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Correlation Between Gene Expression of Interferon Regulatory Factor-5 and Disease Activity Index in Systemic Lupus Erythematosus Iraqi Patients

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Abstract

Systemic lupus erythematosus (SLE) is a heterogeneous autoimmune disease characterized by elevated levels of circulating anti-nuclear autoantibodies and interferon-alpha (INFs-α). Interferon regulatory factor-5 (IRF5) plays an important role in the induction of type I interferon and pro-inflammatory cytokines, and participates in the SLE pathogenesis. This study aimed to investigate the role of IRF5 gene expression levels in a sample of SLE Iraqi patients and its correlation with disease activity, and to identify its diagnostic ability as a biomarker reflecting disease activity. Blood samples were taken from 45 participants diagnosed with SLE cases classified according to the American College of Rheumatology (ACR) criteria. They were scored via the SLE disease activity index 2000 (SLEDAI-2K) to assess the disease activity and according to it, they were subdivided into "SLE (I) group" (SLEDAI-2k ≤5), and "SLE (II) group" (SLEDAI-2k >5), as well as age and gender matched healthy control group. RNA was isolated from whole blood samples and gene expression levels of IRF5 were determined using real-time polymerase chain reaction (PCR). Our results revealed that the expression levels of the IRF5 gene were significantly increased in SLE (I) and SLE (II) patient groups compared with the control group (p<0.05, and p<0.01) respectively, as well as higher in SLE (II) group than the SLE (I) group (p<0.01). Moreover, the expression levels of IRF5 were found to be related positively and significantly to the disease activity index in both SLE patient groups. The analysis of receiver operator curves (ROC) for gene expression levels of IRF5 in SLE (II) group showed a perfect accuracy to distinguish between SLE patients and healthy individuals (AUC=0.989, sensitivity= 95.5%, and specificity= 88.0%). However, in SLE (I) group showed a good accuracy to discriminate between SLE patients and healthy individuals. (AUC=0.769, sensitivity= 69.6%, and specificity= 80.0%). The correlation between gene expression levels of IRF5 with other parameters revealed that a significant positive correlation was found with uric acid and ALP in SLE (I) group, while in SLE (II) group with urea, creatinine, and uric acid. Our conclusion suggests that the up-regulation of IRF5 gene expression levels correlates positively with disease activity in SLE patients reflecting the possibility of using it as an immunological biomarker for diagnosis, and monitoring the disease flare.

Keywords: Interferon regulatory factor (IRF5), gene expression, systemic lupus erythematosus, Iraqi patients, disease activity index.

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العلاقة بين التعبير الجيني لجين العامل التنظيمي للإنترفيرون −5 و فعالية المرض عند المرضى العراقيين المصابين بداء الذئبة الحمامية الجهازية

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الخلاصة

الذئبة الحمامية الجهازية هي احدى امراض المناعة الذاتية غير المتجانسة والتي تتميز بمستويات مرتفعة من الأجسام المضادة الذاتية المضادة للنواة وإنترفيرون ألفا (INFs−α). يلعب العامل التنظيمي للإنترفيرون− 5 (IRF5) دورًا مهمًا في تحريض النوع الأول من الإنترفيرون والسيتوكينات المسببة للالتهابات، ويشارك في التسبب في مرض الذئبة. هدفت هذه الدراسة إلى التحقق من دور التعبير عن جين IRF5 في عينة من مرضى الذئبة العراقيين وعلاقته بنشاط المرض، لغرض تحديد قدرته التشخيصية كمؤشر حيوي يعكس نشاط المرض. أخذت عينات الدم من 45 مشاركًا تم تشخيص إصابتهم بمرض الذئبة والمصنفين وفقًا لمعايير الكلية الأمريكية لأمراض المفاصل (ACR). تم قياس مؤشر نشاط المرض بالاعتماد على SLEDAI−2K index و وفقا لذلك تم تقسيمهم الى مجموعتين: المجموعة الاولى (I) SLE لديهم مؤشر نشاط المرض اقل اويساوي 5 والمجموعة الثانية (II) SLE اعلى من 5، بالاضافة الى مجموعة سيطرة من المتطوعين الاصحاء والمتطابقين معهم بالعمر و الجنس. تم عزل الحمض النووي الريبي من عينات الدم الكاملة و تحديد مستويات التعبير عن جين IRF5 باستخدام تفاعل البوليميراز المتسلسل في الوقت الحقيقي. اظهرت نتائجنا أن مستويات التعبير عن جين IRF5 قد زادت بشكل معنوي في مجموعات المرضى (I) SLE و (II) SLE مقارنة مع مجموعة الاصحاء ($p<0.05$ و $p<0.01$)، على التوالي. وكذلك اعلى في مجموعة (II) SLE من مجموعة (I) SLE ($P<0.01$). علاوة على ذلك، وجد ان مستويات التعبير عن جين IRF5 مرتبطة بشكل إيجابي و معنوي بمؤشر نشاط المرض في كلتا المجموعتين من مرضى الذئبة. أظهر تحليل منحنيات مشغل المستقبل (ROC) لمستويات التعبير عن جين IRF5 في مجموعة (II) SLE دقة مثالية للتمييز بين مرضى الذئبة والافراد الاصحاء (AUC=0.989 و Sensitivity= 95.5% و Specificity= 88.0%). بينما في مجموعة SLE (I) اظهر التحليل دقة جيدة للتمييز بين المرضى والافراد الاصحاء (AUC=0.769 و Sensitivity= 69.6% و Specificity= 80.0%). كشفت العلاقة بين مستويات التعبير عن جين IRF5 مع المعلمات الأخرى عن وجود علاقة إيجابية معنوية مع حمض اليوريك و ALP في مجموعة (I) SLE ، بينما في مجموعة (II) SLE مع اليوريا والكرياتينين وحمض اليوريك. استنتجت دراستنا أن ارتفاع مستويات التعبير عن جين IRF5 يرتبط ايجابا مع نشاط مرض الذئبة، وهذا يعكس إمكانية استخدامه كمؤشر مناعي حيوي للتشخيص و مراقبة تهيج المرض.

