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Assessment of miR-146a Gene Polymorphisms in Patients with Systemic Lupus Erythematosus

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Abstract:

Systemic lupus erythematosus (SLE) is a multifactorial chronic autoimmune disease, with a wide spectrum of effect. The main feature of the disease is the production of a wide variety of autoantibodies as a result of immune tolerance loss. The work aims to evaluate the *miRNA-146a* gene polymorphism potential association with disease activity and chronicity changes in SLE patients. The study included 100 SLE patients and 50 matched controls. The systemic lupus erythematosus disease activity index (SLEDAI) was assessed. The single nucleotide polymorphism (SNP) of *miR-146a* gene (rs2910164) polymorphism was assayed by polymerase chain reaction (PCR) and sequencing technique in patients and control. 100 SLE patients were all females and with a mean of age 31.3 ± 10 years (16-63years) and disease duration of 5.8 ± 3.7years (1 month to 15 years). Most clinical manifestations presented in patients were 52% malar rash, 45% oral ulcers, 54% arthritis, and 45% neurological disorder. Proteinuria, ESR, creatinine and AST were significantly higher (65% vs. 10%, 4.1±36.1 vs. 11.8±9.9 mm/hr, 0.62±0.11 vs. 0.70±0.14 mg/dl and 25.37±26.50 vs. 17.23±3.58 U/L respectively) while the PLT was significantly lower (231.9±88.8 vs. 282.3±67.3 103/mL) ($p< 0.001$) among SLE patients as compared to control. There were no significant variations in all study parameters across *miRNA-146a* genotypes (p greater than 0.05). There was a significant association of the homozygote GG genotype (66.7%) with the active SLE state ($p=0.013$). In conclusion, the results suggest a risk effect for the female gender and adult at a young age in the etiology of SLE. The *miRNA-146a* GG genotype is associated with increasing the disease activity and *miRNA-146a* polymorphism is not associated with the risk in SLE.

Keywords: Autoimmune disease, Anti-nuclear autoantibody, MiRNA-146a, Single nucleotide polymorphisms, Systemic lupus erythematosus.

تقييم تعدد الأشكال الجيني miR−146a في المرضى الذين يعانون من الذئبة الحمامية الجهازية

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الخلاصة

الذئبة الحمامية الجهازية (SLE) هي اضطراب مناعي ذاتي مزمن متعدد العوامل ، له تأثير واسع الطيف. السمة الرئيسية للمرض هي إنتاج مجموعة متنوعة من الأجسام المضادة نتيجة فقدان القدرة على

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التحمل المناعي. الهدف من العمل هو تقييم الارتباط المحتمل لتعدد الأشكال الجيني miRNA−146a مع نشاط المرض والتغيرات المزمنة في مرضى الذئبة الحمراء. شملت الدراسة على 100 مريض بمرض الذئبة الحمامية المجموعية و 50 عنصر سيطرة. تم تقييم مؤشر نشاط مرض الذئبة الحمامية الجهازية (SLEDAI). تم تقييم تعدد الأشكال النوكليوتيد الفردي (SNP) لتعدد الأشكال للجين miR−146a rs2910164) عن طريق تفاعل البلمرة المتسلسل (PCR) وتقنية التسلسل في المرضى والمراقبة.

