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Up-Regulation of microRNA-34a in Women with Systemic Lupus Erythematosus

Wissam L. Abdullah*, Reema M. Abed
Department of Biotechnology, College of Science, University of Baghdad, Baghdad, Iraq

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Abstract

Systemic Lupus Erythematosus (SLE) is a chronic systemic autoimmune disease with multiple contributing factors. It is characterized by the immune system's inability to tolerate autoantigens such as nuclear antigens. The objective of this study is to evaluate the expression of microRNA-34a in relation to the incidence and occurrence of SLE in Iraqi women using quantitative real-time PCR and to determine its potential relationship with various demographic and laboratory parameters and disease activity. This investigation enrolled 100 healthy controls with a mean age of 31.68 years and 100 SLE patients, all of whom were women with a mean age of 32.85 years and a disease duration of 9.00 (6.75) years. The mean of the Systemic Lupus Erythematosus disease activity index (SLEDAI-2k) score was 10.860±3.275. Erythrocyte sedimentation rate, C-reactive protein, urea, and creatinine were significantly higher (50.00 vs. 10.00 mm/h, 16.70 vs. 0.650 mg/dl, 58.00 vs. 33.00 mg/l, and 1.550 vs. 0.700 mg/l, respectively), while hemoglobin, white blood cells, and Complement 3 and Complement 4 were significantly lower (8.40 vs. 13.00 g/dl, 3.500 vs. 6.800 x 10^9 /L, 74.00 vs. 130.00 mg/dl, and 10.00 vs. 13.50 mg/dl, respectively) in patients with SLE compared with controls ($P \leq 0.001$). The analysis of anti-nuclear antibodies (ANA) in patients showed that 89% have ANA and 95% have anti-double-stranded DNA (anti-dsDNA). Also, the findings revealed a significant increase in microRNA-34a expression with fold change (5.19 ±0.48) when compared to the fold change mean in controls (1.00 ±0.00). There is no evidence for any correlation between microRNA-34a fold change and any laboratory or demographic examination of the illness in the present study except for age and BMI. In addition, receiver operating characteristic (ROC) analysis was performed on SLE patients to establish the diagnostic accuracy of microRNA-34a in differentiating SLE patients from controls. The specificity and sensitivity of microRNA-34a were 98% and 100%, respectively. The area under the curve (AUC) was 0.990, and the cutoff point was 1.050. This indicates that the identified microRNA-34a may represent strong biomarkers for SLE disorders.

Keywords: Autoimmune disease, complement, microRNA-34a,Systemic Lupus Erythematosus.

*Email: wissamla86@gmail.com

زيادة تنظيم الmiRNA−34a في النساء المصابات بداء الذئبة الحمراء

وسام لطيف عبدالله* , ريما محمد عبد

قسم التقنيات الاحيائية, كلية العلوم, جامعة بغداد, بغداد, العراق

الخلاصة:

الذئبة الحمراء الجهازية هو مرض جهازي مزمن متعدد العوامل وهو يصيب جهاز المناعة .اذ يتميز بنقص التحمل المناعي للمستضدات الذاتية مثل المستضدات النووية. الهدف من هذه الدراسة هو تقييم تعبير miRNA−34a باستعمال تفاعل البوليميراز

