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Single Nucleotide Pleomorphisms of Different Cytokines among Jordanian Patients with Lupus Nephritis: A Pilot Study

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ABSTRACT

Lupus nephritis (LN) is one of the most common clinical manifestations of systemic lupus erythematosus (SLE) and significantly affects the morbidity and mortality associated with SLE. Both environmental and genetic factors contribute to the development of SLE. There is growing interest in identifying genetic markers, particularly cytokine-related variants, that may indicate susceptibility to SLE, predict future organ involvement, and monitor changes in disease activity. This study aims to examine a wide array of cytokine genetic variants and their association with LN in a cohort of 83 Jordanian samples. Genotyping was performed using the Polymerase Chain Reaction/Sequence Specific Primer (PCR/SSP) technique. Our results show an increased frequency of the G allele at the promoter region (-1082 A/G) of interleukin-10 (IL-10) in LN samples ($p = 0.001$) compared to controls, whereas the A allele predominated in the control group ($p = 0.001$). At the genotype level for the -1082 A/G promoter region of IL-10, the GG genotype was significantly associated with LN ($p < 0.001$), while the GA genotype predominated in controls ($p = 0.027$). No significant associations were observed between other cytokine SNPs (IFN-γ, IL-6, TNF-α) and LN in Jordanian SLE patients. Our pilot study suggests that the G allele and GG genotype at the IL-10 promoter region (-1082 A/G) increase the risk of developing LN in Jordanian SLE patients, while the A allele and GA genotype are more common in controls.

Keywords: lupus nephritis, cytokines, SNPs, Jordan.

INTRODUCTION

The autoimmune disease known as systemic lupus erythematosus (SLE) is heterogeneous, inflammatory, and characterized by excessive synthesis of autoantibodies against a variety of auto-antigens, primarily nuclear components, and the creation of immune complexes [1, 2]. SLE, which can cause symptoms like nephritis, leukopenia, arthritis, skin rashes, and neurological inflammation, is nine times more common in women than in men [3, 4]. As with other inflammatory autoimmune diseases, the exact etiology of SLE remains mysterious. But, environmental, epigenetic, and genetic factors are believed to be crucial in its development[5, 6, 7]. Genome-wide association studies (GWAS) have significantly improved our comprehension of the inherent origins of SLE over the past ten years [8]. Approximately one hundred SLE genetic loci have been found thus far, predominantly in Asian and European populations, and they can explain up to 3 percent of the heritability of SLE [9]. The human leukocyte antigen (HLA), specifically HLA class II genes, have by far the highest link with vulnerability to SLE [10, 11]. In addition, a recent study conducted among Jordanian SLE patients showed that the class II alleles of HLA

[a] have equal contribution
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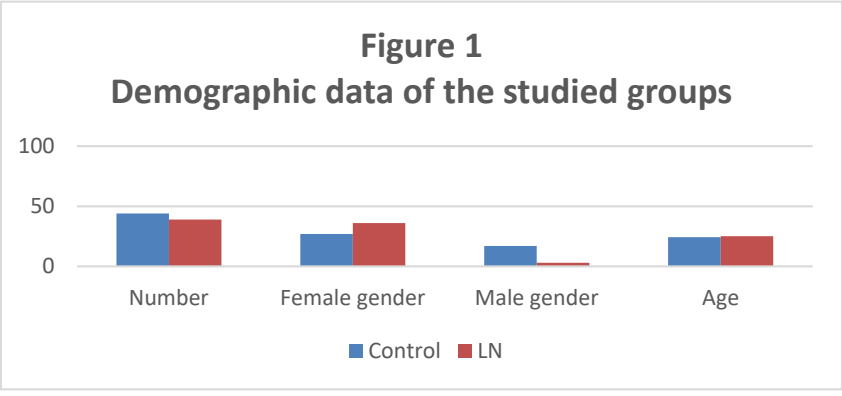
namely DRB1: *0301 and *1501 and DQB1*0601 elevated the risk of developing SLE [12]. Moreover, several meta-analysis studies and review articles encompassing studies across diverse populations and ethnicities support the implication of different cytokines in the pathogenesis of SLE. These cytokines play pivotal roles as mediators of inflammatory and immune responses and are associated with various levels of cytokines produced, being as high, intermediate, or low [2, 13-16]. While the genes responsible for producing these cytokines differ, multiple studies have highlighted the notable influence of single nucleotide pleomorphisms (SNPs) in diverse cytokines on predisposition to SLE. Notably, interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) are pro-inflammatory cytokines that have a fundamental role in regulating humoral and cellular responses, in which both play an essential role in inflammation [14, 17-19]. In addition, several studies showed that SNPs of interleukin-10 (IL-10) which have immunosuppressive properties, along with SNPs of interferon-γ (IFN-γ), both are key T helper-1 cytokines involved in immune cell activation [13, 20-24].

