Original Article

Estimate the Level of MiR-125a-5p and IL-23 in Rheumatoid Arthritis Patients

Noor Mahdi Dakhil, Suhad Hassan Aubaid, Raya Al-Saade, Sahar Adnan Shams Al-din

ABSTRACT

Objective: To evaluate the expression of miR-125a-5p, IL-23, and inflammatory markers in RA patients, assess DAS28 severity, and compare findings with healthy controls to support early diagnosis and treatment.

Study Design: Cross-sectional study

Place and Duration of Study: This study was conducted at the College of Health and Medical Technology, Middle Technical University, Baghdad Iraq from 1st May 2024 31st October 2024.

Methods: One hundred and fifty blood samples, 100 rheumatoid arthritis patients (53 females, 47 males) and 50 healthy controls (28 females, 22 males) were enrolled. RNA was extracted using Trisol; cDNA synthesized and analyzed by real-time PCR using SYBR Green. IL-23 was measured via ELISA. Anti-CCP, RF, and Hs-CRP were analyzed using Cobas e 411, Abbott, and Roche analyzers.

Results: miR-125a-5p expression significantly increased in RA patients, severe 11.16±7.28, moderate 6.34±1.29, mild 5.75±2.46 and control 1.0±0.98. IL-23 levels also increased with disease severity

Conclusion: Elevated miR-125a-5p and IL-23 levels are linked to RA progression and can aid in diagnosis and disease monitoring.

Key Words: Interleukin-23, MiR-125a-5p, Anti-ccp, RF, Hs-CRP, DAS28

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INTRODUCTION

Rheumatoid arthritis is a systemic, inflammatory/ autoimmune, polygenic disease affecting millions of people worldwide. Its etiopathology is attributed to a crosstalk between genetic predisposition, autoimmunity and environmental factors. This heterogeneous disorder is characterized by chronic synovitis and a fluctuating clinical course that may result in long-term disability and reduced quality of life in many patients.[1,2] According to the 2010 Classification Criteria of the American College of Rheumatology (ACR), rheumatoid factor (RF) and/or antibodies against cyclic citrullinated proteins (anti-CCP) is needed for classification.[3-4] Anti-CCP are highly specific for RA and are detected in 60%–70% of RA patients; RF is also present in nearly 70% of patients with RA.[5,6]

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Nowadays, diagnostic tests for RA are not sufficiently accurate, and leading to the late diagnosis of the patients. Therefore, new biomarkers need to be identified to provide a rapid, simple, with high sensitivity and specificity for the diagnosis of RA.[7,8]

MiR-125a-5p located in 19q13.41, known to play a regulatory role in various physiological and pathological processes, including inflammation and immune responses[9] is involved in modulating the production of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6, also IL-23 and IL-10 which are critical mediators of inflammation in RA 15. Its play a role in regulating the migration, proliferation and inflammatory responses of RA-synovial fibroblast, impacts the differentiation and functions of T cells, B cells, macrophages and influence the balance between Th17 cells and T cells (Tregs).[10]

IL-23 is a pro-inflammatory cytokine belonging to the IL-12 cytokine family. IL-23 is essential for the differentiation of Th17 lymphocytes, a subtype of T lymphocyte implicated in chronic inflammatory/ autoimmune mediated diseases, produced by mononuclear (dendritic cells and macrophages) cells in the synovial fluid of RA patients, promotes inflammatory responses by inducing IL-8 and IL-6 production from human fibroblast-like synoviocytes.[11] It plays a key role in both innate and adaptive immunity.[12] The ability of IL-23 to induce IL-17 provides a unique role in the development and the maintenance of autoimmune inflammation.[13]

METHODS

This cross-sectional study in Wasit Province included 150 blood samples (100 RA patients and 50 healthy controls) collected from 1st May 2024 31st October 2024. RA diagnosis was based on ACR criteria and confirmed by rheumatologists. Anti-CCP, RF, and CRP were measured using automated analyzers, IL-23 by ELISA, and miR-125a-5p expression by RT-qPCR normalized to U6**.** Autoimmune disease, malignancy, pregnancy and current infectious disease were included. All participants in this study were informed before collecting samples, and a verbal agreement was obtained from each of them. The subject data, permission form, and the study protocol were examined and approved by a local ethics committee.