المتسلسل الكمي في النساء العراقيات المصابات بمرض الذئبة الحمراء وتحديد علاقته المحتملة بالمعايير السكانية والمختبرية المختلفة ونشاط المرض. تضمنت الدراسة الحالية 100 عينة صحية بمتوسط عمر 31.68 ± 8.308 سنة و 100 مريض مصاب بمرض الذئبة الحمراء كلهم من النساء بمتوسط عمر 32.85 ± 9.992 سنة ومدة المرض 9.00 (6.75) سنة. ان متوسط مؤشر نشاط مرض الذئبة الحمراء الجهازية−SLEDAI (2kهو3.27 ± 10.860 درجة. كان معدل ترسيب كرات الدم الحمراء والبروتين التفاعلي C واليوريا والكرياتينين أعلى بكثير (50.00 مقابل .10.00 مم / ساعة ، 16.70 مقابل 0.650 مجم / ديسيلتر ، 58.00 مقابل 33.00 مجم / لتر و 1.550 مقابل 0.700 مجم / لتر ، على التوالي) بينما كان الهيموغلوبين وخلايا الدم البيضاء والمتمم 3 والمتمم 4 أقل بكثير (8.40 مقابل 13.00 جم / ديسيلتر و 3.500 مقابل 6.800 × 109 / لتر و 74.00 مقابل 130.00 مجم / ديسيلتر و 10.00 مقابل 13.50 مجم / ديسيلتر على التوالي.) (p<0.001) بين مرضى الذئبة الحمراء بالمقارنة مع مجموعة السيطرة. أظهر تحليل الأجسام المضادة للنواة (ANA) في المرضى أن 89 ٪ لديهم ANA و 95 ٪ لديهم حمض DNA مزدوج (مضاد لـ dsDNA). كشفت النتائج عن زيادة كبيرة في تعبير miRNA−34a مع تغيير أضعاف (5.304 ±4.925) بالمقارنة مع متوسط تغيير الطيات في عناصر التحكم (1.00 ± 0.00). لا يوجد دليل على أي علاقة بين تغيير أضعاف miRNA−34a او أي تحليل مختبري أو ديموغرافي للمرض في الدراسة الحالية باستثناء العمر. بالإضافة إلى ذلك ، تم إجراء خاصية تشغيل جهاز الاستقبال (ROC) على مرضى SLE لتحديد الدقة التشخيصية لـ miRNA−34a في التمييز بين مرضى الذئبة الحمراء والأشخاص الاصحاء. كانت خصوصية وحساسية miRNA−34a 98٪ و 100٪ على التوالي. كانت المنطقة تحت المنحنى (AUC)= 0.990ونقطة القطع 1.050. يشير هذا إلى أن miRNA−34a المحدد قد يمثل مؤشرات حيوية قوية لاضطرابات مرض الذئبة الحمراء.

1. Introduction

Systemic lupus erythematosus (SLE) is a heterogeneous autoimmune disorder with a multifactorial etiology and pervasive multisystemic effects. In SLE, the immune system is misdirected against numerous autoantigens. Immune effectors activate signaling pathways, resulting in the destruction of disease-specific tissues [1]. Because of the excessively high levels of autoantibodies, such as anti-double-stranded DNA (anti-dsDNA) antibodies, SLE is classified as a systemic autoimmune illness. In addition to being used for diagnosis, measuring anti-ds-DNA antibodies allows for the monitoring of patient progress and the study of the disease's pathogenesis [2]. The erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and complement protein level are currently employed biomarkers for the diagnosis of SLE [3].

As biomarkers for lupus susceptibility, diagnosis, and surveillance, microRNAs have demonstrated encouraging initial results. Many of them have been studied for their potential as SLE biomarkers. [4]. Patients with SLE have distinct microRNA-34a signatures in comparison

to healthy individuals and patients with other diseases. It has also been found that dysregulation of microRNAs is associated with disease activity and involvement of the main organs [5]. The chromosome 1p36 microRNA-34a gene is an essential immune modulator [6, 7]. MicroRNA-34a is capable of targeting diacylglycerol kinase zeta, which can increase T cell activity via this signal pathway [8]. In addition, microRNA-34a can modulate T cell migration and cell signaling through immunological synapses downstream of "T cell receptors" [9] by targeting five members of the protein kinase C family. MicroRNA-34a can modulate T-cell function and numerous innate and adaptive immunity components [10].

Several autoimmune diseases, such as multiple sclerosis, were found to have dysregulated microRNA-34a expression [11]. A recent investigation by Xie and colleagues [12] suggests that microRNA-34a derived from SLE patients' peripheral blood mononuclear cells may play a role in disease activity. Several disease indices, such as erythrocyte sedimentation rate and C-reactive protein, were directly correlated with the gene's expression levels. This indicates that microRNA-34a may function as a biomarker or a new target for SLE disease. Therefore, this study aimed to determine micrRNA34a gene expression and understand its relationship with demographic and laboratory analysis in women with SLE.

2. Methods

Subjects

One hundred SLE patients were recruited from Baghdad, Iraq (Baghdad Medical City Hospital) for the study. Using the European League Against Rheumatism/American College of Rheumatology (EULAR/ACR) diagnostic criteria for systemic lupus erythematosus [13], the rheumatologists at the unit clinic diagnosed the disease. The clinical and laboratory evaluations were conducted in their entirety. During blood collection, the physician also directly determined the Systemic Lupus Erythematosus disease activity index (SLEDAI-2k) score for each patient. Before participating in the research, all participants provided written informed consent. The local Ethics Commission (CSEC/0223/0015) approved and received written informed consent from all of the participants. Under the direction of medical professionals at Baghdad Medical City Hospital, the University of Baghdad group conducted the study in Baghdad, Iraq. The healthy control group included 100 individuals obtained from the National Blood Transfusion Center.

