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Determination of miRNA -34a Expression in Rheumatoid Arthritis

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ABSTRACT

Background: MicroRNAs are gene expression regulators, associated with several human pathologies. Rheumatoid arthritis (RA) is an autoimmune systemic disease characterised by symmetrical inflammatory polyarthritis. Rheumatoid arthritis mainly impacts smaller joints, although it can also affect large joints.

Objectives: To evaluate the expression profile of miRNA-34a and the serum levels of erythrocytes sedimentation rate (ESR), rheumatoid factor (RF), C-reactive protein (CRP), anticyclic cetrolinated peptide (Anti-CCP) in both patients and control group and to find their association with disease progression in RA patients and evaluate their significance as novel markers for RA.

Subjects and methods: This study included 100 female patients and 100 female healthy controls with mean ages of controls and patients (37.44±23.10 and 49.74±9.775 respectively). ESR, serum CRP, RF and Anti-CCP were investigated. We examined the expression of micro ribonucleic acid 34a (miRNA-34a) in patients with RA and controls by using quantitative time polymerase chain reaction (qRT-PCR). In addition, receiver operating characteristic (ROC) was constructed for RA patients to establish the diagnostic accuracy of miRNA-34a in differentiating RA patients from control people.

Results: ESR, serum CRP, RF and Anti-CCP were 37.44±23.10 mm/h, 22.72±1.44 IU/ml, 16.56±0.842 IU/ml and 28.09±6.68 EU/ml, respectively. The findings revealed a significant decrease in micro ribonucleic acid-34a (miRNA-34a) expression with fold change (0.312). Regarding the ROC curve, specificity and sensitivity of miRNA-34a were 62% and 61.62%, respectively. The area under curve (AUC) was 0.630 and cut-off point was 9.79.

Conclusion: Expression profile of miRNA-34a can be used as novel markers for RA.

Keywords: Non-coding sRNA, Autoimmune disease, Gene expression, Rheumatoid arthritis**.**

INTRODUCTION

Rheumatoid arthritis is an autoimmune systemic illness distinguished by persistent symmetrical polyarthritis of the large and small joints. Between 0.5 and 1 percent of people worldwide are affected by it, and women are 2.5 times more likely to have it than males, primarily between the ages of 30 and 50. Joint discomfort, stiffness, and swelling related to synovial inflammation, as well as effusion, are all symptoms of this condition [1,2,3].

Extra articular symptoms such as fever, tiredness, anemia, interstitial lung involvement, vasculitis, nodules, and osteoporosis may develop, as well as acute-phase reactants increased, such as ESR and CRP [4]. Previous research has suggested that genetic factors, environmental variables, and the immune system all contribute [5–9]. An accurate diagnosis at an early stage is essential for successful treatment, especially in patients' characteristics that have a poor prognosis (such as high disease activity or the presence of cancer, early joint degradation, and autoantibody presence) [10].

The most difficult problem remains is identifying biomarkers to enable early diagnosis, specifically for individuals who cannot acquire a definitive diagnosis at first and are diagnosed with undifferentiated arthritis as their primary diagnosis [11]. MicroRNAs (miRNAs) are irregularly expressed in both of the inflamed synovium and the circulatory system of RA patients, according to mounting evidence [12]. The role of miRNAs in rheumatoid arthritis may make them appropriate as new molecular diagnostic markers for rheumatoid arthritis [13]. Non-coding small RNA molecules known as (miRNAs) are assumed to play a role in regulation of genes post-transcriptionally. MiRNAs attach to complementary-sequence messenger RNAs (mRNAs) and prevent them from translating, preventing the synthesis of certain proteins [14,15].

In individuals with autoimmune disorders, miRNA expression appears to be altered in a variety of cells and bodily compartments, in addition to controlling normal cellular function [11,16]. Enhanced inflammatory pathway signalling, elevated synthesis of pro-inflammatory cytokines, and other processes that contribute to the autoimmune death spiral have all been related to altered miRNA expression [17]. In addition, miRNAs are a component of the complex human gene transcription and translation regulatory mechanism, which can function individually or in groups, have multiple targets, and can target one gene with several miRNAs. miRNAs in clusters are more effective at regulating complicated processes [18]. miRNA-34a has been identified as a cell death regulator with conflicting activities as an apoptosis inducer or defender. A number of studies have indicated the abnormal regulation of miRNAs in RA-affected joints with inflammatory conditions [19].

The purpose of this study was to shed more light on the topic by broadening our knowledge of the

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association between miRNA-34a and rheumatoid arthritis in a sample of Iraqi patients.

