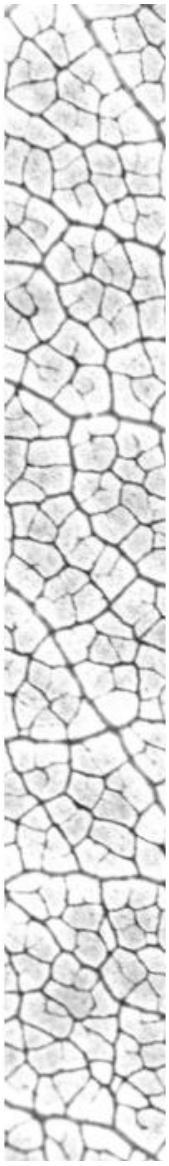

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The association between genetic polymorphisms of Il-6 gene and susceptibility of systemic lupus erythematosus in Iraqi population

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Abstract
Systemic lupus erythematosus (SLE) is a systemic autoimmune disorder that affects many organs and is characterized by cytokine imbalance and hereditary factors that give a predisposition to the diseases development. The present study aimed to determine whether single nucleotide polymorphism (SNP) in the promoter region of IL-6 gene (-174G/ C) plays an important role in the SLE in Iraqi population. Blood samples of 103 SLE patients and 100 healthy individuals were collected. Serological test of serum anti-dsDNA was assayed, and it was very efficient in diagnosis of the disease. The sensitivity and specificity of anti-dsDNA was 96.2% and 100%, respectively, with high predictive value. The Genotyping of IL-6 gene SNP was carried out by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay. The genotype distribution showed significant difference (P<0.05) between controls (GG: n= 69, 69%; GC: n=22, 22%; CC: n=9,9 %) and SLE patients (GG : n= 38, 36.89 %; GC : n=41, 39.80 %; CC : n=24, 23.3%). C allele frequency was significantly increased in SLE patients than in controls (X2=25.204; P<0.05; OR=3.043, 95% CI=1.954-4.738). In conclusion, the frequency of C allele (heterozygous and /or homozygous) in Iraqi SLE patients may indicated that it may be play a big role in pathogenesis of SLE and it can be considered as a major cause of local tissue inflammation that involved in all certain complication of SLE disease.

Keywords: systemic lupus erythematosus, Promoter of IL-6 gene, SNP, Iraq

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INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disease that affects multiple organs. The complex interaction between genetics, environment and hormones causes immune regulation to break down and the tolerance of autoantigens to break down, resulting in the production of autoantibodies, inflammation and destruction of the final organs (Moulton et al. 2017). Clinical heterogeneity of the disease was forced to establish 11 criteria, with the need for 4 criteria for the formal diagnosis of systemic lupus erythematosus (SLE) (Tsokos 2011; Ghosh, 2018).

The etiology of lupus is not known, but many Environmental factors play a role in being a trigger for SLE which include ultraviolet light, smoking (Simard et al. 2009), and viruses (like Epstein– Barr virus (EBV) (Draborg et al. 2016). Increased risk of lupus affected by hormones, estrogen- or prolactin, psychosocial factors (Cooper et al. 2002, Pan et al. 2019), and also associated with using of oral contraceptive, early age at menopause (Costenbader et al. 2007).

In addition, familial aggregation researches show that 11% of patients with SLE have first or second degree relatives with the disease (Cárdenas-Roldán et al. 2013).

Genetic factors play an important role in preparing for SLE. Different genes in chromosome 6 of the main histocompatibility complex (MHC) and non-MHC (polymorphic genes) have a strong effect on SLE sensitivity (Relle & Schwarting 2012, Ceccarelli et al. 2015).

In addition to all these risk factors, the severity of SLE is associated with an imbalance of cytokines responsible for causing autoimmune diseases, infections, and infectious diseases. (Gottschalk et al. 2015).