Laboratory investigations

The demographic information and clinical manifestations for each patient were extracted from their medical records. The laboratory investigations were conducted to evaluate the standard and additional laboratory tests. At the time of sampling, all hematological parameters assessed by erythrocyte sedimentation rate (ESR), C-reactive protein, and complete blood count (CBC) were determined. Fujifilm was programmed to measure blood urea and creatinine in accordance with the manufacturer's instructions. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were carried out in serum that was obtained from patients and controlled by biochemical tests according to the manufacturer’s instructions. Complement (C3) and complement (C4) were estimated by radial immune diffusion (RID). Anti-nuclear autoantibody (ANA) and anti-double-stranded DNA (dsDNA) were determined using the enzyme-linked immunosorbent assay (ELISA) method (Human Company, Germany).

Total RNA extraction

Using the Qubit™ RNA HS Assay Kit (Q32852), total RNA was isolated from both patient and healthy control whole blood (Thermo Fisher, USA). The Qubit 4.0 Fluorometer (Invitrogen, USA) was used to quantify total RNA in samples. Total RNA samples are stored at -70 ^{o}C until processed for downstream applications.

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

The NCBI gene bank database was used to obtain the cDNA sequences of the *microRNA-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 utilized in this study.

Table 1: The primers used in the study

Primers of miRNA	Sequence 5' to 3'
microRNA-34a Forward primer	GGGTGGCAGTGTCTTAGC
microRNA-34a Reverse Primer	CAGTGCGTGTCGTGGAGT
MicroRNA-34a RT Primer	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAGGGC A
U6 Forward primer	GAGAAGATTAGCATGGCCCCT
U6 Reverse Primer	ATATGGAACGCTTCACGAATTTGC

cDNA Synthesis from microRNA

The ProtoScript® First Strand cDNA Synthesis Kit (E6300S) was utilized. cDNA synthesized in a laboratory was used to evaluate microRNA-34a expression levels. This kit includes the M-MuLV enzyme mix and the M-MuLV reaction mix, two optimized mixtures. M-MuLV enzyme mix consists of M-MuLV reverse transcriptase and murine RNase inhibitor, whereas M-MuLV reaction mix includes dNTPs and an optimized buffer. The conditions for cDNA reverse transcription in a thermal cycler include the use of a random primer mix, an incubation phase at 25 °C for five minutes, incubation of the 20µl cDNA synthesis reaction at 42 °C for one hour, and enzyme inactivation at 80 °C for five minutes.

Real Time -Quantitative PCR (RT- qPCR) for microRNA

Quantitative RT-PCR was utilized to estimate the levels of microRNA expression. Luna Universal qPCR MasterMix is a 2X reaction mix optimized for real-time qPCR detection and quantification of target microRNA using the SYBR®/FAM channel of the majority of real-time qPCR instruments. It contains Hot Start Taq DNA Polymerase and a unique and compatible passive reference pigment (New England Biolabs, United Kingdom). Kits used in this investigation, along with their description and origin across numerous instrument platforms and the reaction mix components and quantities, are mentioned in the table below:

Table 2: Quantitative real-time PCR components utilized in a gene expression experiment

Component	20 ul Reaction
Luna Universal qPCR Master Mix	10
Forward primer (10 µM)	1
Reverse primer (10 µM)	1
Template DNA	5
Nuclease-free Water	3

The program for real-time PCR was setup with the indicated thermocycling protocol, as shown in Table 3.

Table 3: The profile of gene expression temperature

Cycle Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	60 seconds	1
Denaturation Extension	95°C 60°C	15 seconds 30 seconds	45
Melt Curve	60-95°C	40 minute	1

Statistical Analysis

For data analysis, version 27 of the Statistical Package for the Social Sciences (SPSS) was used in the current study. Quantitative parametric results were calculated using the mean (median) and interquartile range, whereas qualitative non-parametric data was calculated using the means and standard deviation. Additionally, comparisons were made using the Pearson Chi-square test, and the Spearman correlation test was performed to look into the link between different study variables. If a p-value is less than 0.05 with a 95% confidence interval, it is considered significant. Receiver operating characteristic (ROC) analysis was adopted to estimate the sensitivity and specificity of a parameter as well as its area under the curve (AUC) that predicts its significance [14].