Introduction

Systemic lupus erythematosus (SLE) is a complicated autoimmune disease that can affect multiple organs of the body including skin, joints, central nervous system, heart, kidneys, and so on. SLE pathogenesis is still poorly understood and various factors are contributed to the disease development, involving genetic, epigenetic, hormonal, immunoregulatory, ethnic and environmental factors [1-5]. SLE can affect both sexes, but the incidence in females especially at the childbearing age is higher than the males with a ratio of 9 women for each man suffering from SLE [6]. SLE is a chronic inflammatory disease characterized by heterogeneous clinical manifestations and immunological abnormalities,

especially the overproduction of autoantibodies, primarily to the nuclear materials of the cell, complement activation, and the deposition of the circulating immune complexes (CIC) in various tissues leads to inflammatory [7-9]. Multiple mechanisms may be participated in the pathophysiology of SLE, comprising incomplete clearance of apoptotic and necrotic materials, abnormal exposure to autoantigens, hyperactivity of self-reactive B and T lymphocytes, and increased levels of B-cell stimulatory cytokines [10-12]. Interferon regulatory factor-5 (IRF5) is a protein that is in humans encoded by IRF5 gene [13]. It is a member of the IRF family of transcription factors, composed in humans of nine distinct proteins [14-16]. IRF5 is a DNA-binding protein that regulates inflammatory and immune responses against viruses in both innate and adaptive systems [17,18]. It is mainly expressed in activated B cells, plasmacytoid dendritic cells (DCs), macrophages and monocytes [19,20]. IRF5 plays an important role in regulating type I interferons (IFNs) production and mediating Toll-like receptor (TLR) signaling pathway, as well as the induction of various pro-inflammatory cytokines such as interleukin-6 (IL-6), interleukin-12 (IL-12), and tumor necrosis factor-alpha (TNF-α) [21-24]. In addition to its function in the host immune response to pathogens, IRF5 also mediates DNA damage- and death receptor-induced apoptosis, cell adhesion, and cell cycle, all of these play an important role in autoimmunity [17,20]. Several recent studies have implicated the role of IRF5 in SLE pathogenesis in a number of genome-wide association studies (GWAS) and identified that there is a strong correlation between IRF5 gene polymorphism and risk of SLE, and that IRF5 is an important genetic risk factor that plays a critical role in SLE pathogenesis [15,21,25-29]. Moreover, the expression level of IRF5 in peripheral blood mononuclear cells (PBMCs) of SLE patients was significantly upregulated as compared with healthy donors [17,30]. In monocytes of SLE patients, IRF5 activation was found to be significantly elevated, as determined by its nuclear localization when compared with healthy controls [31]. In addition to being associated with SLE, IRF5 is involved in the pathogenesis of the other autoimmune disease, such as rheumatoid arthritis (RA), inflammatory bowel disease, Sjögren's syndrome (SS), and multiple sclerosis (MS) [28,32]. However, recent studies were performed on SLE patients in some world countries, and there are no studies about the IRF5 gene expression levels in SLE Iraqi patients, therefore in the present study, we determined the expression levels of IRF5 gene in sample of SLE Iraqi patients, and study the correlation between it and disease activity, to investigate its diagnostic ability as an immunological biomarker mirror disease activity.