Numerous studies have shown a clear picture of the imbalance of cytokine production, which contributes to weakened immunity and organ damage and mediates tissue inflammation in SLE patients. (Ohl and Tenbrock, 2011).These Inflammatory cytokines include IL-1, IL-2,

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IL-4, IL-6, IL-16, IL-17, IL-18 (Lauwerys & Houssiau, 2003, Ohl& Tenbrock, 2011, Stypińska & Paradowska-Gorycka 2015), tumor necrosis factor α (TNF-α) (Chen et al. 2019), IFN-γ (Benagiano et al. 2019).This disorder in the regulation of the immune system results in the progressive development of autoantibodies and the formation of immune complex (Choi et al. 2012). SLE is a complex autoimmune disease characterized by various autoantibodies in the blood of patients (Luo et al. 2019).

Sherer et al. (2004) found that 116 autoantibodies have been discovered in SLE patients. The presence of a large amount of autoantibodies directed to self-antigens mainly of nuclear origin (ANAs) consist of various types of autoantibodies is the hallmark of the disease (Dema and Charles 2016).

These antinuclear antibodies characterized by different antigen specificities, and these antigens include single and double strand (ss & ds) DNA, nucleosomes, histones, proteins of centromere, and many type of extractable nuclear antigens (ENA) (like Smith antigen (Sm antigen), ribonucleoprotein (RNP), Ro, and La) (Cozzani et al. 2014).

The most important type of these ANAs autoantibodies is that it is against dsDNA which is a distinct feature of SLE. IgG-class dsDNA antibodies are very important in diagnosing the disease. It is also associated with lupus nephritis (de Leeuw et al. 2017). Deoxyribonucleoprotein antibodies (anti-DNP) are one of the common ANAs encountered in SLE (consisting of the DNA and proteins (Mainly consisting of histones)), which are found in SLE patients with a higher prevalence rate for arthritis (Tang et al. 2010).

IL-6 is a multifunctional cytokine (28 kDa protein) produced in endothelial cells, monocytes, T and B lymphocytes, and fibroblasts which promote activation and differentiation of a differentiation factor of B cells, T cells, macrophages and, neutrophils (Naka et al. 2002).

The IL-6 gene is located on the short arm of chromosome 7. The IL-6 gene consists of five exons and four introns. IL-6 gene translation and post-translation modification results in a long-chain protein consisting of 128 amino acids (May et al. 1988).

Increased serum levels of IL6 in some studies are associated with flares of disease in patients with active SLE (Grondal et al. 2000, Chun Jain et al. 2007, Jain et al. 2016).

In addition to systemic effects, IL-6 has been involved in driving the production of autoantibodies, as well as it plays an important role in local inflammation which includes lupus nephritis and participates in the multiplication of mesangial cell, which is a feature of the complications of proliferative nephritis SLE (Tsai et al. 2000, Tackey et al. 2004).

Studies have suggested an association between the IL-6 gene polymorphisms and the susceptibility of SLE (Cui et al. 2010, Godarzi et al. 2011, Katkam et al. 2017).

Table 1. Criteria for the Diagnosis of Systemic Lupus Erythematosus (SLE) from American College of Rheumatology (ACR) (four of the 11 criteria are needed for the formal diagnosis of SLE) (Hochberg, 1997)

Criterion	Definition
Malar rash	" A rash on the cheeks and nose, often in the shape of a butterfly"
Discoid rash	"A rash that appears as red, raised, disk-shaped patches"
Photosensitivity	" A reaction to sunlight that causes a rash to appear or get worse"
Oral ulcers	"Sores in the mouth"
Arthritis	"Joint pain and swelling of two or more joints"
Serositis	"Inflammation of the lining around the lungs (pleuritis) or inflammation of the lining around the heart that causes chest pain, which is worse with deep breathing (pericarditis)"
Kidney disorder	"Persistent protein or cellular casts in the urine"
Neurologic disorder	"Seizures or psychosis"
Blood disorder	"Anemia (low red-cell count), leukopenia (low whitecell count), lymphopenia (low level of specific white cells), or thrombocytopenia (low platelet count)"
Immunologic disorder	"Positive test for anti–double-stranded DNA, anti-Sm, or antiphospholipid antibodies"
Abnormal antinuclear antibodies	"Positive antinuclear-antibody test"

The aim of this study was to find out whether a polymorphic variant of IL-6 (at -174G/ C) of promoter region of gene plays an important role in SLE Iraqi patients.