3. Results and discussion

Characteristics of the study population

This case-control study included 100 SLE women with a mean age of 32.85 ±9.992 years and 100 female healthy controls with a mean age of 31.68 ± 8.308 years, as shown in Table 4. Ninety-one percent of patients did not have a family history of SLE. There were significant differences in BMI between patients (23.106±3.791) and control groups (20.345±1.879). The median (IQR) disease duration for patients was 9.00 (6.75). The mean disease activity index for Systemic Lupus Erythematosus (SLEDI-2K) was 10.860±3.275. In this study, all laboratory parameters for women with SLE and healthy controls were listed in Table 4. There were significant differences ($P < 0.0001$) in ESR and CRP between patients and controls. The complete blood count showed significant variation ($P < 0.0001$) in Hb levels and WBC counts between patients and the control group, while there were no significant differences in platelet count ($P = 0.373$). Also, the biochemical tests revealed a non-significant association in ALT and AST serum levels between patients ($P = 0.754$) and control groups ($P = 0.617$). The immunological analysis showed significant variation in C3 and C4 levels in patient groups compared with control groups ($P < 0.0001$). The analysis of ANA in patients showed that 89% have ANA and 95% have anti-dsDNA.

Table 4: Baseline characteristics of SLE patients and healthy controls

Parameters		Patients N=100 mean±SD,N% or	Controls n=100 mean±SD,N% or	*p*-value
Ages(years)		32.85 ±9.992	31.68 ± 8.308	0.369NS
Family history	**Yes**	9(8.9)%	-	0.000**
	No	91(90.1)%		
BMI		23.106±3.791	20.345±1.879	<0.0001**
Disease duration		9.00(6.75)	-	-
SLEDI-2K		10.860±3.275	-	-
ESR (mm/h)		50.00(33.00)	10.00(4.00)	<0.0001**
CRP mg/dl		16.70(1.78)	0.650(0.80)	<0.0001**
Hb g/dl		8.40(1.75)	13.00(2.10)	<0.0001**
WBC x10^9/L		3.500(1.30)	6.800(2.17)	<0.0001**
PLTx10^9/L		250.500(147.00)	250.500(134.00)	0.373NS
Urea mg/l		58.00(43.75)	33.00(16.00)	<0.0001**
Creatinine mg/l		1.550(0.70)	0.700(0.30)	<0.0001**
ALT U/L		34.00(30.50)	34.00(31.00)	0.754NS
AST U/L		23.00(22.85)	23.00(22.05)	0.617NS
C3 mg/dl		74.00(8.00)	130.00(42.00)	<0.0001**
C4 mg/dl		10.00(4.00)	13.50(11.00)	<0.0001**
ANA positively		89%	-	
Anti-dsDNA positively		95%	-	-

N: number within each parameter; %: percentage and mean standard deviation; BMI: body mass index. WBC stands for white blood cells, and Hb for hemoglobin.

Systemic lupus erythematosus (SLE) is a heterogeneous autoimmune disease characterized by increased production of autoantibodies directed against self-antigens, variable clinical manifestations, and an unpredictable course of episodes [15]. Despite advances in our comprehension of the pathophysiology of SLE, SLE patients continue to have a high risk of organ damage and mortality. Predicting novel instruments enables earlier SLE diagnosis, leading to earlier disease monitoring and treatment selection [16]. Several inflammatory markers, including CRP, can be measured in SLE patients [17, 18]. CRP levels can function as an indicator of subclinical inflammation in infections and chronic diseases [19]. CRP levels can be normal or elevated in SLE patients, indicating an inflammatory response, as demonstrated by [18]. Additionally, these results are consistent with [20]'s report that both C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) can be elevated in systemic lupus erythematosus (SLE) exacerbation and infection, but they are of limited use in distinguishing between the two conditions in febrile SLE patients.

