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Evaluation the Serum Level and Gene Expression of IL-6 in Iraqi Systemic Lupus Erythematous Patients

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Abstract: The chronic systemic autoimmune disorder known as systemic lupus erythematous (SLE) can be distinguished serologically by both cellular and humeral immunologic abnormalities such as direction of many autoantibodies against non-organ specific intracellular antigens. The SLE is created by a combination of several environmental and genetic factors. The current study included 54 Iraqi females with SLE who were diagnosed by specialists in Baghdad Teaching Hospital / Medical City and Kadhimiya Teaching Hospital from June 2022 until September 2023 and 46 healthy women with an age range of 10-50 years old. This study aimed to evaluate the serum level and gene expression of interleukin-6 (IL-6) in SLE patients using ELISA technique and real time-PCR in addition to the possibility of their relationship with the disease pathogenesis. The demographic study showed that the highest age group of patients was 40.74% (21-30). Results show that the level of IL-6 serum were highly significant (p< 0.01) increased in patients group compared to control group. Additionally, IL-6 gene expression was increased about eight folds compared to control group. These results pointing to the association of IL-6 with SLE. The present study recommend to use IL-6 as a biomarker for SLE.

Keywords: Interleukin-6, SLE, Serum level, Gene expression.

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Introduction

Systemic lupus erythematosus (SLE) is a systemic immune disease that affects more than one organ if it occurs (1). It is also considered as a chronic autoimmune disease characterized by autoantibody production and multisystem inflammation (2). The cells and tissues in any part in the body may be attacked by the immune system, leading to tissue damage and inflammations (3).

Interleukin-6 (IL-6) can be released from macrophages, lymphocytes, endothelial cells, fibroblasts and dendritic cells. It is a pro-inflammatory cytokine with multiple functions (4). Naïve B-lymphocyte maturation into plasma cell can be directly induced by IL-6, and cytotoxic T-lymphocyte differentiation can be also facilitated by this cytokine throughout IL-2 and IL-2R upregulation (5). Because of such functions, systemic autoimmunity and pathologic inflammatory responses are promoted by IL-6 (6). SLE patients were reported to have higher S. IL-6 levels than healthy individuals in one study (7), indicating that IL-6 can be engaged in the pathogenesis of SLE. Furthermore, there is an evidence showing that the higher expression of S. IL-6 is significantly correlated with SLE

activities. Current study aimed to determine the relation between IL-6 level and gene expression as well as performing laboratory and clinical investigations among some Iraqi female SLE patient.

Materials and method

Study subjects

Patients group included 54 Iraqi females with SLE with an average age of 10-50 years were diagnosed by specialists in Baghdad Teaching Hospital / Medical City and Kadhimiya Teaching Hospital from June 2022 until September 2023. Patients with cancer, renal failure, diabetic and other autoimmune disease were excluded from this study. Apparently healthy individuals (46 females) were included within the present study as control group, they were matched with patients in age and sex to exclude the effect of these two factors on the study results.

Laboratory investigation

Laboratory investigations included estimation of total serum IL-6 levels and IL-6 gene expression by taking 5-10 ml of venous blood from each healthy women and SLE patients. Each sample has been separated into two tubes. The first tube used to extract RNA. In the second tube used to determine the level of IL-6 in the patients and control, determine the level of dsDNA, determine the level of complement 3 (C3) and complement 4 (C4).

ds-DNA measurement

The serum ds-DNA was measured by ds-DNA Screen ELISA Kit (Demeditec, Germany) based on direct ELISA assay.

C3 and C4 measurement

The human complement proteins C3 and C4 in blood serum was measured by Human Complement (C3 or C4) ELISA Kit based on sandwich ELISA technique (Shanghai YL Biont, China).

Measurement of interleukin-6 serum level

For serum IL-6 estimation, the ELISA method was applied in accordance with the manufacturer's guidelines Human IL-6 (Interleukin 6) ELISA Kit (Elabscience, USA).

Gene expression

RNA extraction

Total RNA was extracted from all samples using the TransZol Up Plus RNA Kit (ER501-01) according to the manufacturer instructions, 200 *µl* of chloroform was added to the tube then vortexed and incubated at room temp. then centrifuged at 10,000 rpm for 15 min. at 2-8 ℃. Into a new RNase free tube, the upper aqueous phase containing RNA was transferred and an equivalent volume of 96–100% ethanol was added and then centrifuged. 500 µl of clean buffer 9 (CB9) to a spin column was added and spinned at 12,000 rpm for 30 seconds then repeated this step. 500 µl of wash buffer 9 (WB9) that (WB9 with ethanol added) was added and then centrifuged. The spin column was placed in the clean 1.5 ml RNase-free tube and it was incubated for 1 minute at room temperature after adding 50-200 µl of RNase-free Water. The RNA was eluted by centrifuging at 12,000 rpm for 1 minute then the extracted RNA was Stored at -20°C.