SUBJECTS AND METHODS

Patients and controls

One hundred RA patients were involved in the study with ages ranging from 22 to 65 years years old, were obtained from Iraq/ Baghdad City (Medical City, Baghdad Hospital) and diagnosed as RA patients on the basis of (ESR, CRP, RF and Anti- CCP) tests. Additionally, in this study 100 female healthy controls were obtained from National blood transfusion center, with ages ranging from 20 to 55 years old.

Blood collection

Five millilitre of study samples were collected from each of patients and control and the drawn blood were placed into tubes contain 1 ml lysis buffer for genetic analysis and stored at -20 °C for further steps.

Extraction and analysis of miRNA

With the EasyPure® Blood Genomic miRNA Kit, miRNA was isolated from both patient and healthy control whole blood (Transgen, China).

The spectrophotometer Nano drop (Q5000)(Quawell,China) was used to quantify and evaluate the purity of miRNA in samples (UV-VIS). The acceptable purity range for miRNA is between 1.7 and 2.

Primer design and its preparation for miRNA-34a gene expression

The NCBI Gen Bank database was used to obtain the cDNA sequences of the miRNA-34a gene, as well as miRNA-*U6* as a housekeeping gene.

The primers lyophilized were dissolved in nuclease-free water as per the assembly specifications to create a stock solution with a concentration of (100 µM) for each primer.

This stock solution was then frozen at -20°C to create a primer working solution by dilution of 10 µL of primer standard solutions with 90 µL of nuclease-free water, resulting in a 10 µM working solution. Table 1 summarizes the sequences of the primers 1414tilized in this study.

Table (1): The primers used in the study

Primers of miRNA	Sequence (5′→3′ direction)
miRNA-34a F.P	GCAGTGGCAGTGTCTTAG
miRNA-34a R.P	GGTCCAGTTTTTTTTTTTTTTACAAC
Universe miRNA reverse	GTGCAGGGTCCGAGGT
miRU6 F.P.	AGAGAAGATTAGCATGGCCCCT
miRNA-universe R.P.	GCGAGCACAGAATTAATACGAC

Expression of miRNA-34a

Synthesis of cDNA from miRNA

The Transgen, China's EasyScript® Step removal gDNA and Synthesis cDNA SuperMix technique was used. The synthesis cDNA was employed to evaluate the miRNA-34a expression levels. Reverse transcription reactions must be carried out in an environment free of RNase. All reagents were thawed, as well as the miRNA templates, and each solution was gently blended. The component of the reaction was shown in table 2.

Table 2. Reverse transcription reaction components and reaction volume utilized to create cDNA from miRNA

Components	Volume (µl)
Total miRNA	5
Anchored Oligo(dT)18 Primer(0.5µg/µl)	1
Random Primer(0.1µg/µl)	1
2×EX Reaction Mix	10
*Easy Script®*RT/RI Enzyme Mix	1
gDNA Remover	1
RNase-free Water	1
Total volume	20

In accordance with table 3, tubes were set up in a thermal cycler program. Synthesized cDNA was employed directly as a PCR template or for long-term preservation at -20°C.

Table 3: The conditions for cDNA reverse transcription in a thermal cycler steps

	Step1	Step2	Step3
Temperature/ °c	25	42	85
Time	10min	15min	5seconds
	Random Primer (N9)	Anchored Oligo(dT)18	Inactivate reverse transcriptase enzyme

Quantitative real time PCR (qRT–PCR):

The qRT-PCR was used to estimate the miRNA expression levels. Quantitative time qRT-PCR SYBR Green assay (TransStart® Top Green qPCR SuperMix for gene expression) was employed to validate the expression of the target miRNA. This procedure was performed in accordance with the manufacturer's instructions in a reaction volume of 20 µl. (Transgen, China). The Cephied Real-time PCR System (Smart Cycler Technologies) and qPCR Soft software were used to carry out qRT-PCR by determining the threshold cycle using the 2xqPCR Master Mix Kit's components, levels of gene expression and the change fold were measured. Each reaction was carried out in a duplicate. Table 4 determines the volume of each component that was required.

Table 4. Quantitative real-time PCR components utilized in a gene expression experiment

Component	Volume (µl)
Master mix Syper Green	10
Forward primer	1
Revers primer	1
CDNA	3
Nuclease free water (N.F.W)	5
Total volume	20

The thermal profile, which is displayed in table 5, was used to program the cycling protocol for the following optimum cycles.