100 مريض بمرض الذئبة الحمامية المجموعية كانوا جميعهم من الإناث ومتوسط أعمارهم 31.3 ± 10 سنوات (16−63 سنة) ومدة المرض 5.8 ± 3.7 سنوات (من شهر واحد إلى 15 سنة). معظم المظاهر السريرية التي ظهرت في المرضى كانت 52٪ طفح جلدي ملاريا ، 45٪ تقرحات فموية ، 54٪ التهاب مفاصل و 45٪ اضطراب عصبي. كانت البيلة البروتينية و ESR والكرياتينين و AST أعلى بشكل ملحوظ (65٪ مقابل 10٪ ، 4.1 ± 36.1 مقابل 11.8 ± 9.9 مم / ساعة ، 0.62 ± 0.11 مقابل 0.70 ± 0.14 مجم / ديسيلتر و 25.37 ± 26.50 مقابل 17.23 ± 3.58 U / L على التوالي) بينما كان PLT أقل بشكل ملحوظ (231.9 ± 88.8 مقابل 282.3 ± 67.3 103 / مل) (p <0.001) بين مرضى الذئبة الحمراء مقارنة بمجموعة التحكم. لم تكن هناك اختلافات كبيرة في جميع معلمات الدراسة عبر الأنماط الجينية ميرنا −146 أ (p أكبر من 0.05). كان هناك ارتباط كبير بين النمط الجيني GG متماثل الزيجوت (66.7 ٪) مع حالة SLE النشطة (p = 0.013). في الختام ، تشير النتائج إلى وجود تأثير خطر للجنس الأنثوي والبالغ في سن مبكرة في مسببات مرض الذئبة الحمراء. يرتبط النمط الجيني miRNA−146a GG بزيادة نشاط المرض ولا يرتبط تعدد الأشكال miRNA−146a بالمخاطر في مرض الذئبة الحمراء.

Introduction

Systemic lupus erythematosus is a multifactorial chronic autoimmune disorder, with a wide spectrum of effect [1]. The main feature of the disease is the production of a wide variety of autoantibodies as a result of immune tolerance loss; which leads to the attack of the self-antigens such as host nuclear-antigens, immune complexes, DNA, and cellular elements [2, 3]. In addition, this disease is a heterogeneous disease with various clinical and laboratory features. Regarding that makes a diagnosis, assessment of disease activity, and treatment hard and challenging for physicians [4].

Genetic factors possess an essential role in SLE development[5]. In the last few years, the rate of study focusing on genetic discovery increased remarkably, and genome-wide association studies (GWAS) are the greatest contribution in that field [1]. GWAS identified a number of single nucleotide polymorphism (SNPs) markers[6]. Lupus is a polygenic disease with estimated heritability reaching to up 66% with approximately 180 identified susceptible loci, these loci account for 30% of the total heritability [1]. Most of the established genetic variants are located in the intron (non-coding) regions making it a challenge for the scientist to understand the functional implications of SLE pathogenesis [7]. Recently, researchers that focus to understand the genetic loci identified a good number of variants related to SLE with other biological factors [5].

MicroRNAs (miRNAs) are small single-strand RNA (21-25 nucleotides) and represented 30% of all transcriptomes in humans, although are non-coding and involved in gene expression regulation [8]. The miRNAs can link to the target gene through the "3′-untranslated region" (3′-UTR), more is responsible for the "messenger RNA" (mRNA) degradation [9]. Hundreds of "miRNAs" have been identified to regulate the expression of different gene types, and a less number of miRNAs that shown to have a role in the regulation of immune system response [10]. More evidence shows the miRNAs ability to alter the events of various cellular pathways such as development and differentiation, also increased

attention to exploring the role of "miRNA" in the immune system is started [11]. The miRNAs were found to be related to the prognosis of SLE [12] and many other diseases [11]. While the TRAF-6 mRNA is degraded by miRNA-146 activity and prevents the production of new protein, the already existing protein can proceed to work. According to, miRNA146a could be described as a tool for letting down inflammation [10]. Multiple studies have demonstrated the vital role of *miRNA146a* in SLE incidence increase and development of the disease pathogenesis through deregulation of several immunological signaling pathways [13]. Interestingly, the effects of the *miRNA-146a* polymorphisms on the miRNA structure, maturation, and function led to dysregulation in the expression of target genes especially genes involved in the immune system [14], which correlated the miRNA dysregulation with various diseases such as SLE [15], rheumatoid arthritis [16] and autoimmune thyroid disorder [17].This study aimed to evaluate the *miRNA-146a* gene polymorphism potential association with disease activity and chronicity change in SLE patients.