Molecular detection of miRNA-221 was carried out according to Taq Man™ MicroRNA Assayfor 20 samples (5 mild, 5 moderate, 5 severe, and 5 control),

Total RNA was extracted from whole blood using the TRIzol® reagent kit following the manufacturer's instructions. Briefly, 500 µl TRIzol® was added to 250 µl blood, followed by 0.2 ml chloroform. After mixing and phase separation, samples were centrifuged, and the aqueous phase was transferred. RNA was precipitated with isopropanol, washed with 75% ethanol, air-dried, and dissolved in 50 µl of dissolving solution. RNA was stored at -70 °C and quantified using a Nanodrop spectrophotometer by measuring absorbance at 260/280 nm.

Amplifying of the has- miR125a-5p and housekeeping gene U6 were carried out using primers (Table 1). Primers have been diluted with 300 µl distilled water, then 10 µl from each primer added to 90 distal water to make concentration 100 in whole blood patients and control. These primers were provided by Macrogen Company, Korea:

MiRNA cDNA synthesis step for miRNA125a5p by using cDNA master mix was used. After that, these qPCR master mix component placed in qPCR premix standard plate tubes then the plate mixed by vortex centrifuge for 3 minutes, and placed in Real-Time PCR system. Thermocycler conditions convert RNA into cDNA (RT step) firstly the temperature 50°C for 1 hour and for heat inactivation 95°C for 5 min.

A RT Quantitative PCR master mix was prepared using the GoTaq® qPCR Master Mix (Promega), based on SYBR Green dye detection. The mix was dispensed into qPCR premix standard plate tubes, vortexed and centrifuged for 3 minutes, then loaded into the Real-Time PCR system. The thermocycling conditions were: initial denaturation at 95°C for 5 min (1 cycle), followed by 40 cycles of denaturation at 95°C for 20 sec, annealing at 56°C for 30 sec, extension at 72°C for 30 sec, and a final hold at 4°C.

The qPCR also used in quantification of housekeeping gene (U6) used in normalization of miRNA125-a-5p expression analysis. qPCR master mix was prepared according to kit .After that, these qPCR master mix component placed in qPCR premix standard plate tubes that contain the other qPCR SYBR green dyeamplification components, then the plate mixed by vortex centrifuge for 3minutes, then placed in Real-Time PCR system .

Statistical analysis was performed using SPSS-28.0. Anova and independent t-test were used to compare groups, and Pearson's correlation assessed relationships between variables. ROC analysis was used to determine cut-off values. DAS28 scores were interpreted based on EULAR and APLAR criteria: >5.1 indicates active disease, <3.2 indicates low activity, and <2.6 indicates minimal disease activity.

RESULTS

Table 2 revealed highly significant differences ($P<0.01$) between RA patients and healthy controls: Anti-CCP (43.8±19.7 vs. 8.09±1.94), Hs-CRP (15.9±5.2 vs. 0.91±0.33), ESR (45.79±17.7 vs. 12.06±1.84), and RF (27.0±11.1 vs. 5.2±1.67). Table 3 shows IL-23 levels significantly differed ($P<0.01$) among groups based on DAS28 severity: Control (107.8±23.6), Mild (129.8±10.8), Moderate (269.7±59.8), and Severe (751.5±94.7). Post hoc analysis indicated significant differences ($P<0.01$) between most groups, except between moderate vs. mild and mild vs. control ($P>0.01$), which were not statistically significant.

The findings demonstrated that the result, IL-23 level increases with development of the disease, as seen by considerably greater mean level seen in the patient samples compared to the control samples as seen in Figure1A.The current research successfully identified IL-23 level linked to RA. Figure 1B show the DAS28 classification of patient and compare with control. Patient suffering from RA were divided into four groups according to the DAS28 index. The ANOVA statistical test results showed that the mean level of IL-23 statistically varied between the four different subgroups in terms of disease activity. The levels of IL-23 were increasing by elevating the DAS28 index.