Table 5. The profile of gene expression temperature

Stages	Processes	Temperature	Time	Cycle
Stage 1	Denaturation	94°C	30 sec	1
Stage 2	Denaturation	94°C	5 sec	40
	Annealing	56°C	10 sec	
	Extension	72°C	20 sec	
Stage 3	Melting curve	65-95 °C	1 min	1

The real-time cycler program was used to calculate the threshold cycle (Ct) for each sample. A housekeeping gene was used to standardize the expression data of chosen genes.

For data analysis, the ΔΔCt method by **Kenneth and Thomas** [20] was employed, and the results were presented as folding change in gene expression as mentioned in the study of **Aljoubory and Altaee** [21], where the difference in Ct values (ΔCt) between each target gene and the housekeeping gene was computed for each sample.

Ethical considerations:

The local Ethics Commission (CSEC/0122/0001) approved and received written informed consent from all of the participants. Under the direction of medical professionals at (City of Medical, Baghdad Hospital), the University of Baghdad group conducted the study in Baghdad, Iraq.

This work has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans.

Statistical analysis

The Statistical Package for the Social Sciences (SPSS), version 20.0. (IBM, Chicago, IL), was used for statistical analysis. Data were presented as mean and standard deviation and were compared by the student's t-test. A receiver operating characteristic (ROC) curve analysis was used to determine the best cut-off point of miRNA-34a for predicting RA. The accuracy of the test was measured using the area under the curve (AUC). $P<0.05$ was regarded as statistically significant.

RESULTS

Baseline characteristics of rheumatoid arthritis patients and healthy control

Totally 100 RA female patients with the mean age of 49.74±9.775 years and 100 female healthy controls with mean age of 37.44±23.10 were recruited in the study. The patients' demographics and clinical characteristics are summarized in the table 6.

Table 6. Baseline rheumatoid arthritis patients and healthy control characteristics

Parameters	(mean± SD)		*p*- value	Normal value
	Control No.=100	Patients No.=100		
Ages(years)	37.44±23.10	49.74±9.775	p≤0.0001**	-
ESR mm/h	-	37.44±23.10	-	0-20
RF IU/ml	-	22.72±1.44	-	<15
CRP IU/ml	-	16.56±0.842	-	<15
Anti-CCP EU/ml	-	28.09±6.68	-	<20

****: significant**

Quantitative Expression of miRNA-34a

A real-time PCR quantification was done to measure miRNA-34a expression. The (**miRNA-U6** *gene*) was used as a housekeeping gene to normalize the gene expression, and the ΔCt value and folding ($2^{-\Delta\Delta Ct}$) method were used to the gene expression quantify. In figures 1 and 2, a typical RT-qPCR plot was shown.