Lupus nephritis (LN), a severe and prevalent clinical feature of SLE that reached 50.4% in Arab countries including Jordan, as well as for African Americans, Hispanics, and Europeans LN was estimated to reach 68.9%, 60.6%, and 29.1% respectively[25-27]. Our study aims to examine a wide array of cytokine SNPs among Jordanian LN patients in the promoter areas of the following cytokine DNA segment: TNF-α (−308 A/G) (rs1800629) and IL-6 (−174 G/C) (rs1800795), three polymorphic regions of IL-10: (−1082 A/G) (rs1800896), (−819 C/T) (rs1800871) and (−592 A/C) (rs1800872), as well as IFN-γ +874 (rs62559044) in comparison with healthy controls.

Sample collection

A total of eighty-three individuals took part in this study, 39 LN patients (36 females, 3 males,) and 44 healthy controls (27 females, 17 males) (Figure 1). All the patients were Jordanians who were enrolled at the Jordan University Hospital in Amman with IRB approval number (146/2021). As well as the control group was collected from Al-Zaytoonah University Health Center with IRB approval number (4/2/2020-2021). The patients diagnosed with LN had a mean age of 25.15 with a standard deviation (SD) of ±10.78 years, while the control group had a mean age of 24.25 with SD ± 6.15 years. The inclusion criteria for all participants were as follows: they had to be between the ages of 22 and 50, all participants had to be of Jordanian nationality with no relative degree among them. In this study, healthy volunteers with a family member affected by SLE or any other autoimmune disorder were excluded. However, patients with SLE and LN were selected based on the standards outlined by the American College of Rheumatology (ACR) [28]. Following the volunteer's agreement by signing a consent document, 3 ml of blood was gathered using EDTA tubes.



DNA extraction and cytokine SNPs genotyping

After collecting a three-milliliter complete blood sample, the DNA was extracted by the Wizard® Genomic DNA extraction Kit by Promega, USA, according to the previously outlined protocol [29]. The cytokine SNPs detection of IL-6 (−174G/C), TNF-α (−308A/G), three polymorphic regions of IL-10: (−1082A/G), (−819C/T), (−592A/C), and IFN-γ (+874) was carried out through polymerase chain reaction/sequence-specific primers (PCR/SSP) technique of One Lambda, USA Genotyping Tray following the manufactures' protocol.

Statistical analysis

The Statistical Package for Social Sciences (SPSS) program (IBM SPSS Statistics 22, USA) was used for statistical analysis of the data in the current study. Alleles, genotypes, and haplotypes frequency were calculated according to the rate of recurrence of each allele, genotype, and haplotype among the total number of alleles, genotypes, and haplotypes for each SNPs loci, and the categorical variables were presented as frequency (%). Cross-tabulation analysis was used evaluate the link among various cytokines alleles, genotypes and haplotypes and LN in comparison to controls. Statistical significance within the two studied groups was calculated by using Chi-square (X^2) test with P-value less than 0.05[30].

To assess the variables linked to LN, a two-step binary regression analysis (LN vs. control) was used to estimate the 95% Confidence Interval (CI) odds and ratio (OR). Genotypes for TNF-*α*, IL-6, IFN-γ, and IL-10 were used as independent factors in the regressions. Results were considered significant if the P value was less than 0.05.