Materials and Methods

Study groups

Patients: The current study includes 45 patients diagnosed with SLE disease (42 females and 3 males), who fulfilled 4 or more of the 1997 American College Rheumatology (ACR) revised criteria for the classification of SLE [33]. The patients were attending the rheumatology unit of Baghdad Teaching Hospital during the period from November 2020 until February 2021. The disease activity score was assessed by the systemic lupus erythematosus disease activity index 2000 (SLEDAI-2K) score [34], and according to it they were subdivided into “SLE (I) group” (SLEDAI-2k ≤5) and “SLE (II) group” (SLEDAI-2k >5). The group of SLE (I) consists of 23 patients (21 females and 2 males) whose ages ranged from (18-55) with a mean of 36.04±11.64 years and their disease duration ranged from (0.25-23)years with a mean of 4.5 years. While the group of SLE (II) consists of 22 patients (21 females and 1 male) whose ages ranged from (14-53) with a mean of 34.32±10.71 years and their disease duration ranged from (0.1-31) years with a mean of 4.19 years. A full history was taken from all the patients and a proper physical examination was done by specialist physician for each one. The following routine laboratory tests were done for every patient: general urine examination (GUE), blood urea, serum creatinine, 24-hour urinary protein,

complete blood count (CBC), erythrocyte sedimentation rate (ESR), antinuclear antibodies (ANA), anti-double strand DNA (Anti-ds DNA), and complements concentration (C_3 & C_4). Clinical and immunological characteristics of SLE patients are summarized in Table 1, as well as the medication used at the time of recruitment.

Control group: This group consists of 25 healthy volunteers (23 females and 2 males) their ages ranged from (19-52) with a mean of 33.64±9.74 years. All the routine laboratory tests were also done for every participant as shown in Table 1. This group was used for comparison.

Table 1: Demographic data**,** Clinical and immunological characteristics of SLE patients and controls

Characteristic		SLE patients	Controls
Demographic Data			
Samples number		45	25
Age (year), mean ±SD (range)		35.2±11.1 (14-55)	33.64±9.74 (19-52)
BMI (Kg/m^2), mean ±SD		27.87±6.27	26.25±5.26
Gender, female/male		42/3	23/2
Disease duration (year), mean (range)		4.34 (0.1-31)	-
Family history with SLE, n (%)		7 (16%)	-
Clinical and immunological manifestations			
Vasculitis, n (%)		(2) 4%	-
Arthritis, n (%)		(14) 31%	-
Myositis, n (%)		(0) 0%	-
pleurisy, n (%)		(3) 7%	-
Proteinuria, n (%)		(10) 22%	-
Hematuria, n (%)		(1) 2%	-
Urinary casts, n (%)		(2) 4%	-
Oral ulcers n (%)		(15) 33%	-
Alopecia, n (%)		(7) 16%	-
Fever, n (%)		(8) 18%	-
New rash, n (%)		(9) 20%	-
Thrombocytopenia, n (%)		(11) 24%	-
Leucopenia, n (%)		(4) 9%	-
Anemia, n (%)		(20) 44%	
Low complement, n (%)		(16) 36%	-
(+) ANA, n (%)		(30) 67%	
(+) ds-DNA antibodies, n (%)		(27) 60%	-
SLEDAI-2k score, mean ±SD (range)		7.49±4.31 (2-18)	-
Medications			
Prednisolone Treatment	Yes, n (%)	(36) 80%	-
	No, n (%)	(9) 20%	
Hydroxychloroquine Treatment	Yes, n (%)	(31) 69%	-
	No, n (%)	(14) 31%	
Azathioprine Treatment	Yes, n (%)	(10) 22%	-
	No, n (%)	(35) 78%	
Methotrexate Treatment	Yes, n (%)	(2) 4%	-
	No, n (%)	(43) 96%	

The results were expressed as mean ± SD (mean± standard deviation), range (minimum-maximum), or number (percentage). BMI= body mass index, ANA= anti-nuclear antibodies, ds-DNA antibodies= double strand deoxy nucleic acid antibodies, SLEDAI-2K= Systemic lupus erythematosus disease activity index 2000.

Exclusion criteria

Patients with other autoimmune diseases, immune suppressant conditions such as cancer, acute inflammation, other chronic diseases: renal failure, liver diseases, anemia, pancreatitis, gout, diabetes, and patients who had a history of smoking or alcohol drinking were excluded.

Samples collections

Blood samples: About five milliliters of venous blood were collected from overnight fasting patients and healthy volunteers. The blood was immediately divided into two parts, the first one (0.5 ml) was transferred into a sterile Eppendorf tube containing 1 ml of TRIzol reagent that is used for RNA isolation. The second one (2 ml) was transferred into an EDTA tube, then the blood samples were stirred carefully for a few seconds to avoid blood clotting and they were used for ESR and CBC determination.

Sera samples: The reminder blood was transferred into a glass tube with a gel separator, the blood samples were allowed to clot for 10 minutes at 37^{o} C in a water bath, then they were centrifuged at 3000xg for 10 minutes. The obtained clear serum was dispensed in several aliquots, and stored frozen at -20^{o} C until being used to estimate the different parameters included in the study. Hemolyzed sera were excluded. The study protocol conforms to the ethical guidelines, endorsed by the College of Science, University of Baghdad Ethics Committee.