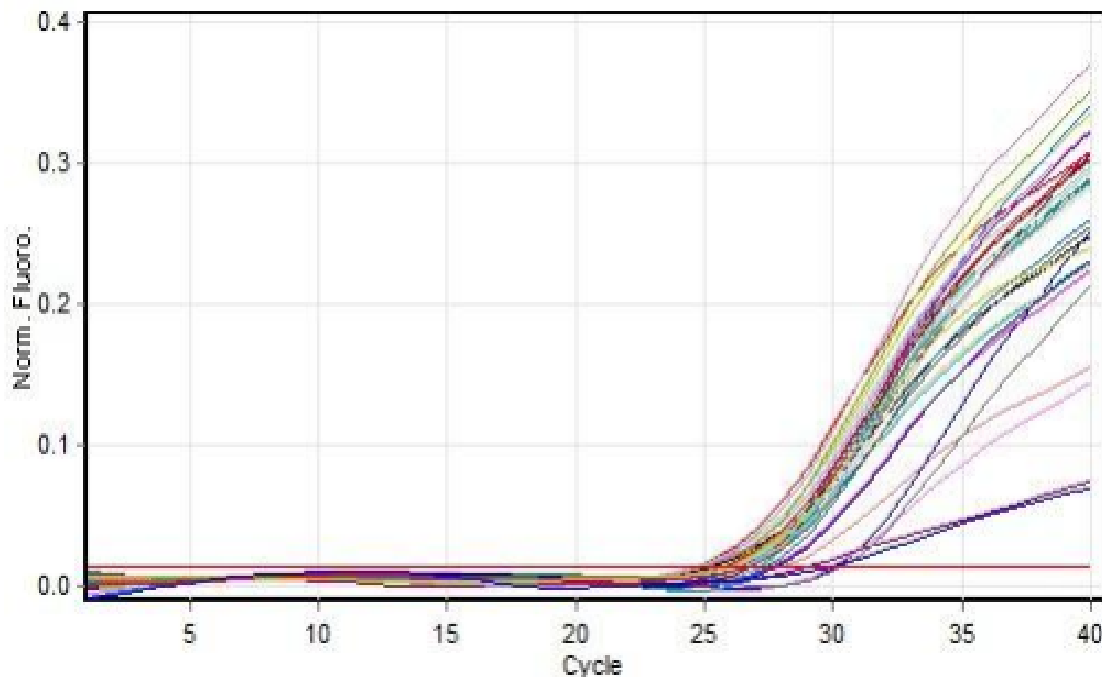

Figure 1: RT-qPCR amplification plots of the miRNA-34a gene

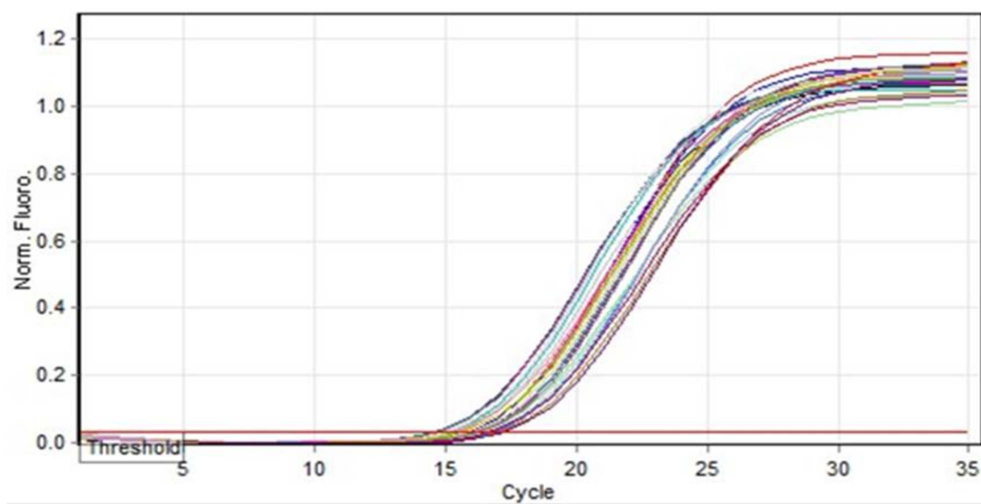

Figure 2: RT-qPCR amplification plots of the miRNA-U6 gene

Figure 3 displays a typical melt curve for the *miRNA* gene for samples subjected to RT-PCR analysis. A single peak was detected for the amplicons. These results can be explained by the fact that the melt curve indicated a single, pure amplicon for each sample, and it was believed that the intercalating dye assay had great specificity for amplification.

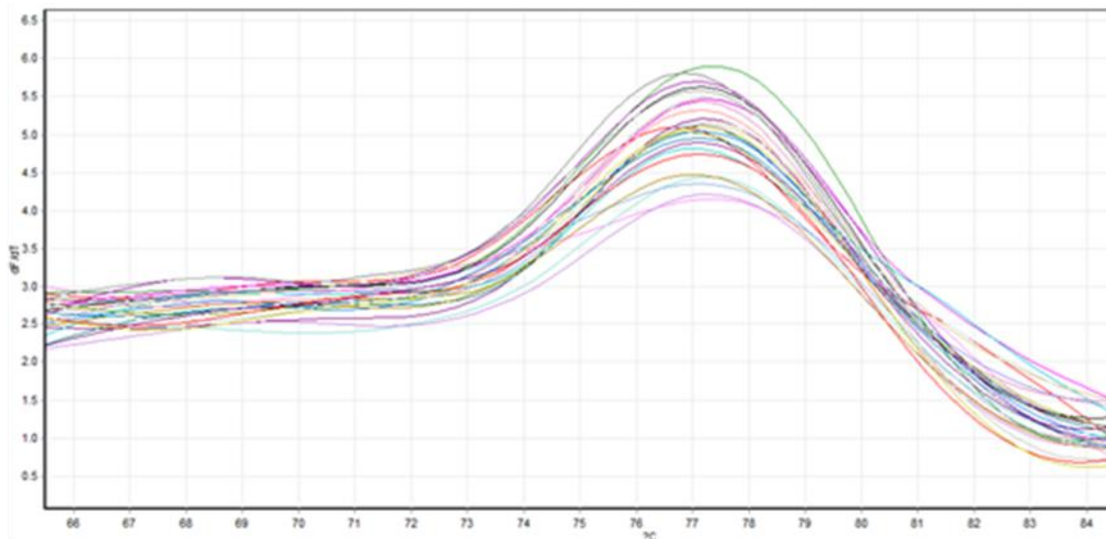

Figure 3. After RT-qPCR analysis, the amplicons of the miRNA-34a gene exhibit single peaks on the melting curve