A study conducted in China reported that 29% of SLE patients had a deficiency in white blood cells [21]. Other studies suggested 25.03% of SLE patients in Pakistan [22], 22.5% of patients in Oman [23], 23.5% of patients in the UAE [24], and 51% of SLE patients in the UAE, according to other studies. The complement proteins (C3 and C4) have decreased, with a significant difference that has been demonstrated in SLE patients compared to a healthy control. Further studies were performed in China [25]. The United States of America [26], Egypt [27], and Iraq [28] shared the findings of the present study. A decrease in complement proteins in SLE is a consequence of excessive production of autoantibodies and cytotoxicity mediation [29]. Therefore, the ANA, anti-ds-DNA, and complement protein tests are considered complementary factors in the diagnosis of SLE and in determining the treatment plan [25].

In the second assessment of previous immunological factors for the same Iraqi patients after a period of time since their diagnosis and treatment, the results showed that ANA-positivity is lost over time. This seroconversion in ANA positivity was previously assumed [30]. A Swedish longitudinal prospective cohort study reported Secondly, anti-dsDNA states the greatest decrease in the frequency of autoantibodies with the second assessment among the ANA and complement proteins. An observation that has been corroborated by previous studies performed in Brazil [31] and Sweden [32]. All studies shared the same suggestion, despite the differences in the measurement methods and the populations involved in the studies [33]. This transformation in anti-dsDNA to negative after treatment suggests it can be used for SLE disease monitoring [34]. The levels of complement proteins (C3 and C4) in the serum of SLE patients also become typical in most of them over time. Research reported a similar finding and suggested that this is due to the decrease in the number of autoantibodies after treatment [29].

Expression levels of microRNA-34a

A real-time PCR quantification was done to measure the expression of microRNA-34a. The miRNA-U6 gene was used as a housekeeping gene to normalize gene expression and also used the Livak formula to quantify gene expression. In Figures 1 and 2, a typical RT-qPCR plot is shown.