Results

TNF-α (-308A/G) gene pleomorphism

The TNF-α (-308A/G) allele and genotype occurrences are shown in Table 1 for both the control and LN patient groups. The results showed that the frequency of the G allele was 78.4% in the control group while 88.5% in LN patients without any statistical significance. The frequency of A allele was 21.6% and 11.5% among control group and LN patients respectively. Additionally, at the genotype level, the most frequent genotype was GG with frequency of 63.6% and 82.1% among control group and LN patients respectively, followed by GA genotype with frequency of 29.5% and 12.8% among control group and LN patients respectively. Overall, the TNF-α (-308A/G) allele and genotype polymorphisms did not vary significantly between the two groups (Table 1).

Table 1: The allele and genotype occurrence of the TNF-α (-308A/G) SNPs.

TNF-α (-308A/G) SNPs			
Allele	**Control N=88 (Frequency %)**	**LN N=78 (Frequency %)**	**P Value**
G	69 (78.4%)	69 (88.5%)	ref
A	19 (21.6%)	9 (11.5%)	0.084
Genotype	N=44 (Frequency %)	N=39 (Frequency %)	
G/G	28 (63.6%)	32 (82.1%)	0.061
G/A	13 (29.5%)	5 (12.8%)	0.065
A/A	3 (6.8%)	2 (5.1%)	0.747

N: Number, LN: lupus Nephritis, (P value <0.05).

IL-6 (-174 G/C) gene pleomorphisms

The IL-6 (-174 G/C) allele and genotype occurrences are shown in Table 2 for both the control and LN patient groups. The results showed that the frequency of the G allele was 79.5% in the control group and 80.8% in LN patients without any statistical significance. While the frequency of C allele was 20.5% and 19.2% among control group and LN patients respectively. Additionally, at the

genotype level, the most frequent genotype was GG with frequency of 68.2% and 76.9% among control group and LN patients respectively. Our findings revealed that the occurrence of allele and genotype polymorphisms of the promotor IL-6 (-174 G/C) did not significantly vary between the LN and control groups. (Table 2).

Table 2: The allele and genotype occurrence of IL-6 (-174 G/C) SNPs.

FIL-6 (-174 G/C) SNPs			
Allele	**Control N=88 (Frequency %)**	**LN N=78 (Frequency %)**	**P Value**
G	70 (79.5%)	63 (80.8%)	ref
C	18 (20.5%)	15 (19.2%)	0.844
Genotype	N=44 (Frequency %)	N=39 (Frequency %)	
G/G	30 (68.2%)	30 (76.9%)	0.375
G/C	10 (22.7%)	3 (7.7%)	0.060
C/C	4 (9.1%)	6 (15.4%)	0.379

N: Number, LN: lupus Nephritis, (P value <0.05).

IFN-γ (+874 T/A) gene pleomorphisms

The **IFN-γ (+874 T/A)** allele and genotype occurrences are shown in Table 3 for both the control and LN patient groups. The results showed that the frequency of the T allele was 58% in the control group and 69.2% in LN patients while the frequency of the A allele was 42% and 30.8% among control group and LN patients, respectively. While at the genotype level, the most frequent genotype was TT with frequency of 34.1% and 51.3% among control group and LN patients respectively, followed by TA genotype with frequency of 47.7% and 35.9% among control group and LN patients respectively. Our results show that the two groups did not differ significantly at the allele or genotype level of the IFN-γ +874 T/A polymorphisms (Table 3).

Table 3: The allele and genotype occurrence of IFN-γ (+874 T/A) SNPs.

IFN-γ (+874 T/A) SNPs			
Allele	**Control N=88 (Frequency %)**	**LN N=78 (Frequency %)**	**P Value**
			Control-LN
T	51 (58%)	54 (69.2%)	ref
A	37 (42%)	24 (30.8%)	0.133
Genotype	N=44 (Frequency %)	N=39 (Frequency %)	
T/T	15 (34.1%)	20 (51.3%)	0.113
T/A	21 (47.7%)	14 (35.9%)	0.276
A/A	8 (18.2%)	5 (12.8%)	0.502

N: Number, LN: lupus Nephritis, (P value <0.05).

