of the sedimentation process.

The results show a similarity to the study conducted by Bawazir, (26), Who reported that hypertension and diabetes are the most prevalent diseases among patients with RA in their research. In patients with Type-2 diabetes, inflammatory mediators, including CRP, (IL-6), and (TNF)-a are often found at high levels. The same mediators are also found in the blood of patients with RA long before the disease becomes clinically apparent.

This suggests that these mediators play an essential role in the progress of RA (27). Nazary *et al*. (28) performed a comparison of thyroid function between 400 rheumatoid arthritis patients and a control group.The research observed greater thyroid dysfunction in RA patients. Mahagna *et al.* (29), determined RA patients had a comparatively less risk of developing hyperthyroidism.

The current results agree with Mun *et al*. (30) results, which showed a high level of statistical significance of RF when comparing patients to the control group. RF may have been found in rheumatic and non-rheumatic patients and healthy people (31). RF positive has been observed in healthy individuals. It is believed that genetic and environmental factors contribute to the global differences in the distribution of RF. The RF identified in healthy individuals differ from those observed in RA patients, as their titres are low to moderate. RF can occasionally be detected in healthy older adults, indicating that they might result from the immune system's age-related changes (32,33).

Seronegative rheumatoid arthritis is less common than seropositive arthritis due to a variety of factors. RF may not be present in the early stages of RA, immune cells may need time to produce antibodies, and certain individuals produce anti-citrullinated protein antibodies (ACPAs), which are associated with RA, instead of RF (34,35) This factor serves as a titer, indicating the depth of medication response rather than

predicting the duration or severity of the disease (36).Also, the current study agrees with the Morovic-Vergles *et al.* (37), who showed RA patients had greater CRP values, indicating more inflammation. CRP is widely available, easily performed, and inexpensive, making it the preferred biomarker to measure the activity of RA (38).

The molecular results support the Iraqi study conducted by Jumaah *et al.* (39) which showed that the study found that miRNA-499 gene had higher levels of expression in patients compared to the control group. This suggests that *miRNA-499* could prove useful as a promising therapeutic target for the disease. Ayeldeen *et al.* (40), found similar findings, concluding that *miRNA-499* gene is highly upregulated in patients with RA in comparison to healthy individuals. This *miRNA-499* gene could be helpful as a diagnostic marker. Overexpression of *miRNA-499*gene is linked to inflammatory reactions. It may promote inflammation in RA-affected joints by upregulating pro-inflammatory signaling pathways and cytokines. A disruption in gene expression patterns may result from elevated levels of the *miRNA-499* gene in RA, possibly impacting the expression of genes that play a role in inflammation and immune responses, and other processes associated with the development of RA.

Several variables, such as the individual's genetic background, disease stage, and the presence of other *miRNAs* or regulatory molecules, may modify the impact of *miRNA-499* overexpression in RA. *miRNA-499* can control the formation of anti-CCP antibodies by regulating the expression of the *PADI4* gene. (41). RA may now be diagnosed using standard criteria, although many patients present with undifferentiated arthritis at initial presentation due to an absence of biomarkers, which is difficult to find. The increasing amount of information about the involvement of miRNAs in RA and their abnormal expression in the inflamed synovium and circulation of RA patients (42). suggests that they might be used as novel RA molecular diagnostic markers. An Iraqi study by Jumaah *et al.* (39) found a positive correlation between IL-17 and age with *miRNA-499* gene expression, which is in contrast to this result. and a negative relationship with the duration of the disease, CDAI, ESR, and ACCP. Increased production of miRNA-*499* may disrupt the balance between regulatory T cell (T regs) and effector T cell, leading to decreased immunological tolerance and the continuation of autoimmunity. A Study has revealed the atypical expression of *miRNAs* in individuals with RA, with a focus on T cell differentiation (Th17), the production of inflammatory cytokines, and B cell activation. Moreover, several *miRNAs* show various expression patterns throughout the disease course, facilitating the identification

During the development of chronic inflammatory diseases and long-term exposure to environmental antigens According to Chalan *et al*. (44), the immune system undergoes immunosenescence as a result of the alteration of its innate and adaptive components with age. The expression of *miRNA* may be influenced by the immunological environment rather than RA itself. This was seen while examining the absence of consistent findings about

differential *miRNA* expression across various populations (45). These data validate the pleiotropic impact of some *miRNAs*, which varies depending on the specific cells or tissues in which they are found.

Conclusion

The overexpression of *miRNA-499* may have an essential purpose in developing rheumatoid arthritis Furthermore, It has the ability to serve as a diagnostic marker.

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