Methods

Expression of IRF5 gene

RNA Extraction

Total RNA was extracted from fresh whole blood samples using TRIzol^{TM} Reagent (Invitrogen Company, USA). According to the manufacturer's protocol includes the following: adding 0.5 ml blood sample into 1ml TRIzol^{TM} reagent for complete lysis. After that 200µl chloroform was added and then centrifuged the sample tube. The homogenate was separated into forms three phases: upper aqueous phase, interphase and organic phase. Total RNA remains exclusively in the aqueous phase, then transferred into a new fresh tube. The RNA was precipitated using an equal volume of cold isopropyl alcohol. The pellet was washed with 1ml 70⁒ cold ethanol. After ethanol evaporation by air, the RNA pellet was resuspended by adding 50µl of RNase-free water and incubated at 60 ^{o} C for 10 minutes by using a thermomixer. Total RNA samples were stored at -20^{o} C until processed for downstream application.

CDNA synthesis

Equal amounts of total RNA (250 ng) that were isolated from each blood sample were reverse transcribed into complementary DNA (cDNA) via ProtoScript® First Strand cDNA Synthesis Kit, (NEW ENGLAND, BioLabs Company, UK) according to the manufacturer's instruction. Each transcription was performed in a reaction buffer containing 250 ng of total RNA (5 µl for each sample), 2 µl of $d(T)_{23}$ VN (50 µM), 10 µl reaction mix (dNTPs and optimized buffer), 2 µl of MULVEnzyme (reverse transcriptase and RNase inhibitor) and the volume of mixture reaction completed up to 20 µl by adding 1 µl of RNase free water. The mixture was incubated at 42 ºC for 1 hour using a thermocycler, then the enzyme was inactivated by incubating the mixture at 80 ºC for 5 minutes. The concentration of extracted RNA and synthesized cDNA were measured by the fluorometer Qubit 4.0 instrument

(Invitrogen Company, USA) using Qubit ™ RNA HS Assay kit and Qubit ™ dsDNA HS Assay kit (ThermoFisher, USA) respectively.

Quantitative Real-Time polymerase chain reaction (qRT-PCR) Analysis

The Real-Time PCR analysis was performed to determine the IRF5 gene expression in blood samples. Real-Time PCR detection system, (Bioer, Japan) and the Luna® universal qPCR Master Mix kit (NEW ENGLAND, BioLabs company, UK) were used. The primers used in this study were designed and provided by Macrogen Company, Korea. They are listed in Table 2.

Table 2: Primers sequence that used in the study.

Primer		Sequence	Amplicon
IRF-5	F	5'TACCTCTGGGTTTCCTGGAAG3'	151 bp
	R	5'GAGTTCTTTCCCTGCTCATGG3'	
β-actin (HKG)	F	5'GGGCGGCACCACCATGTACC3'	211 bp
	R	5'GACGATGGAGGGGCCCGACT3'	

According to the manufacturer's protocol. The qRT-PCR reaction for each sample was carried out in 20 µl as total volume, which consisted of 5 µl of the cDNA, 10 µl master mix (1x), o.5 µl of each forward and reverses primers (0.25 µM), and 4 µl of nuclease-free water. For each sample, there are two qRT-PCR tubes one for IRF5 gene and the other for β-actin as a housekeeping gene (HKG) in this study. The detection of quantity depends on the fluorescence power of SYPER green dye which detects gene amplification. Table 3 demonstrates the conditions of the thermocycling program.

Table 3: Real-Time PCR conditions

Cycle step	Temperature	Time	Number of cycles
Initial denaturation	95° C	60 seconds	-
Denaturation	95 ° C	15 seconds	40-45
Extension	60 ° C	30 seconds	
Melt curve	60-95 ° C	40 minutes	-

The results of qRT-PCR were analyzed by the relative quantification of gene expression level (fold change) according to Livak method [35,36]. Based on comparing the distinct cycle determined by threshold values Ct at a constant fluorescence level. The target gene was normalized to an endogenous control (HKG) and relative to the calibrator which is the target gene in the healthy control group. The fold-change was calculated for each sample using the following equations:

$\Delta Ct_{sample} = Ct_{gene} - Ct_{HKG}$, $\Delta\Delta Ct_{sample} = (\Delta Ct_{sample}) - (\text{average } \Delta Ct_{control\ group})$,

$\text{Fold-change}_{sample} = 2^{-\Delta\Delta Ct}$

Other laboratory tests

Complete blood count (CBC) was done by Abbott Hematology auto-analyzer (Cell-DYN-Ruby, USA). Erythrocyte sedimentation rate (ESR) was determined by the Westergren method. Urinalysis was determined by routine techniques. Protein urea was quantified by 24-h urine collections. Anti-nuclear antibodies (ANA) and ds-DNA antibodies (ds-DNA) were determined using ELISA method by Naissa Immuno auto-analyzer (Neomedica, Europe). While C3 and C4 were determined using turbidimetry method by Hipro Immuno auto-analyzer (Hipro, China). Other biochemical tests include total serum protein, albumin, glutamate oxaloacetate transaminase (GOT), Glutamate Pyruvate Transaminase (GPT), serum

uric acid, total cholesterol (TC), triglyceride (TG), and high-density lipoprotein (HDL) were measured using kits (LINEAR CHEMICALS company, Spain). Low-density lipoprotein (LDL) was calculated using the Friedewald equation LDL= [TC-HDL-(TG/5)] [37]. Very low-density lipoprotein (vLDL) was calculated by dividing TG by five. While blood urea and serum creatinine (BioSystems company, Spain), and alkaline phosphatase (BIOLABO company, France). All the assays were performed by colorimetric methods according to the manufacturer's instructions.