MATERIALS AND METHODS

a) **Patients**: One hundred and three systemic lupus erythematosus patients, who admitted to Alsader Teaching Hospital in al-Najaf, were included in this study, after taking their consent. The project was ethically approved by the committee of Faculty of Science at the University of Kufa.

All these patients had been previously diagnosed as having definite SLE according to the 11 classification criteria of SLE (at least 4, with involvement, for all of these patients, renal and hematologic disorder) (Hochberg, 1997).

b) **Controls**: It consists from 100 healthy individuals; all were without autoimmune disorders and clinical manifestation of any disease.

Blood Samples

Four ml of venous blood was collected from clinically suspected patients and control.

a) Two ml was allowed to clot at room temperature then centrifuged at 3000 rpm for 5 minutes the serum was used freshly for the serological test of serum anti-dsDNA.

b) Two ml of venous blood was collected in EDTA tubes and store at -20°C until used for PCR test.

Serological test of serum anti-dsDNA

Serum anti-dsDNA was tested using a rapid latex agglutination test kit for the detection of antibodies in serum to double stranded (ds) DNA in the SLE patients and controls. The kit provided by AVITEX Inc. (SLE test, Omega, UK).

Table 2. PCR reaction conditions for IL-6 gene amplification

Initial Denaturation	Numbers of Cycles	The compositions of each cycle			Extension Step
		Denaturation	Annealing	Extension	
95ºC for 5min	30 cycles	94 ºC for 30 sec	52 ºC for 45 sec	72 ºC for 45 sec	70 ºC for 5 min

DNA isolation and RFLP-PCR technique

Genomic DNA was isolated using protocol from Genomic DNA Mini Kit (Geneaid Biotech. Taiwan) protocol procedure, which was specially designed to purify DNA from frozen blood.

A sequence of single nucleotide polymorphic polymorphisms in the region of promoter in IL-6 gene (-174G/ C) was amplified using the primer-pairs: forward primer -'5TTG TCA AGA CAT GCC AAA GTG-3' and reverse primer -'5TCA GAC ATC TCC AGT CCT ATA-3'. These primers have already been published previously (Lorente et al. 2016), and synthesized by AccuOligo® Bioneer Corporation, USA. All these primers were reached to us as lyophilized form with different concentration. We dissolved them with appropriate volume of TE buffer for each one (according to the manufacturer) in order to get 100 pmole/µl (stock solutions). Working solution with10 pmole/µl was prepared from stock solutions.

Polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP) was performed to detect IL-6 promoter polymorphism (-174G/ C). All DNA samples were amplified individually using primers and corresponding cycling conditions (as described in **Table 2**) by using a thermal cycler (Labnet/USA).

The PCR product (300-bp) was digested with N1aIII restriction enzyme (New England Biolabs, USA, 10 units was sufficient, and generally 1µl was used) at 37°C overnight. One of these results was yielded after enzyme digestion for each sample:

1. PCR fragment remains intact (300-bp) when the patient has two normal alleles, GG (homozygous genotype patient).
2. Two fragments with different length (169 and 131-bp) when the patient has two mutant alleles, CC (homozygous mutant genotype patient).
3. Three fragments (300,169 and 131 bp) when the patient has one normal and one mutant allele, GC (A heterozygous genotype patient) had 131-bp.

Finally, the gel electrophoresis method was done which included preparing the agarose gel (2% agarose gel), loading 5 µl of each samples and running the gel (Sambrook and Russell 2001).