Table (3) also showed DAS28 classification of patient and compare with control for has-miRNA125-a-5p expression. The results showed that the mean folding of has-miRNA125-a-5p statistically varied between the four different subgroups in terms of disease activity. The levels of has-miRNA125-a-5p folding were increasing by elevating the DAS28 index

Figure 2A shows the comparison of has-miR-125a-5p expression between RA patients and healthy controls. The results indicate a significantly higher mean level of has-miR-125a-5p in RA patients, suggesting its upregulation with disease progression. Figure 2B presents the DAS28-based classification of RA patients into four subgroups, compared with the control group. ANOVA analysis revealed significant differences in IL-

23 levels among the subgroups, with levels increasing in parallel with DAS28 scores. Additionally, has-miR-125a-5p expression increased with higher disease activity. The Mean, SD and median of ΔCt, ΔΔCt and Fold change of has-miRNA125-a-5p in Patient compare with control (Table 4). To identify the changes that happened in the expression levels of the selected miRNAs in blood 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. The overall analysis of the results showed a significant increase in the expression levels in patients compared to healthy controls ($p< 0.01$). Table 5 displays the Spearman correlation results, showing a strong positive correlation between miR-125a-5p and CRP, ESR, Anti-CCP, RF, DAS28-ESR, and DAS28-CRP, indicating its potential as a biomarker for RA severity.

Table No.1: Primer Sequence with their product size

Primers	**Sequence**		**Reference**
has-miR-125-a5p	RT primer	5′TGTCAGGCAACCGTATTCACCGTGAGTGGTTCACAG-'3	15
	has- miR-128b	5′-UCCCUGAGACCCUUUAACCUGUGA-'3	
	F	5′-TGTCAGGCAAGTATTCACC'3	
	R	5′-CGTCAGATGTCCGAGTAGAGG'3	
U6 (snRNA)	F	5′-CTCGCTTCGGCAGCACATAT -'3	16
	R	5′- TTGCGTGTCATCCTTGCG-'3	

Table No.2: Distribution of Biomarkers level according to studied groups

Test	**RA patient**	**Control**	**t test**	**p.value**
Anti-CCP	43.8±19.7	8.09± 1.94	17.97	HS (<0.01)
CRP	15.9±5.2	0.91± 0.33	28.25	HS (<0.01)
ESR	45.79±17.7	12.06±1.84	18.8	HS (<0.01)
RF	27.0±11.1	5.2±1.67	17.5	HS (<0.01)

Table 3: Serum levels of IL-23 and blood gene folding of has-miRNA125-a-5p according to disease severity in the studied groups

Groups	Sever group (F1) N= 40	Moderate group (F2) N=36	Mild group (F3) N= 24	Control (F4) N=50	ANOVA
IL-23 (Pg/ml)	751.5±94.7	269.7±59.8	129.8±10.8	107.8±23.6	HS (<0.01)
Post hoc test (P-Value)	F1 vs F2 ,F3,F4 =HS < 0.01 F2vs F3 = NS > 0.01		F2 vs F4 =HS < 0.01 F3 vs F4 = NS > 0.01		
has-miRNA125-a-5p	11.16±7.28	6.34±1.29	5.75±2.46	1.0 ±0.98	HS.<0.01
Post hoc test (P-Value)	F1 vs F2 ,F3 = HS < 0.01 F3 vs F4 = NS > 0.01		F2 vs F3, F4 = HS < 0.01 F4 vs F1 = HS < 0.01		

Table No.4: Mean, median and standard deviations (SD) of the studied miRNA PCR values (ΔCt, ΔΔCt and fold change)

has-miRNA125-a-5p				**P. value**
Groups		**Patient (No. = 15)**	**Control (No. = 5)**	
ΔCt	MEAN	0.40	3.13	< 0.01
	SD	0.77	2.40	
	MEDIAN	0.56	2.00	
ΔΔCt	MEAN	-2.73	0.00	< 0.01
	SD	0.77	2.40	
	MEDIAN	-2.57	-1.13	
Fold change (2^-ΔΔCT)	MEAN	7.75	1.74	< 0.01
	SD	5.29	1.10	
	MEDIAN	5.95	2.19	