Statistical analysis

The results were presented as mean ± standard deviation (mean± SD). The differences among the studied groups were assessed by applying analysis of variance one-way (ANOVA), and followed by post hoc Tukey analysis to test the differences between every two groups within ANOVA. The degree of correlation between parameters was calculated by Pearson's correlation test. The percentage of significance was obtained by r and *p* values. The *p*-value is considered significant if it is < 0.05, and highly significant if it ≤ 0.01. Receiver Operator Characteristics curve (ROC) analysis was constructed for IRF5 gene expression level to evaluate its diagnostic yield for SLE disease, the areas under the curve were considered exceptional (1-0.9), excellent (0.9-0.81), good (0.8-0.71), fair (0.7-0.61), and poor (0.6-0.5). The statistical analyses were performed using software statistical package for social science (SPSS) version 26.0 (IM SPSS, Chicago, IL, USA) and GraphPad Prism, version. 9.3.1 (San Diego, California, USA).

Results and Discussion

General characteristic of the study

The study enrolled a total of 70 participants, 25 healthy controls and 45 SLE patients. All patients had met the American College Rheumatology (ACR) criteria (at least 4 out of 11 criteria). The disease activity score was calculated for each patient using the SLEDAI-2K score, and according to it, they were subdivided into two subgroups, SLE (I) group and SLE (II) group. The detailed information on the studied groups was presented in Table 4.

It is clear from Table 4 that there were no statistical differences in age, gender, and BMI among the three studied groups, as well as in disease duration between the SLE patient groups. While there is a significant difference in SLEDAI-2K score between the SLE patient groups. The number of SLE patients with family history in SLE (I) group and SLE (II) group are 3 (13%) and 4 (19%), respectively.

Table 4: Distribution of the studied groups according to the number, gender, age, BMI, SLEDAI-2K score, family history with SLE and disease duration

Group	Control	SLE (I)	SLE (II)	P_a	P_b	P_c
Samples number	25	23	22	-	-	-
Age (year), mean ±SD (range)	33.64±9.74 (19-52)	36.04±11.64 (18-55)	34.32±10.71 (14-53)	0.718	0.974	0.852
Gender, Female, n (%) Male, n (%)	23 (92%) 2 (8%)	21 (91%) 2 (9%)	21 (95%) 1(5%)	0.915	0.644	0.877
BMI (Kg/m^2), mean ±SD	26.25±5.26	28.92±5.80	26.77±6.68	0.267	0.950	0.446
Disease duration (year), mean (range)	-	4.5 (0.25-23.0)	4.19 (0.10-31.0)	-	-	0.975
Family history with SLE, Yes, n (%) No, n (%)	-	3 (13%) 20 (87%)	4 (18 %) 18 (82)	-	-	-
SLEDAI-2k score, mean ±SD (range)	-	4.0±0.74 (2-5)	11.14±3.33 (6-18)	-	-	<0.01

The results were expressed as mean ± SD (mean± standard deviation), range (minimum-maximum), or number (percentage). Analysis of variance one-way (ANOVA) was used for the comparison among the studied groups. $\boldsymbol{P_a}$ = the SLE (I) group compared with control group, $\boldsymbol{P_b}$ = the SLE (II) group compared with control group, $\boldsymbol{P_c}$= the SLE (I) group compared with SLE (II) group, $p > 0.05$= non-significant differences, $p < 0.05$= significant differences, $p \leq 0.01$= high significant differences. BMI= body mass index, SLEDAI-2K= Systemic lupus erythematosus disease activity index 2000.

Expression of IRF5 gene

To investigate the role of IRF5 in the pathogenesis of sample in SLE Iraqi patients. We analyzed the mRNA expression levels of IRF5 gene in whole blood samples of SLE patients and controls. The results indicated that the expression levels of IRF5 gene were significantly increased in SLE patients. The mean mRNA expression levels of IRF5 in SLE (I) group and SLE (II) group showed a 1.36-fold and 2.2-fold increase ($p<0.01$, $p<0.05$), respectively, with respect to control group, as well as there was a significant difference was found between SLE patient groups ($p<0.01$) as shown in Figure 1.