Primer design

The primers were designed using the Primer 3plus V4, and double checked by the University of California, Santa Cruz (UCSC) programs and with their reference sequences in the database of the National Center for Biotechnology Information (NCBI) as illustrated in (Table 1).

Table (1): Primers sequences that were designed for IL-6 gene expression

Primer	Sequences (5′→3′ directions)	primer sizes bp	Products sizes bp	Ta °C
IL-6 (Gene expressions)				
Forward	AGTCCTGATCCAGTTCCTGC	20	196	58
Reverse	CTACATTTGCCGAAGAGCCC	20		
GAPDH Glyceraldehyde 3-phosphate dehydrogenases				
Forwarded	GAAATCCCATCACCATCTTCCAGG	24	160	58
Reversed	GAGCCCCAGCCTTCTCCATG	20		

Synthesis the cDNA form RNA

The complementary DNA (cDNA) synthesis by Easy Script® One Step gDNA Removal & cDNA Synthesis Super Mix AE311-02 Kits. The total RNA was reverse transcribed to cDNA. According to manufacturer's instructions, the operation was performed in a reaction volume of 20 µl (Table 2).

Table (2): Strand cDNA synthesis reaction component

Component	Volume reaction
Total RNA	5 µl
Anchored Oligo(dT)18 Primer (0.5 µg / µl)	1 µl
Random Primer (0.1 µg / µl)	1 µl
2xES Reaction Mix	10 µl
***EasyScript*® RT/RI Enzyme Mix**	1µl
gDNA Remover	1µl
RNase-free Water	1µl
Total volume	20µl

Quantitative real time PCR

The PCR master mix preparation and Real Time PCR Program thermal cycling conditions for IL-6 gene expression illustrated in (Table 3, 4).

Table (3): Reaction mixture of quantitative real-time PCR for IL-6 gene expression

Components	20 µl rxn
***2xTransStart*® Top Green qPCR Super Mix**	10
Nuclease free water	6
Forward Primer (10 µM)	1
Reverse Primer (10 µM)	1
cDNA	2

Table (4): Thermal profiles of GAPDH and IL-6 gene expressions

Steps	Temp. (°C)	Time (sec)	Cycle
Enzyme activations	94	60	1
Denaturations	94	10	45
Annealings	58	15	
Extensions	72	20	
Dissociations	62 °C-95 °C		1

Statistical analysis

For detection the effect of difference factors on the study parameters, the Statistical Analysis System (SAS 2012) program was applied. To compare between means significantly, the T-test and Least significant difference LSD test and

analysis of variation (ANOVA) were used. To compare between percentages (0.05 and 0.01 probabilities), the Chi-square test was used in this study. The (SAS 2012) program was used for estimate of correlation coefficient between variables.

Results and discussion

The clinical presentations of SLE patient and control group are shown in table (5). The SLE patient age ranged between (10-50) years. The percentage age for the studied groups was 20.37% (10-20), 40.74% (21-30), 25.92% (31-40) and 12.96% (41-50) respectively. These result showed a significant differences in the ages among the studied groups (p=0. 0026).

Table (5): Distribution of sample study according to onset of disease

Onset of disease/Years	Percentage (%)
10-20	20.37
21-30	40.74
31-40	25.92
41-50	12.96
Chi-Square-χ^2	14.322 **
P-value	0.0026
** (P≤0.01).	

The demographic distribution in this study showed that the number of old aged women with SLE were less than those in fertilizing periods (> 10 and < 40) years, which can be attributed to anxiety and psychiatric disorders or associated to steroid hormone, particularly estrogens and progesterones which affect the immune system. Low levels of progesterone in women affected with SLE indicate a relative imbalances in favor of estrogen, which can be involved in immune reactivity in certain patients (8). The highest number of women affected by SLE were within the age group (10-40) years in comparison with other age groups, which coincided with some studied conducted in Southern and Eastern Asia which demonstrated that all ages may be affected by SLE, but most frequently the age group (10-40) years(9).