Table 5: Correlation among studied parameters in RA groups

		MICRO RNA	DAS 28 CRP	DAS 28 ESR	CRP(mg\dL)	IL -23	RF	ANTI CCP	ESR
MICRO RNA	R	1							
DAS 28 CRP	R	.614**	1						

DAS 28 ESR	R	.593**	.967**	1					
CRP(mg\dL)	R	.659**	.958**	.975**	1				
IL -23	R	.565**	.887**	.865**	.830**	1			
Rheumatoid titer	R	.607**	.952**	.946**	.943**	.892**	1		
ANTI CCP	R	.554*	.939**	.968**	.951**	.891**	.947**	1	
ESR	R	.618**	.970**	.959**	.944**	.919**	.956**	.955**	1

**. Correlation is significant at the 0.01 level

Table No.6: Estimation of has-miRNA125-a-5p cut-off values, sensitivity, specificity in studied groups

TEST	Cut-off	SN (%)	SP (%)	AUR	P-Vales
NPT	3.44	85 %	95 %	0.90	<0.01

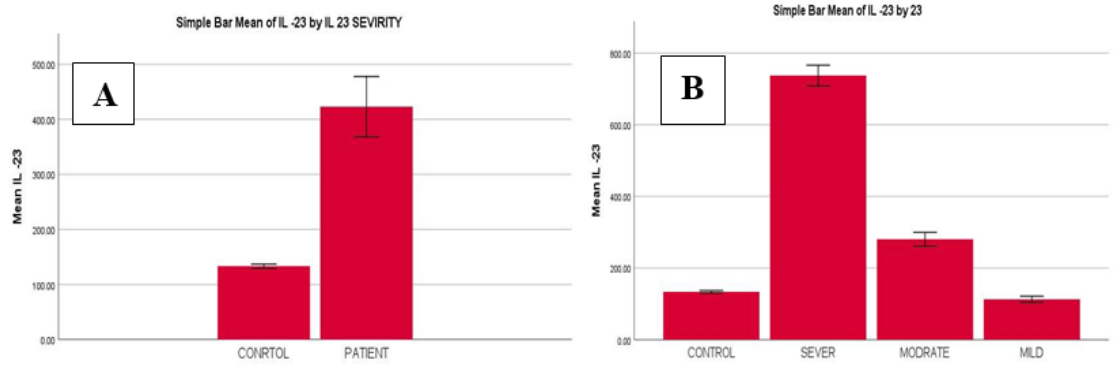

Figure No. 1: Comparison of levels of circulating IL-23 (A) Between rheumatoid arthritis (RA) patients and healthy controls. (B) Between severity group based on DAS28 classification into mild, moderate, severe and control

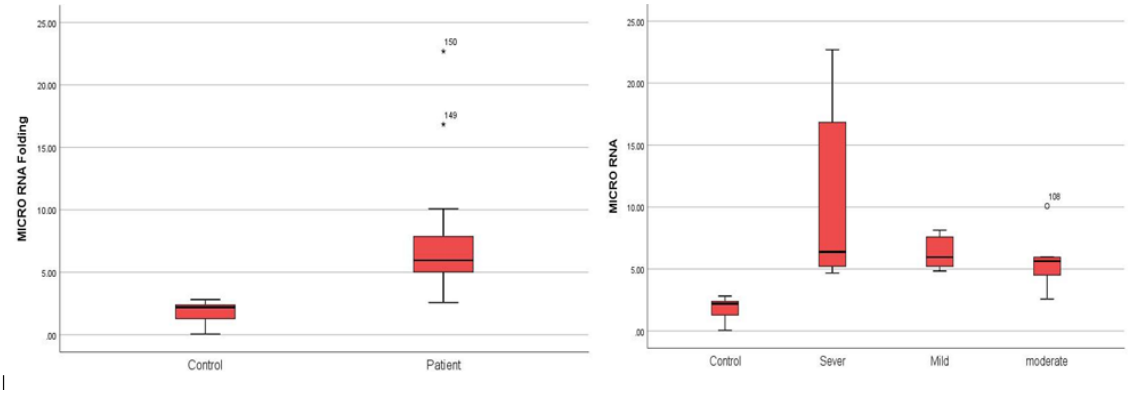

Figure No. 2: Comparison of levels of circulating miR-125a-5p between rheumatoid arthritis patients and healthy co