Group	**ΔCt** (Mean ±SD)	**$2^{-\Delta\Delta Ct}$ (Fold)** (Mean ±SD)
Control (n=25)	4.36±0.26	1.00±0.18
SLE (I) (n=23)	3.92±0.45	1.36±0.41
SLE (II) (n=22)	3.21±0.44	2.20±0.65

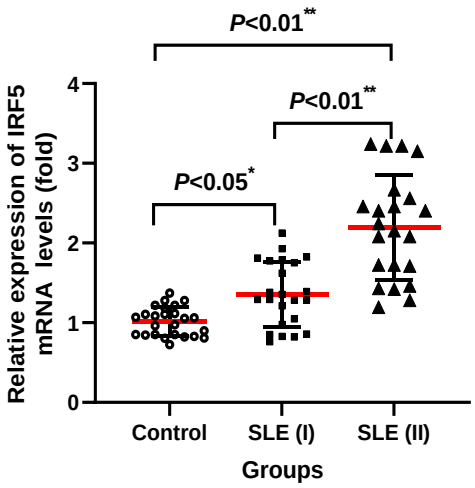

Figure 1: Relative expression of IRF5 mRNA levels (fold) in blood samples of the studied groups, $p > 0.05$= statistically non-significant differences, * Statistically significant differences at $p < 0.05$, ** Statistically significant differences at $p \leq 0.01$.

Our findings are in agreement with the study, which indicated that gene expression of IRF5 in peripheral blood mononuclear cells (PBMCs) increased by 55% in childhood-onset SLE patients in comparison with healthy controls [30]. As well as compatible with Feng *et al*. (2010) who found that the IRF5 expression levels and alternative splicing were significantly up-regulated in peripheral blood mononuclear cells (PBMCs) of SLE patients compared with healthy donors. In humans, IRF5 exists as multiple isoforms (v1-v11) that are generated from alternative spliced transcripts. Each isoform with distinct cell-type specific expression, cellular localization, regulation and function [17,38]. IRF5 isoforms (v2, v9, v10) that are generated from exon 1B are susceptible to SLE, whereas increased IRF5 expression in the absence of 1B does not confer risk [15,39]. IRF5 plays an important role in the inflammatory response in human via regulating the type I interferon and pro-inflammatory cytokines expression, including interleukin (IL6), interleukin (IL12), tumor necrosis factor-alpha (TNF-α), and pathogenic antibody production. Dysregulation of many of these cytokines was associated with disease pathogenesis, and IRF5 predominantly expressed in immune cells such as dendritic cells, B cells and monocytes responsible for their production. Although the

biological mechanism(s) by which IRF5 participates in SLE pathogenesis is still unclear. But could be elucidated by Cham *et al.* (2012) who reported that the IRF5 functions downstream of Toll-like receptor (TLR) and other microbial pattern-recognition receptors. The circulating immune complexes that involved autoantibodies which correlated with SLE disease seem like to act as a chronic endogenous stimulus to this signaling pathway, and subsequently facilitating interferon-alpha production [40,41]. Type I IFN pathway bridges the innate and adaptive system in the stimulation of immune response and seems to play a crucial role in the etiology of SLE [42]. Several studies have suggested that type I IFN plays a central role in the beginning and development of SLE. The elevated serum levels of IFN- alpha and up-regulated expression levels of type I IFN-inducible genes have been shown to be associated with the activity and severity of SLE disease [16,43,44]. In addition to IRF5 function of regulating type I INF expression, IRF5 is participated in other signaling pathways, involving, macrophage polarization, IgG switching in B cells, and apoptosis. And its role in the pathogenesis of SLE may therefore not be limited to dysregulated control of expression type I interferon [45] On the other hand Poole *et al*. (2016) investigated the effects of IRF5 overexpression in B cells on apoptosis, and they hypothesized that overexpression of IRF5 would decrease activation-induced apoptosis, and this would reduce tolerance and likely contribute to the progression of SLE disease [46]. Additionally, Ban *et al*. (2018) proposed that a loss of negative regulation of IRF5 causes its hyperactivation, resulting in the hyperproduction of type I interferon and other cytokines and finally in the improvement of SLE [47].

Correlation between expression of IRF5 gene and disease activity

Our results revealed that the IRF5 expression levels in SLE (I) and SLE (II) groups were significantly and positively correlated with disease activity index (SLEDAI-2K), (r=0.461, p=0.027) and (r=0.494, p=0.019), respectively as shown in Figure 2.