IL-10 gene pleomorphisms

The three SNPs promoter regions for IL-10, namely (-1082 A/G), (-819 C/T), and (-592 A/C), exhibit genotype and allele frequencies, as presented in Table 4. Our findings showed a significant difference in IL-10 allele frequency at -1082 G to A region between the control group and LN patients. With a frequency of (61.8%) in LN compared to controls (34.1%) and a P value (P<0.001), the occurrence of IL-10-1082 G allele was noticed in LN patients rather than in controls. In contrast, the IL-10 (-1082 A) allele variants were more predominant in controls, with a frequency of (65.9%) compared to LN (38.2%) and a P value (P<0.001). Furthermore, concerning the genotype level of IL-10 position -1082, our findings

revealed a statistically significant variation in the occurrence of the G/G genotype, which was found to be in frequency of 52.6%) in LN as opposed to a frequency of 13.6%) in the control group with a P value of (P<0.001), and the G/A genotype, which was found to be in frequency of 18.4% in LN as opposed to a frequency of (40.9%) in the control group with a P value of (P=0.02). Conversely, no differences were observed between the LN and control group in the allele and genotype occurrence of IL-10 at the two positions (-819 C/T, -592 A/C).

Additionally, there were noticeable changes in the genotype frequencies of IL-10 (-1082 AG, -819 CT, -592 AC), the genotype GCC/GCC was more frequent in LN (52.6%) in comparison to the control group (13.6%) with P value (P<0.001), and GCC/ACC was more commonly observed in the control group (25%) compared to LN (2.6%) with P value (P=0.027) (Table 4). As well as, our results revealed a significant difference at the haplotype level between controls and LN patients in IL10 GCC to be predominate in LN patients with a frequency of (61.8%) compared to the control group (34.1%) with P value (P<0.001) while the ACC haplotype was found in higher frequency in controls (37.5%) compared with a frequency of (22.4%) LN patients with P value (P=0.036).

Table 4: The allele, genotype, and haplotype occurrence of IL-10 (-1082 A/G), (-819 C/T), (-592 A/C) SNPs.

IL-10: (-1082 A/G), (-819 C/T), (-592 A/C) SNPs			
Allele	**Control N=88 (Frequency %)**	**LN N=76 (Frequency %)**	**P Value Control-LN**
−1082			
G	**30 (34.1%)**	**47 (61.8%)**	**0.001**
A	**58 (65.9%)**	**29 (38.2%)**	**0.001**
−819			
C	63 (71.6%)	64 (84.2%)	0.054
T	25 (28.4%)	12 (15.8%)	0.35
−592			
A	25 (28.4%)	12 (15.8%)	0.054
C	63 (71.6%)	64 (84.2%)	0.054
Genotype	N=44 (Frequency %)	N=38 (Frequency %)	
−1082			
G/G	**6 (13.6%)**	**20 (52.6%)**	**0.001**
G/A	**18 (40.9%)**	**7 (18.4%)**	**0.027**
A/A	20 (45.5%)	11 (28.9%)	0.124
−819			
C/C	26 (59.1%)	28 (73.7%)	0.165
C/T	11 (25%)	8 (21.1%)	0.673
T/T	7 (15.9%)	2 (5.3%)	0.124
−592			
A/A	7 (15.9%)	2 (5.3%)	0.124
C/A	11 (25%)	8 (21.1%)	0.673
C/C	26 (59.1%)	28 (73.7%)	0.165
Genotype	N=44 (Frequency %)	N=38 (Frequency %)	
GCC/GCC	**6 (13.6%)**	**20 (52.6%)**	**0.001**
GCC/ACC	**11 (25%)**	**1 (2.6%)**	**0.004**
GCC/ATA	7 (15.9%)	6 (15.8%)	0.988
ACC/ACC	9 (20.5%)	7 (18.4%)	0.817

IL-10: (-1082 A/G), (-819 C/T), (-592 A/C) SNPs			
Allele	**Control N=88 (Frequency %)**	**LN N=76 (Frequency %)**	**P Value Control-LN**
ACC/ATA	4 (9.1%)	2 (5.3%)	0.507
ATA/ATA	7 (15.9%)	2 (5.3%)	0.124
Haplotype	N=88 (Frequency %)	N=76 (Frequency %)	
GCC	**30 (34.1%)**	**47 (61.8%)**	**0.001**
ACC	**33 (37.5%)**	**17 (22.4%)**	**0.036**
ATA	25 (28.4%)	12 (15.8%)	0.054

N= number, LN: Lupus Nephritis. (P value <0.05).