Statistical Analysis: Statistical analyses of all results were carried out by the help of SPSS version 23 software statistical package using Chi-square test (with P value at level of significance less than 0.05) to compare values of results between groups. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV), for anti-dsDNA test were calculated according to Lalkhen and McCluskey (2008) (which described in terms of TP: true positive, TN: true negative, FN false positive: and FP: false positive) as the following:

Sensitivity = TP / (TP + FN) ×100
Specificity = TN / (TN + FP) ×100
PPV= TP/ (TP + FP) ×100
NPV=TN/ (TN + FN) ×100

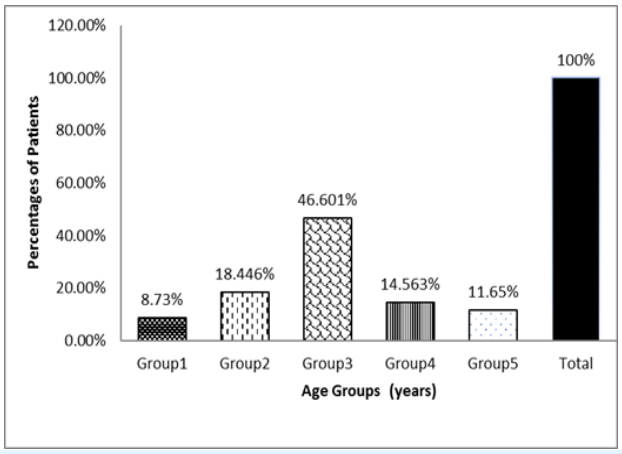

Fig. 1. Age distribution of systemic lupus erythematosus (SLE) patients (Group1: 15-22 years, Group2: 23- 30 years, Group3: 31-38 years, Group4: 39- 46 years, and Group5: 47-54 years

RESULT AND DISCUSSION

The study included one hundred and three systemic lupus erythematosus patients and 100 healthy individuals without autoimmune disorders and clinical manifestation of any disease. In Iraq the SLE disease has wide prevalence, so that led to attempt to give a greater awareness for this major health problem by providing many sensitive laboratory tests necessary to diagnose SLE.

All SLE patients according that involved in this study were female (100%). Their age ranged between 15 and 54 years, age mean was 34.4 ± 11.66 years (mean ± SD) compared to controls, where age mean was 32.7 ± 10.2. Assessment of age presentation of SLE patients revealed that 9 (8.737%) patients were seen in age group 15-22 years, 19 (18.446%) in age group 23- 30, 48 (46.601%) in age group 31-38 years, 15 (14.563%) in age group 39- 46, and 12 (11.65%) in age group 47-54 years. The estimated incidence of SLE increased significantly (P< 0.05) in the third age group (31-38 years) in comparison with the other groups (**Fig. 1**). There was no significant difference (P> 0.05) in the distribution of age groups between the patients and controls.

The high prevalence of females in this study agree with another studies in Iraq (Noori et al. 2013, Al-Rawi et al. 2014), where females represented 91% and 98%,

Table 3. The results of the anti-dsDNA latex agglutination test

Subjects (N)	Anti-dsDNA test results		Total	P value
	Positive	Negative		
Healthy controls (N=100)	0	100 100%	100 49.26%	P < 0.001*
SLE patients (N=103)	99 96.11%	4 3.88%	103 50.738%	
Total	99 48.768%	104 51.231 %	203 100%	

Data were expressed as number of patients (result values also represented as a percentage (%).
*P < 0.05 is significant. Abbreviations: dsDNA: double-stranded DNA

respectively, of total patients involved in these studies in Iraq. SLE is characterized by a high prevalence of females (9: 1 ratio of female to male cases) (Petri, 2002). The female predominance was recorded in the largest American study (1,103 patients) (Ginzler et al. 1982) and European series (1,000 patients) (Cervera et al. 1993) where females represented 88% and 91% respectively.

Female dominance characterizes SLE cases (especially during reproductive years) indicating that many factors related to female reproductive years, for example, estrogen cause low levels of B-cell production, and poor B-cell tolerance, which plays an important role in pathogenesis of SLE (Cohen-Solal et al. 2008, Schwartzman-Morris and Putterman 2012). This is consistent with the results of the current study, which showed that the peak of SLE incidence for female is during reproductive years.