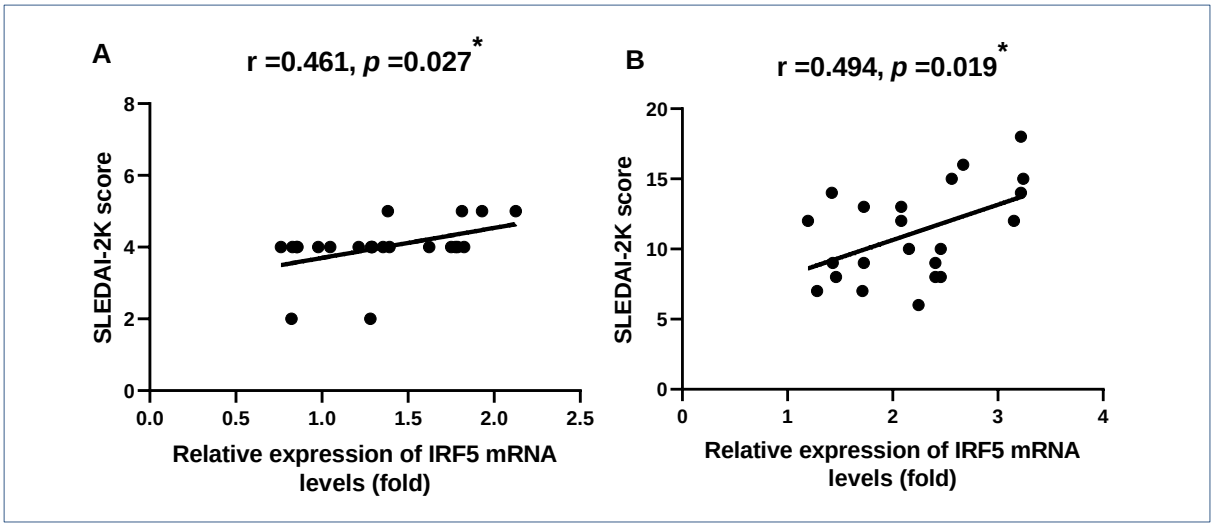

Figure 2: (A and B) The correlation between relative expression of IRF5 gene and SLEDAI-2K score in SLE (I) group and SLE (II) group, respectively. r = Pearson coefficient. p> 0.05= statistically non-significant correlation, * Statistically significant correlation at p< 0.05, ** Statistically significant correlation at $p\leq$0. 01.

Shu *et al*. (2017) found a positive correlation between IRF5 expression and INF-α expression levels in peripheral blood mononuclear cells (PBMCs) in childhood-onset SLE patients [30], and these findings support to explain our results about the positive correlation between expression levels of IRF5 and disease activity. This could be elucidated by numerous

studies that have suggested that the raised serum levels of IFN- alpha have been shown to be correlated with the activity and severity of SLE patients [16, 43, 44].

Correlation between expression of IRF5 gene and other parameters

The correlation between IRF5 gene expression levels and other parameters in both SLE patient groups is summarized in Table 5. The results indicated that the gene expression levels of IRF5 in SLE (I) group were positively correlated with uric acid (r=0.420, *p*=0.046), and ALP (r=0.429, *p*=0.041). However, in SLE (II) group expression levels of IRF5 were positively correlated with urea (r=0.6, *p*=0.003), creatinine (r=0.639, *p*=0.001), and uric acid (r=0.710, *p*=0.0001).

Table 5: The correlation between relative expression of IRF5 mRNA levels (fold) with other parameters.

parameter	Relative expression of IRF5 mRNA levels (fold)			
	SLE (I) group n= (23)		SLE (II) group n= (22)	
	r	*p*-value	r	*p*-value
Demographic Data				
Age (year)	0.120	0.584	0.219	0.328
Gender (F/M)	-0.239	0.273	0.072	0.72
BMI (Kg/m^2)	0.043	0.845	0.019	0.932
Disease duration (year)	-0.052	0.815	-0.246	0.269
Family history with SLE	-0.58	0.792	0.236	0.291
Hematological parameters				
WBC X 10^3 /UL	-0.358	0.094	0.319	0.148
RBC X 10^6 /UL	-0.223	0.307	-0.395	0.069
Hb (gm/dl)	-0.371	0.081	-0.406	0.061
PLT X 10^3 /UL	0.222	0.309	0.374	0.086
ESR (mm/1 hr)	0.406	0.055	0.109	0.629
Biochemical parameters				
Urea (mg/dl)	0.207	0.344	0.6	0.003**
Creatinine (mg/dl)	0.113	0.607	0.639	0.001**
Uric acid (mg/dl)	0.420	0.046*	0.710	0.0001**
GOT (U/L)	0.046	0.833	0.345	0.116
GPT (U/L)	0.227	0.298	-0.10	0.965
ALP (U/L)	0.429	0.041*	0.218	0.33
Total serum protein (g/l)	0.129	0.559	-0.290	0.190
Serum albumin (g/l)	-0.301	0.162	-0.414	0.056
Globulins (g/l)	0.334	0.108	0.112	0.620
Albumin /globulins	-0.358	0.094	-0.319	0.148
Total cholesterol (mg/dl)	0.108	0.623	0.269	0.226
Tri glyceride (mg/dl)	0.068	0.759	0.327	0.137
vLDL (mg/dl)	0.065	0.768	0.327	0.137
HDL (mg/dl)	-0.174	0.427	0.155	0.490
LDL (mg/dl)	0.139	0.527	0.253	0.257

r =Pearson coefficient. *p*> 0.05= statistically non-significant correlation, * Statistically significant correlation at *p*< 0.05, ** Statistically significant correlation at *p*≤0. 01. BMI= body mass index, WBC= white blood cells, RBC= red blood cells, Hb= hemoglobin, PLT= platelets, ESR= erythrocyte sedimentation rate, GOT= glutamate oxaloacetate transaminase, GPT= Glutamate Pyruvate Transaminase, ALP= alkaline phosphatase, vLDL=very low-density lipoprotein, HDL= high- density lipoprotein, LDL= low-density lipoprotein.