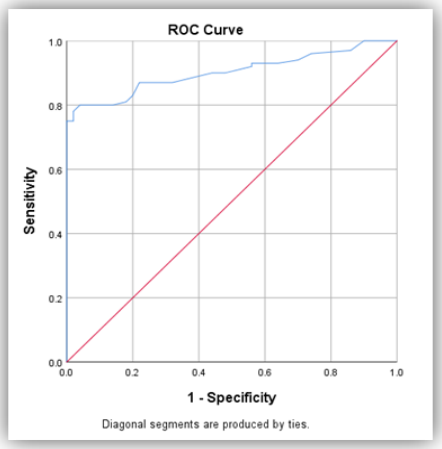

Figure No. 3: ROC Curve chart for the association has-miRNA125-a-5p Marker in the RA studied group

Receiver Operating Characteristic (ROC) analysis was used to identify the optimal cutoff value for the diagnostic test. Table 6 and Figure 3 illustrate the balance between sensitivity and specificity, along with the statistical significance of the test parameters. The ROC analysis demonstrated a high diagnostic accuracy of hsa-miR-125a-5p expression for RA, with an area under the curve (AUC) of 0.90 ($p < 0.01$). The optimal cutoff value was 3.44, with a sensitivity of 85% and specificity of 95%.

DISCUSSION

MIR-125a-5p were significantly elevated in RA patient compare with healthy control subjects, this result similar to that found an increase blood level of miR-125a-5p in RA patients.[16,17] Ormseth et al[18] showed there was a significant over expression miR-125a-5p, in

peripheral blood of RA patients compared to control. When analyzing the with previous studies[19,20] confirmed that the upregulation miR-125a-5p expression appears significantly up-regulated of RA patients compared with healthy controls these studies match with our study. On the other hand, the results of this study indicated that the levels of miR-125a-5p are positively correlated with CRP, ESR, Anti CCP, RF, DAS 28, IL-23, and DAS 28 CRP indices in RA patients, this finding match with other studies that found similar correlation.[19,20] These result is proposing the increase expression of mir-125a-5p is positively associated with the disease activity. The elevated mir-125a-5p expression is to increase the inflammatory process citrullination in RA patients according to(23) that found expression miR125a-5p with other microRNAs were higher in RA patients. Therefore, these findings suggest that the mir-125a-5p may be involved in the occurrence and progression of RA disease. IL-23 result in this study were significantly elevated in RA patient compare with healthy control subjects, these results are consistent with the previous studies that revealed a significant increase in the mean of IL-23 level among the RA patients as compared to the controls[23], other studies found the circulating IL-23 concentrations were significantly high in patients with RA compared with controls and there was a significant positive correlation between serum IL23 levels in patients with RA and individual disease activity parameters.[24,25] Furthermore, study revealed no correlation between IL-23 and disease activity measured by DAS 28 score despite of gradual increase in the median level of serum IL-23 in RA patients with remission, low activity, moderate activity and severe activity, this increased serum level failed to achieve a significant difference[26] which not corresponding with our study. IL-23 is crucial for the maintenance and expansion of Th17 cells. Zaky et al[26] showed that a negative correlation between miR-125a-5p expression and the percentage of Th17 cells in human CD4+ T cells as well as a positive correlation between miR-125a-5p expression and the percentage of T-regulatory in human CD4+ T cells. In summary, these data demonstrate that the up regulation of miR-125a-5p expression act as a compensatory mechanism to limit excessive inflammation that caused by high level of IL-23 in blood of RA patient.

CONCLUSION

Elevated miR-125a-5p expression in RA patients suggests its role in disease progression and potential as a diagnostic marker. Additionally, increased IL-23 promotes IL-17 production, contributing to systemic inflammation and elevated CRP and ESR levels.

Author's Contribution:

Concept & Design or acquisition of analysis or interpretation of data:	Noor Mahdi Dakhil, Suhad Hassan Aubaid
Drafting or Revising Critically:	Raya Al-Saade, Sahar Adnan Shams Al-din
Final Approval of version:	All the above authors
Agreement to accountable for all aspects of work:	All the above authors

Conflict of Interest: The study has no conflict of interest to declare by any author.

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