The use of estrogen from external sources, like hormone therapy, oral contraceptives, and postmenopausal hormones, which represented exogenous estrogen; also it has a major effect on the pathogenesis of SLE (Costenbader et al. 2007, Zandman-Goddard et al. 2012).

Other causes of female dominance are the number of X chromosomes, and genetic variants (on the X chromosome) because they are associated with a higher risk of developing SLE. The presence of two an effective X chromosome appears to give a greater risk of developing SLE than a single X chromosome (Scofield et al. 2008, Weckerle and Niewold 2011).

Serological study (anti-dsDNA)

Among the 103 patients of SLE disease, 99 (96.11%) cases were positive for anti-dsDNA (**Table 3**), while 4 (3.88%) cases and all the controls (100%) were negative. There was a significant difference (P<0.05) between positive and negative results.

One of the best serological tests of SLE is Anti-double-stranded DNA (dsDNA) (Conti et al. 2015). Antibodies to dsDNA involved in the pathogenesis of lupus nephritis by accumulation in the glomerular and tubular basement membrane and bound to surface cell antigens or the basement membrane of glomerular glomerular components through chromatin (Wang& Xia 2019). The sensitivity of anti-dsDNA was 96.2% and specificity of this serological test was 100% with the high predictive value (**Table 4**). These results were agreed with many studies results (Eriksson et al. 2011, Wichainun et al. 2013, Almeida González et al. 2015).

Table 4. Sensitivity, Specificity, and Predictive Values of anti-dsDNA latex agglutination test

Anti-dsDNA latex agglutination test	SLE patients (N=103)	Healthy controls(N=100)	Total
Positive	99	0	**99**
Negative	4	100	**104**
Total	103	100	**203**
Sensitivity	Specificity	PPV	**NPP**
96.2%	**100%**	**100%**	**96.2%**

Data were expressed as number of patients. Abbreviations: dsDNA: double-stranded DNA; PPV: positive predictive value; NPV: negative predictive value

Table 5. The Results of the genotypic distribution of IL-6 (at -174G/ C of promoter region) in Systemic lupus erythematosus (SLE) patients

Genotype	Controls (N=100) (49.26%)	patients (N=103) (50.738%)
GG	69 (69%)	**38 (36.89 %)**
GC	22 (22 %)	**41(39.80 %)**
CC	9 (9%)	**24 (23.3 %)**
P-value	**0.000022***	
Alleles frequency	N(%)	**N(%)**
G allele	160(80%)	**117(%56.796)**
C allele	40(20%)	**89(%43.2)**
X2	**25.204**	
P-value	**0.0000***	
OR (95%CI)	**3.043(1.954-4.738)**	

Data were expressed as number of patients (Result values also represented as a percentage (%)).
*P <0.05 significant. Abbreviations: X2= chi-square, OR= odds ratio,CI= confidence interval.

Molecular Study

To study the effect of IL-6 -174G/ C polymorphisms on the susceptibility and prognosis of SLE, the SNP was analyzed in both the 100 healthy subjects and 103 SLE patients by using PCR-RFLP analysis.

IL-6 polymorphism in controls

Among the 100 healthy subjects; 69 (69%) had found as homozygous genotype (GG alleles),22 (22%) had found as heterozygous genotype (with both GC alleles), and 9 (9%) found as homozygous genotype (CC alleles); (GG : n= 69, 69%; GC : n=22, 22%; CC : n=9 ,9 %, **Table 5** and **Fig. 2**).

IL-6 promoter polymorphism in SLE patients

Among the 103 SLE patients , 38 (36.89 %) had found as homozygous genotype (GG alleles), 41 (39.80 %) had found as heterozygous genotype (with both GC alleles), and 24 (23.3%) found as homozygous genotype (CC alleles); (GG : n= 38, 36.89 %; GC : n=41, 39.80 %; CC : n=24 , 23.3%, **Table 5** and **Fig. 2**), with a significant difference with that of subjects in control group (p<0.05). These findings are consistent with those of Mellor-Pita et al. study (2009) and Yang et al. study (2014) which indicated that the IL-6 -174G/ C polymorphisms could be candidates for SLE susceptibility.