Patients and Methods

Subject

A case-control study was conducted on one hundred SLE female patients. Cases were referred to the outpatient clinic at Baghdad Teaching Hospital (the Rheumatology Unit) from October 2020 – to February 2021. The diagnosis of the disease was handled by the rheumatologists at the unit clinic by following the European League against Rheumatism/American College of Rheumatology (EULAR/ACR) diagnostic criteria for SLE [18]. The clinical and laboratory assessments were all performed. Furthermore, the score of the Systemic Lupus Erythematosus disease activity index (SLEDAI) for every individual patient was determined directly by the physician during the blood draw [19]. Depending on the disease activity score results, SLE patients were classified into two groups: active disease (scores≥4) and inactive disease (scores<4) [20]. Fifty healthy controls were enrolled in this study; the controls were recruited from the Units of Healthcare in Baghdad. The physicians at the Units ascertained their health status, which did not have any autoimmune diseases or treatment by immunosuppressive agents.

All the participants have been provided with written informed consent to be included in the study. The protocol of the study was approved by the Ethics Committee at the Iraqi Ministry of Higher Education and Scientific Research (No. CSEC/0121/001 on January 29, 2021).

Inclusion and Exclusion criteria

We applied available data to common inclusion criteria including age >16 years, positive for EULAR/ACR) diagnostic criteria for SLE [18]. The study excluded patients with other immune diseases or suffering from overlapped autoimmune diseases, patients with juvenile SLE (early-onset) that their age less than 16 years old, or with lupus nephritis (LN).

Laboratory Investigations

The demographic data information and clinical manifestation were collected from the medical record for each patient. The laboratory investigations were performed to assess the immunological and routine tests. All the hematological parameters that assess by complete blood count (CBC), Erythrocyte sedimentation rate (ESR), and the amount of proteinuria were determined at the time of sampling. The anti-nuclear autoantibody (ANA) was determined by enzyme linked immunosorbent assay (ELISA) technique (Human Company, Germany) [21]. The blood urea and creatinine were determined by automated Fujifilm according to manufacturer instructions.

Genotyping

The monarch genomic DNA purification kit manufactured by New England Bio labs corporation (England) was used to isolate total genomic DNA from blood treated with the anticoagulant EDTA. Qubit 4.0 was used to assess DNA concentration and purity, which was given as ng/µl, and DNA purity that ranges between 1.7- 1.9. The genomic DNA was stored at -4^{0}C for later use. The polymorphism rs2910164 in the *miRNA-146a* gene was evaluated using a Polymerase Chain Reaction (PCR). To amplify a 360-bp fragment, a forward (**5-GTTTATAACTCATGAGTGCC-3**) and reverse primer were utilized (**5-GAACTCACACTCCTTATACCT-3**). The PCR reactions were carried out in a 25µl volume that has included a 12.5µl mixture of the GoTaq Green Master Mix (Promega, USA), 0.75 µl of the forward primer (10pmol/µl), 0.75 µl reverse primer (10pmol/µl), 2 µl of the DNA template and 9 µl of the distilled nuclease-free water. Then denaturized at 95°C for 30 cycles, followed by 30 sec. of annealing at 55ºC, 72°C of extension for 45 sec. and 7 minutes of termination at 72°C, starting at 95°C C for 5 minutes. The products amplified by PCR were subjected to forward and reverse Sanger DNA sequencing (Macrogen Corporation; South Korea). Geneious software (version 11.0.5) was used to reveal *miRNA 146a* SNPs after alignments with reference-DNA-sequences available in the National Center for Biotechnology Information (NCBI).

Statistical Analysis

The Statistical Package for the Social Sciences (SPSS) version 22 has been used in the current study for dealing with obtained data. Mean (median) and interquartile range has been used to calculate parametric results(quantitative) while non-parametric data(qualitative) was calculated by means and standard deviation. Additionally, the Pearson Chi-square test was used also for comparisons, and Spearman correlation was used to test the correlation between different study parameters. P-value is significant if ≤ 0.05 at a confidence interval of 95%.