Our results of the positive correlation with urea, creatinine, and uric acid could be explained by the presence of some patients with lupus nephritis as shown in Table 1. Consequently, the presence of any criterion associated with renal disorder such as proteinuria and hematuria lead to elevate the score of disease activity. Therefore, patients with lupus nephritis ranked the high score of disease activity among the patients, subsequently increasing their expression of IRF5. Moreover, our result indicated that the IRF5 expression levels correlated positively with ALP in SLE (I) group, and this could be explained by Imran *et al*. (2021) who found that overall, 28 of the total 135 (20.7%) patients had liver abnormalities, including liver function tests and those detected using ultrasonography. About nine patients had raised alkaline phosphatase (ALP) or gamma-glutamyl transferase (GGT) levels are higher two-fold than the upper limit of normal values [48]. Takahashi *et al.* (2013) concluded that dysfunction of the liver in the presence of SLE can be triggered by many factors, but when existing at the time of SLE onset, either SLE itself or drugs can be the reason. Autoimmune hepatitis should be considered when liver dysfunction is rather severe [49].

ROC curve analysis of expression of IRF5 gene

In order to estimate the ability of the gene expression levels of IRF5 to distinguish between the active SLE patient and healthy subjects as a diagnostic immunological biomarker. We analyzed it using ROC curve analysis.

The result showed that the gene expression levels of IRF5 in SLE (II) group had a high ability to differentiate SLE patients from healthy individuals. The AUC, sensitivity, and specificity were 0.989, 95.5%, and 88.0%, respectively, at the cutoff value of 1.2492-fold, which was the perfect value of SLE correct prediction. While SLE (I) group had a good ability to discriminate SLE patients from healthy individuals. The AUC, sensitivity, and specificity were 0.769, 69.6%, and 80.0%, respectively, at the cutoff value of 1.199-fold, which was the good value of SLE correct prediction, as shown in Figure 3.

According to our results of ROC curve analysis, it showed that gene expression levels of IRF5 in SLE (II) group could represent a perfect predictor for SLE diagnosis, while in SLE (I) group could represent a good predictor for SLE diagnosis.

Items	SLE (I) (n=23)	SLE (II) (n=22)
AUC	0.769	0.989
SE	0.074	0.011
p-value	0.001	<0.0001
95% CI	0.625-0.913	0.968-1.000
Cut-off value	1.199	1.2492
Sensitivity	69.6%	95.5%
Specificity	80.0%	88.0%
PPV	76.2%	87.5%
NPV	74.1%	95.7%
Accuracy	75.0%	91.5%

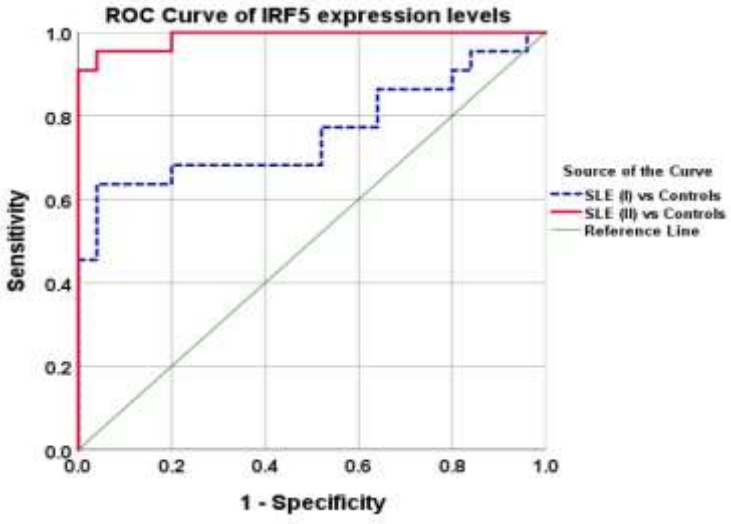

Figure 3: Receiver operator curve (ROC) analysis for the predictive value of relative expression of IRF5 mRNA levels (fold) in SLE (I) group (n=23), and SLE (II) group (n=22) versus healthy controls (n=25). AUC= area under the curve, SE= standard error, CI= confidence interval, PPV=positive predictive value, NPV= negative predictive value.

Conclusion

Our study results suggest that the expression levels of the IRF5 gene may play an important role in SLE pathogenesis, and it may be useful in the diagnosis of SLE patients based on ROC analysis. The up-regulation of IRF5 gene expression levels associated positively and significantly with disease activity in SLE patients reflect the possibility of using it as a potential immunological biomarker for diagnosis, monitoring the disease course, and response to therapy.

Ethical Clearance

The Research Ethical Committee at scientific research by ethical approval of both environmental, health, higher education, and scientific research ministries in Iraq.

Conflict Ofinterest

The authors declare that they have no conflict of interest.

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