Godarzi et al. study (2010) showed beyond doubt that −174 G/ C polymorphism was associated with the presence of nucleus antibodies in all patients concluding

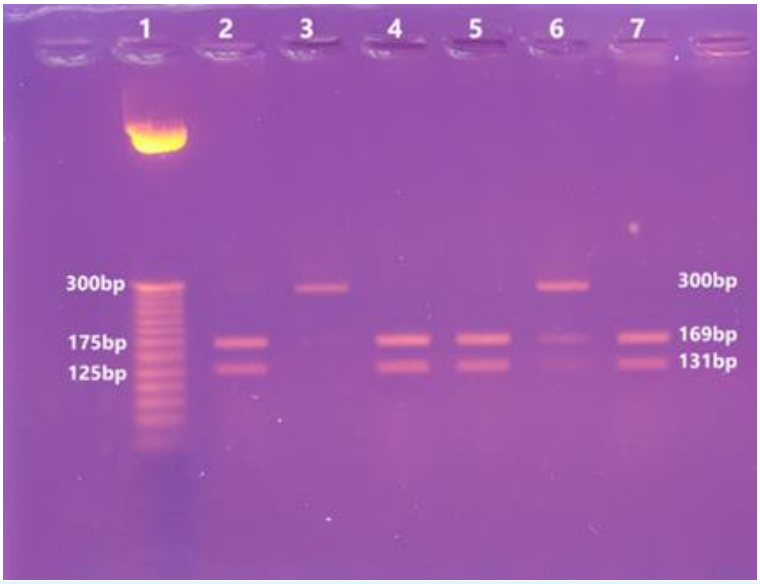

Fig. 2. Electrophoresis image of IL-6 gene polymorphism (-174G/ C) (Lane 1 : DNA 25 bp ladder; lane 2, 4, 5&7: CC (131-bp, 169-bp), lane 3: GG (300-bp); lane 6: GC (131-bp, 169-bp and 300-bp)

that IL-6 −174 GC is important for the production of disease-associated autoantibodies in the Iranian population. While the study of Talaat et al. (2016) found that both GG genotype and G allele of IL-6 gene polymorphisms (-174G/ C) could be considered risk factors for SLE.

IL-6 is mainly organized at the transcriptional level by the regulatory elements in the promoter region of the gene. In this region, -174G/ C is important regulator of transcription (Hulkkonen et al. 2001). Serum IL-6 levels are increased in SLE patients and have correlated with disease activity or anti-dsDNA levels (Esposito et al. 2009).

IL-6 is closely related to specific pathological manifestations of SLE. Elevated levels of IL-6 in cerebral spine fluids have been found in SLE patients and may provide an effective diagnostic scale (Hirohata et al. 2009).

Bettelli et al. (2006) mentioned that IL-6 play important role in the regulation of hepatic synthesis of acute phase reactors, and participates in T helper cell differentiation 17 (Th17), which is understood to be pivotal in inducing autoimmune diseases.

C allele frequency was significantly increased in SLE patients than in controls ($X^2=25.204$; $P<0.05$; OR=3.043, 95% CI=1.954-4.738). In harmony to our results, Asano et al. (2013) have found that a strong correlation was observed in the frequency of the allele C between SLE patients and control groups in residents of northeastern Brazil.

These results differed with Abbas et al. study (2011) which found that the frequency of the allele G was higher than the C allele in both patients (83.3% vs. 16.7%) and controls (85% vs. 15%). SLE patients with the GG genotype showed significantly higher frequencies and increased risk of manifestations features when the disease appears.

CONCLUSIONS

The current study revealed that polymorphism of IL-6 gene and C allele frequency (heterozygous and /or homozygous) in Iraqi SLE patients may play a big role in the pathogenesis of SLE and it can be considered as a major cause of local tissue inflammation that involved in all certain complication of SLE disease.

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