Results and Discussion

The studied population consisted of one-hundred female SLE patients and fifty healthy control with mean ages of 31.3±10 (years) and 34.0±12 (years), respectively. Meanwhile, active SLE represented 60% and inactive 40% of total SLE patients, adult-onset (16-50 years) consisted of 94% of SLE patients, and late-onset 6% (> 50 years). The variation was significant ($P<0.001$). The majority of patients (82%) had no history of SLE in their families. The mean disease duration for patients was 5.8±3.7 years. Demographic and laboratory parameters of the female SLE patients and healthy control enrolled in this study were described in Table 1. Most clinical manifestations presented in this study were (52%) malar rash, (45%) oral ulcers, (54%) arthritis, and (45%) neurological disorder

Table 1: Demographic, clinical, and laboratory parameters in all studied groups.

Parameters	Mean ± SD or N (%)		*P*
	Patients (N=100)	**Controls (N=50)**	
Age	31.3±10.2	34.0±12.4	0.230
Age groups			
Adult-onset	94 (94%)		
Late-onset	6 (6%)		
P	**<0.001***		
Disease duration(years)	5.8±3.7		
Family History	18(18)		
SLEDAI-2K	7.7±5.3		
Active SLE	60(60%)		
Inactive SLE	40(40%)		
Proteinuria	65(65%)	5(10%)	**<0.001***
WBC (10^3/mL)	6.66 ± 2.63	6.38 ± 1.52	0.514
HB (g/dl)	10.91 ± 1.77	10.59 ± 1.86	0.301
PLT (10^3/mL	231.9 ± 88.8	282.3 ± 67.3	**<0.001***
ESR (mm/hr.)	42.1±36.1	11.8±9.9	**<0.001***
Urea (mg/dl)	25.57 ± 7.47	28.80 ± 6.32	0.10
Creatinine (mg/dl)	0.62 ± 0.11	0.70 ± 0.14	**<0.001***
ALT (U/L)	20.91±31.42	17.24 ± 3.50	0.411
AST (U/L)	25.37±26.50	17.23 ± 3.58	**<0.001***
ANA positivity	90(90%)		

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, S: serum, ANA: antinuclear antibody. Normal ranges: WBCs (10^9/L), 4.00-10.00; HB (g\dl), 12-17; Platelets (10^3/µl), 150-450; urea (mg\dl), 15-45; Creatinine (mg\dl), 0.3-0.7. *Correlation is significant at the 0.05 level (2-tailed) calculated by ANOVA.

The rs2910164 SNP had G/C genotypes (Figure1) located on chromosome 5. The genotype frequencies were incompatible with Hardy-Weinberg equilibrium (HWE) in SLE patients and control, and no significant difference (P=0.12) was noticed. Despite the GG, GC, and CC genotypes frequencies were being higher in the patients than in the controls (52.5 vs. 45%; 32.5 vs. 25% and 15 vs. 5%) but the insignificant association was observed for all genotypes, although the differences for both alleles were insignificant (P=1.00) as shown in Table 2.