Correlation of S. IL-6 levels with C3, C4 and dsDNA in SLE patients

Table (6) shows the correlation between S. IL-6 levels and C3, C4 and dsDNA in SLE patients. There was a highly significant positive correlation between S. levels of IL-6 and ds-DNA in SLE patients. There was a significant negative correlation between IL-6 with C3 and C4.

Table (6): Correlation of S. IL-6 levels with C3, C4 and dsDNA i

Group	Mean ± SE			
	C3	C4	dsDNA	IL-6
Patients	111.57 ±4.01	0.0278 ±0.001	4.84 ±0.79	70.62 ±12.96
Control	126.08 ±4.49	0.0489 ±0.003	2.75 ±0.25	27.22 ±3.23
X	12.174 *	0.0053 **	1.996 *	42.956 *
P-value	0.0201	0.0001	0.0403	0.0433
* (P≤0.05), ** (P≤0.01).				

In SLE patients, cytokine imbalance has been found (10). Nevertheless, different cytokines can play variable roles in SLE. However, the role of each singular cytokine and its correlation with the severity of the disease remains confusing (11). This indicates that the auto-antibodies in the serum was increased, which is part of the immune activation (this has been proven by the increasing ds-DNA, IL-6 in this study). The low levels of C3 and C4 in SLE patients means that the patients has increasing autoantibodies(12).

In addition, ds-DNA may become low after the disease improved with treatment, so the anti-DNA antibody can provide the basis for treatment monitoring (13). Where the percentage of ds-DNA is high in most of the cases in autoimmune diseases, including SLE (14). The explanation for the positivity of anti-dsDNA in the patient group is antinuclear antibody, but what distinguishes it is that it is mainly associated with SLE. B cells are released anti body, is D type switehed into IgG antibodies, and bind to the nucleus. Also, when apoptosis occurs, the cells fragment and release the remaining molecules from the cellular organelles, as well as the macrophages that phagocytosis some microorganisms, and as a result the antibodies are released and combine with antigens (forming immune complexes). The complement component works to degrade these complexes, perhaps most SLE patients have a deficiency in the complement component, and these antibodies will accumulate and appear higher in patients (15).

Interleukin-6 (IL-6) can be released from macrophages, lymphocytes, endothelial cells, fibroblasts and dendritic cells. It is a pro-inflammatory cytokine with multiple functions (16). Naïve B-lymphocyte maturation into plasma cell can be directly induced by IL-6, and cytotoxic T-lymphocyte differentiation can be also facilitated by this cytokine throughout IL-2 and IL-2R upregulation (17). Because of such functions, systemic autoimmunity and pathologic inflammatory responses are promoted by IL-6 (18). SLE patients were reported to have higher S. IL-6 levels than healthy individuals in many studies (19), indicating that IL-6 can be engaged in the pathogenesis of SLE.

Molecular analyses (quantitative expression of IL-6 gene)

The mean of Ct value of IL-6 gene in patients group was (32.101) and in control group was (35.187), the Statistical analyses show high significant difference between gene folding change of patients group (8.276) and in control group (1.0) is shown in (Table 7).

Table (7): Fold of IL6 expression depending on 2-ΔCt method.

Groups	Means Ct of IL6	Means Ct of GAPDH	ΔCt (Means Ct of IL-6- Means Ct of GAPDH)	$2^{-\Delta Ct}$	experimental group/ Control group	Fold of gene expression
Patient	32.101	17.437	14.664	0.000039	0.000039/0.000005	8.276
Control	35.187	17.474	17.713	0.000005	0.000005/0.000005	1.000

An up-regulation of IL 6 gene expression was observed in the blood samples of SLE patients, and it was more pronounced in patients with immunological factors than control groups. Many mechanisms can demonstrate the positive correlations between S. IL-6 levels and SLE

disorder. Serious immune complex depositions can be caused by autoantibody overproductions, resulting in a great promotion of SLE pathogenecity. It was indicated that IL-6 can cause increased production of autoantibodies via promotion of the autoreactive B cell proliferations and differentiation of naive B lymphocytes into plasma cell. In addition, IL-6 can up-regulate the recombination activating gene expression, also leading to overproduction of autoantibodies in SLE (20).

Conclusion

It can be concluded that there is a clear association between IL-6 levels and SLE. The relationship between IL-6 and other laboratory parameters may act as a biomarker to observe and predict the severity of the disease, which can help SLE patients to increase their treatment and diagnostic options.

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