The ΔCt mean of *miRNA-34a* gene was increased in RA patients when compared to the ΔCt means in controls, but the difference was not significant, table 7. Furthermore, the relative expression ($2^{-\Delta\Delta Ct}$) of *mi*RNA-34a was reduced by 0.312 folds in RA patients,

Table 7. Fold of miRNA miRNA32a expression Depending on 2-ΔCtMethod

Groups	Means Ct of *miRNA32a*	Means Ct of miRU6	ΔCt (Means Ct of *miRNA32a* - Means Ct of miRU6)	$2^{-\Delta Ct}$	experimental group/ Control group	Fold of gene expression
Group 1 Patient	26.87	15.98	10.890	0.000527	0.000527/0.001689	0.312
Group 2 control healthy	25.07	15.86	9.210	0.001689	0.001689/0.001689	1.000

The characteristic Receiver operating (ROC) curve

Analyses of receiver operating characteristic curves were carried out to determine the diagnostic accuracy of miRNA-34a in distinguishing RA patients from control participants. The analysis revealed that miRNA-34a at the appropriate cut-off value was 9.97, which allowed for a considerable difference between patients and controls (AUC = 0.630; 95 % CI = 0.552–0.708; $p < 0.001$) with a sensitivity and a specificity of 62% and 61.62 %, respectively as shown in figure 4. The Youden index was used to modify the cut-off point (YI = 0.38). This ROC curve was created to determine the predictive values of miRNA-34a expression as an acute phase marker in RA. The ROC curves were substantially above the midline, indicating excellent sensitivity and specificity.

Figure 4: Receiver operating characteristic (ROC) curve analysis of miRNA-34a for differentiating rheumatoid arthritis (RA) patients from healthy controls

DISSCUTION

In rheumatoid joints, the prolonged active inflammation typically causes irreversible damage of the articular cartilage and subchondral bone. ESR and CRP are regarded appropriate biochemical indicators for monitoring the disease activity of rheumatoid arthritis throughout its long term [22]. RF measurement is not specific for the diagnosis of rheumatoid arthritis, as 3 to 5 percent of healthy people have it, as do other autoimmune diseases and non-autoimmune disorders [23]. In our study, RA patients had high levels of ESR, CRP, RF, and Anti-CCP markers. These findings were consistent with **Serdaroğlu *et al.*** [24] and **Vanichapuntu *et al.*** [25].

They demonstrated that none of these biomarkers permitted for accurate monitoring of rheumatoid arthritis activity. Anti-CCP antibody assay's advent as a new serological indicator for rheumatoid arthritis facilitated early diagnosis and altered therapy recommendations [26]. In conjunction with RF, the anti-CCP test provides a more accurate early diagnosis of RA and has prognostic value. It was also mentioned that this biomarker was connected to radiographic damage in RA [27].

The outcome reflects the miRNAs found in the samples. And the patients' group is correlated with a reduced copy number of miRNAs, suggesting its lower expression in the study group (RA) compared to the control group. These findings demonstrate that miRNA-34a expression is reduced in patient group, implying that the miRNA-34a could be used as a biomarker for early diagnosis of rheumatoid arthritis. This result was comparable to that of **Niederer *et al.*** [19], who observed that synovial fibroblasts from RA patients had decreased miR-34a expression levels compared to osteoarthritis patients. Numerous research on miRNA-34a demonstrate that this miRNA is involved in a variety of cancers and autoimmune disorders. miRNA-34a is known to be dysregulated in patients with immune-related disorders such as inflammatory bowel disease (IBD), psoriasis, rheumatoid arthritis (RA), and others [28]. Numerous vital cellular processes, such as tumorigenesis, apoptosis, and proliferation, can be controlled by miRNA-34a. Prior studies has shown that several tumours had low levels of miRNA-34a, suggesting that this gene is an effective tumour suppressor [29].

CONCLUSION

This study concluded low expression of micrRNA 34a in rheumatoid arthritis patients, making it a useful biomarker for the condition.

Consent for Publication: I attest that all authors have agreed to submit the work.
Availability of data and material: Available
Competing interests: None
Conflict of interest: There aren't any overlapping interests between the authors.

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