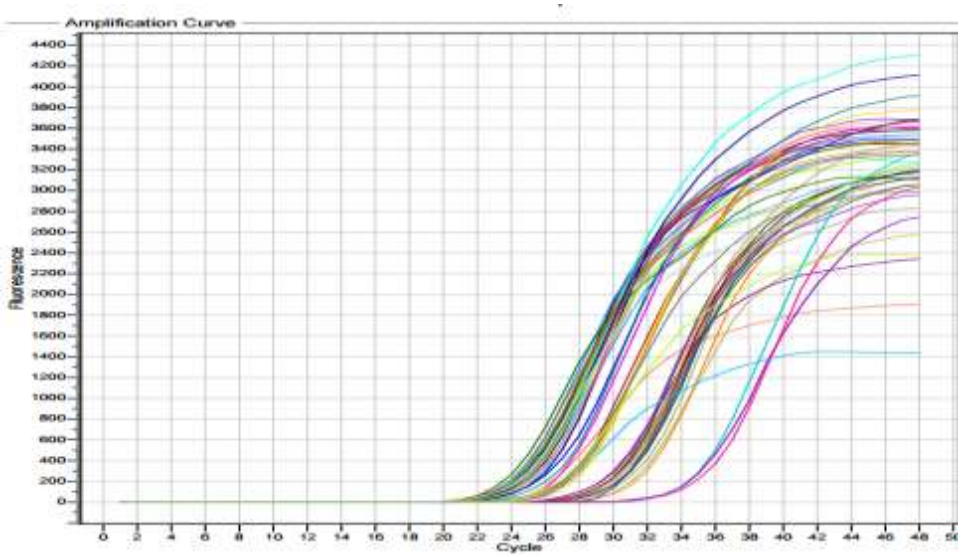

Figure 1: Quantitative real-time PCR amplification curve of the microRNA-34a

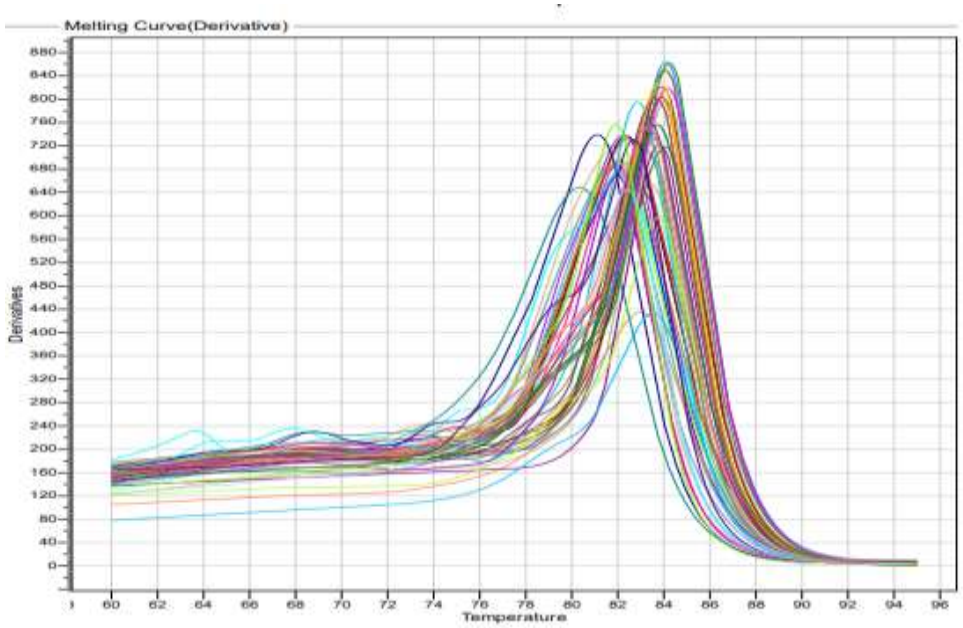

Figure 2: Quantitative real-time PCR melting curve of microRNA-34a

The fold change mean of the microRNA-34a gene was increased in SLE patients (5.19 ±0.48) when compared to the fold change mean in controls (1.00 ±0.00), with significant differences (P = 0.0001), as shown in Table 5.

Table 5: Fold change of microRNA-34a expression with patients and control groups.

Groups	No.	Mean±SD of micro-RNA34a Fold Change
Patients	100	5.19 ±0.48
Control	100	1.00 ±0.00
p-value	---	≤0.0001****

****p- ≤ 0.01**

The microRNA family of noncoding RNAs is associated with immune system homoeostasis, and its genetic variants and gene signature dysregulation are associated with a number of immunological disorders, including SLE [35–37]. Our study showed an increased level of expression of microRNA 34a in SLE patients compared with healthy controls. These results agreed with [38], which demonstrated higher expression of MiR-155 and microRNA-34a in renal tissues in LN-SLE patients as compared to IgA nephropathy patients. Also, these results agreed with [39], which showed that microRNA-34a was highly expressed in the osteoarthritis (OA) synovial tissues of OA patients and that synovial cell apoptosis was depressed with microRNA-34a knockdown in an in vitro study. We also agree with Kong L et al., who explored the significance of microRNA-34a in the pathogenesis of T2DM [40], and the expression of microRNA-34a was elevated in the islets of non-obese diabetic mice as pre-diabetic insulin resistance developed [41]. We also agree with [42], which showed that microRNA-34a was overexpressed in T2DM and DN patients. Therefore, these data suggest microRNA-34a might play an important role in SLE development.

Determination of Pearson correlation coefficient between microRNA-34aand parameters.

Table (6) showed a Pearson correlation coefficient and p-value between microRNA-34a and all parameters under study in patients with systemic lupus erythematosus. Micro-ribonucleic acid 34a was significantly negatively correlated with ages and BMI (r = -0.022; p

= 0.436), significantly negatively correlated with ESR (r = -0.273; p = 0.007), and urea (r = -0.0008; p = 0.952), while it was significantly weakly positively correlated with age (r = 0.26; p = 0.008), while microRNA-34a has no significant correlation with other parameters under study.

Table 6: Pearson correlation coefficient (r) between microRNA-34a and parameters under study

Parameters	Folding of microRNA-34a in SLE patients	
	r_s	*p*-value
Ages(years)	-0.250	0.012*
BMI	-0.231	0.021*
Disease duration	0.119	0.240
SLEDI-2K	-0.124	0.210
ESR mm/h	0.076	0.453
CRP mg/dl	0.083	0.408
Hb g/dl	-0.187	0.063
WBC x10^9/L	0.070	0.482
PLTx10^9/L	0.064	0.5237
Urea mg/l	-0.192	0.055
Creatinine mg/l	0.128	0.204
ALT U/L	-0.051	0.614
AST U/L	-0.031	0.760
C3 mg/dl	-0.106	0.293
C4 mg/dl	0.008	0.931

Values are expressed as N: number in each parameter; %: percentage in each group; and mean ± SD. The abbreviations are: WBC: white blood cell; Hb: hemoglobin; ANA: antinuclear antibody. Normal ranges: WBCs (109/L), 4.00–10.00; HB (g/dl), 12–17; platelets (103 /µl), 150–450; urea (mg/dl), 15–45; creatinine (mg/dl), 0.3–0.7. * The correlation is significant at the 0.05 level.