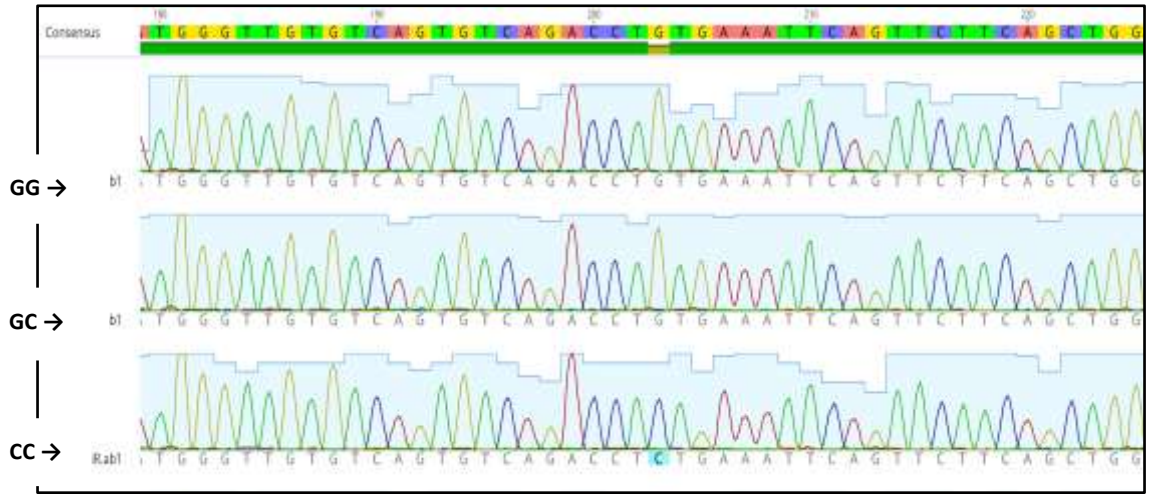

Figure 1: DNA sequence chromatogram of mi-RNA 146a gene SNP (G/C: rs2910164) showing three genotypes: GC, GG, and CC. In addition, the reference sequence (rs2910164)) is also given.

Table 2: The genotypes and alleles frequency of rs2910164 SNP in SLE patients and healthy control

Rs2910164	SLE (N=40)	Controls (N=20)	OR	CI (95%)	*P*
	N (%)	N (%)			
Genotypes					
GG	21 (52.5%)	9(45%)	1.35	0.47-3.88	0.78
GC	13 (32.5%)	10(25%)	0.48	0.15-1.41	0.26
CC	6 (15%)	1(5%)	3.35	0.39-28.47	0.4
Alleles					
G	55(68.75%)	28(70%)	0.94	0.42-2.13	1.00
C	25(31.25%)	12(30%)	1.06	0.47-2.40	1.00
HWE p	0.12	0.39			

N: number of each group, OR: odds ratio, CI: confidence interval, HWE: Hardy-Weinberg equilibrium, p≤o.o5 is significant.

The correlation performed between the disease's clinical manifestations, disease activity, and the miRNA-146a rs2910164 SNP genotypes have been illustrated in Table 3. No significant association has been noticed between the clinical manifestations of the disease and rs2910164 SNP (genotypes and alleles). Regarding the disease activity, results estimated a probable association between the GG genotype and the disease activity, It was noticed that the correlation is significant (P=0.013).

Table 3: Correlation analysis of miRNA-146a rs2910164 SNP genotypes for prediction of SLE clinical manifestation and disease activity

Clinical manifestation	SLE(N=40)	miRNA-146a (N=40)			*P*	r
	N (%)	GG(N=21)	GC (N=13)	CC(N=6)		
Malar rash	18 (45)	10 (55.5)	6 (33.3)	2 (11.2)	0.6	0.9
Oral ulcers	6 (15)	5 (83.3)	1 (16.7)	-	0.1	0.3
Arthritis	23 (57.5)	11 (47.8)	8 (34.8)	4 (17.4)	0.5	-0.1
Neurologic disorder	14 (35)	8 (57.1)	6 (42.9)	-	0.3	-0.2
SLEDAI-2K (Active)	27 (67.5)	18 (66.7)	7 (25.9)	2 (7.4)	**0.013***	0.4
Family History	5 (13)	1 (20)	4 (60)	-	0.645	0.074
Leukopenia	9 (23)	8 (89)	1 (11)	-	0.251	0.183
Anemia	22 (55)	13 (59.1)	6 (27.3)	3 (13.6)	0.635	0.076
Thrombocytopenia	8 (20)	6 (75)	1 (12.5)	1 (12.5)	0.799	0.041
ESR	35 (88)	18 (51.4)	11 (31.4)	6 (17.2)	0.979	0.004
Proteinuria	32 (80)	17(53.1)	12 (37.5)	3 (9.4)	0.5	-0.12
ANA	25 (63)	16 (64)	5 (20)	4 (16)	0.108	-0.254

*Correlation is significant at the 0.05 level (2-tailed) calculated by ANOVA, r: the Pearson correlation coefficient analysis.