The results of the correlation study showed a non-significant association between microRNA-34a and all parameters under study except age and BMI. A small number of research papers adopted the association between the chosen microRNA-34a expression and studied parameters of disease. Recent research by Xie and colleagues suggests that microRNA-34a derived from SLE patients' peripheral blood mononuclear cells may play a role in disease activity. Several disease indices, such as erythrocyte sedimentation rate and C-reactive protein, were directly correlated to their gene expression levels [12]. Our results agree with [43], which demonstrated that many clinical diseases, environmental influences, and genetic factors may also contribute to the development and progression of heart failure; they also agree with [44],

which showed that serum Human β-Defensin2(HBD-2) concentrations are elevated in rheumatoid arthritis (RA) patients than in control participants and that these levels are correlated with disease activity; and they disagree with [45], which demonstrates that the level of IL-28B in serum was lower in people with ankylosing spondylitis (AS) than in the control population. Furthermore, microRNA-34a could limit Tregs differentiation with immune tolerance breakdown and Tregs-Th17 cells' subsequent imbalance [46]. These studies suggested that microRNA-34a could be a new potential target or a potential biomarker for SLE.

Receiver Operating Characteristic (ROC)curve

Receiver operating characteristic (ROC) curves Analyses were carried out to determine the diagnostic accuracy of microRNA-34a in distinguishing SLE patients from control participants. The analysis revealed that microRNA-34a at the ideal cutoff point was 1.050, which could significantly differentiate patients from control subjects (AUC = 0.990; 95% CI = 0.9740–1.000; P<0.001) with a specificity and sensitivity of 100% and 98%, respectively, as shown in Figure 4. This ROC curve was created to determine the predictive values of microRNA-34a expression as a marker of an acute phase in SLE. The ROC curves were substantially on the diagonal above, indicating excellent sensitivity and specificity.

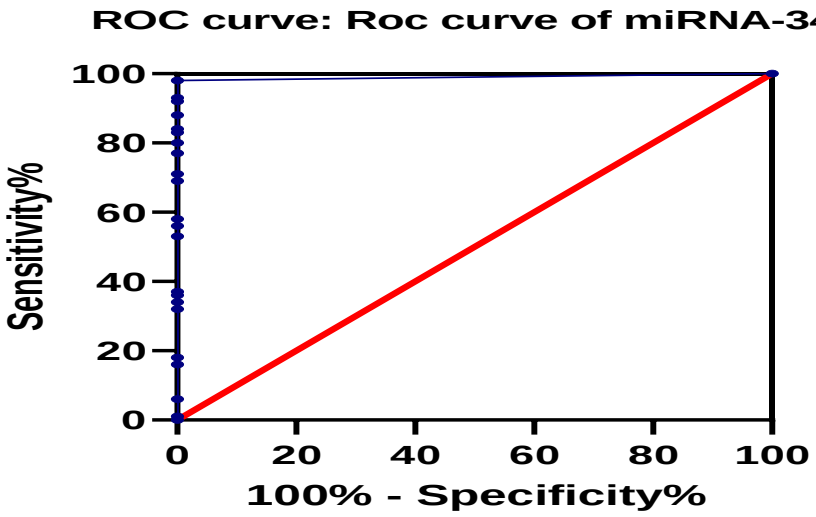

Figure 4: Receiver operating characteristic (ROC) curve analysis of microRNA-34a for differentiating SEL patients from healthy controls

4. Conclusion

Patients with systemic lupus erythematosus exhibited elevated levels of microRNA-34a expression in this study. The increased expression of microRNA-34a may indicate the recurrence of a disease, suggesting that patients should be monitored more closely. This information may provide a foundation for a better understanding of the pathogenesis of SLE and supports the need for additional research to determine whether these changes are consistent across populations and whether the identified microRNA-34a may represent novel biomarkers for SLE disorders. In the current study, there was no evidence of a correlation between the microRNA-34a fold change and any laboratory or demographic investigation of the disease, with the exception of age and BMI.

Conflict of Interest

The authors report having no conflicts of interest.

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