The presented study data suggest a risk effect for female gender and age in SLE etiology. Concerning age, SLE does regard as a disease that often afflicts younger-aged more than elderly-aged people. The age range recorded at diagnosis time is 16-63 years with a mean of 28.9 years. So the twentieth decade of age is considered a crucial risk factor for SLE exception before puberty age [22]. The female gender is an additional risk factor, and most studies agree that SLE occurs more frequently in women than in men [23-25]. Two important explanations may justify the female-gender dominancy in SLE; the interaction between the environment, genotype, and sex hormones during individual development, and all cases were adult and this is similar to previous research clarified, that females with SLE have a higher prevalence than males after puberty age due to high levels of circulating estrogen [25, 26].

SLE is a chronic and clinically heterogeneous disease, it affects multi organs. Patients represent their clinical characteristics in many different ways. The chronicity of the disease reflexes its complex etiological pathogenesis, which highlights the importance of genetic factors and individual susceptibility to environmental factors [27]. In the current investigation immunological disorders 100%, renal disorders 70%, and 68% hematological disorders 68% were the most clinical manifestation of more than 50% of the total cases. In agreement with the previous study reported the same results [28].

Proteinuria has an essential prognostic role on renal outcomes (progression to "chronic kidney disease" (CKD), Remission of lupus nephritis (LN), kidney transplantation, or renal replacement therapy) in lupus patients. Regarding CKD progression, proteinuria is considered a risk factor due to its pro-fibrotic and pro-inflammatory effects on the kidney [29]. The present study finds a significant difference in the positivity of ANA between SLE patients and controls with positivity reaching 91% in patients. Another observation that has also been noticed in many studies is that few studies reported a 100% frequency. The majority reported 95% to 99% even though the lower frequency of ANA positivity is frequent in cross-sectional studies [30, 31].

MiR-146a is an important genetic element and effector mediator, that regulates the response of the innate and adaptive immune systems [32]. Many human gene expressions are regulated by miRNA-146a[33]. The miRNAs regulation role in the immune system occurs in

delicate tune, it is crucial to avoid uncontrolled immune responses leading to auto self-damage related to many different autoimmune diseases [34, 35]. Several studies have shown that polymorphisms in the miRNAs gene affect its expression, biogenesis, and maturation which might represent an important risk determinant in disease susceptibility including carcinogenesis and inflammation [36, 37]. The rs2910164 SNP has been determined as a functional variant, which affects the transcription and expression level of miR-146a. Thereby, it contributes to the pathogenesis of several inflammatory and autoimmune diseases including SLE [38].

The current study shows that genotypes and alleles of rs2910164 SNP in miRNA-146a were insignificantly associated between the SLE patients and healthy controls. Many types of research [39, 40] and meta-analysis studies [41, 42] support our result. The *MiR-146a* gene polymorphisms demonstrated a significant positive correlation with *SLEDAI* that GG genotypes were more present in SLE patients with an active state than in inactive. As well as the gene polymorphisms show a negative correlation with the clinical manifestations, hematological parameters, and standard immunological parameters. The previous studies agree with these findings that study the miRNA expression profile in PBMCs and show an association with the disease activity of SLE [43].

Conclusions

The results suggest a risk effect for the female gender and adult at a young age in the etiology of SLE. The *miRNA-146a* GG genotype is associated with increasing the disease activity and *miRNA-146a* polymorphism is not associated with the risk in SLE.

Ethical Clearance

This research was ethically approved by the Research Ethical Committees of the Ministry of Environmental and Health and the Ministry of Higher Education and Scientific Research, Iraq.

Conflict of Interest

The authors declare that they have no conflict of interest.

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