4.2 Discussion

Systemic lupus erythematosus (SLE) is a complicated autoimmune disorder. Although the precise etiology of SLE is still unknown, it is broadly recognized that both genetic susceptibility and ecological factors are protagonists in disease development [31]. The genetic components of SLE have been clarified by recent research, which has focused in particular on the IL-10 gene and its variations [32]. IL-10 gene, which is located on chromosome number one at the (1q31-1q32) locus is considered an important factor in the pathogenesis of SLE [33]. Research has shown that patients with SLE often exhibit elevated levels of IL-10, which can impact the production of autoantibodies and contribute to disease progression [34]. Indeed, lowering IL-10 expression with focused therapies has shown promise in ameliorating clinical symptoms in SLE patients, highlighting the reputation of IL-10 in the disease process [13].

The IL-10 (-1082 A/G) SNPs is the most genetic variant found in the IL-10 gene that has gained significant attention from researchers[21]. However, different studies have suggested a potential link between the IL-10 (-1082 G) variant and the likelihood of developing SLE [32, 35]. To explain the complexity of genetic factors influencing SLE risk, a meta-analysis found that the IL-10 promoter (-1082 G) variant is associated with SLE in Asian populations but not in Caucasians [34].

Our current study focuses on genetic variations in large panel cytokines among LN patients compared to a control group, investigating the role of cytokine SNPs in LN pathogenesis. This study examined SNPs for: TNF-α (−308A/G), IL-6 (−174G/C), three polymorphic regions of IL-10: (−1082A/G), (−819C/T) and (−592A/C), in addition to IFN-γ (+874 T/A**)** using advanced genotyping techniques. A significant increase in the IL-10 (-1082 G) allele variants was observed in LN patients, indicating a potential genetic susceptibility among Jordanian SLE patients. In contrast, the IL-10 (-1082 A) allele variant was more common in controls, suggesting a protective genetic factor against SLE and LN. Our results are compatible with the results obtained from a meta-analysis study conducted on seventeen populations including Europeans and Asians. The study presented that the IL-10 (-1082 G) allele was associated with both European and Asian SLE patients [25]. In addition, at the genotype level, the IL-10 genotype at the position (-1082 G/G) was linked to LN with (P<0.001), so it may be considered a susceptible genotype for LN development among Jordanian SLE patients; while the IL-10 (-1082 G/A) genotype is thought to be protective, since it was much more common in the control group than in the LN group with (P=0.027). On the other hand, our findings align with the results from diverse populations, including Egyptians, Bulgarians, and Iranians, all of which demonstrated a significant increase in the occurrence of the IL-10 (-1082 G/G) genotype in patients with LN [3, 20, 36]. From the other side, some studies in various ethnic populations (Asians, Europeans, Brazilians and Chinese) showed that IL-10 (-1082 G/A) genotype frequencies are related to SLE development but not LN, since these studies are not linked with any clinical manifestation of SLE disease, while our

study was focused on SLE patients with LN manifestation[21, 32, 37, 38]. Moreover, in this study, the homozygous (GCC/GCC) genotype in the IL-10 promoter region was found to be significantly correlated with LN (P<0.001), whereas the frequency of heterozygous (GCC/ACC) genotype was significantly related to controls (P=0.004), so it may be suggested as a protective genotype since it was much more frequent in the control groups. Additionally, the IL-10 haplotype of ACC was also marked as a protective haplotype (P=0.036), whereas the IL-10 haplotype of GCC is associated with LN disease (P<0.001). Furthermore, consistent with our findings, a study conducted on LN patients demonstrated a link between the GCC haplotype and LN in Iranian and European populations [15, 36]. Notably, associations between specific IL-10 genotypes and haplotypes with LN disease severity further emphasize the intricate genetic landscape underlying SLE pathogenesis. Additionally, our study did not identify significant associations between certain cytokine SNPs (TNF-α, IL-6, IFN-γ) and LN among Jordanian SLE patients, consistent with findings from other populations like Mexican, Thai, and Polish groups [16, 39, 40]. Overall, the results from different ethnicities are debatable but, these collective insights highlight the intricate genetic interplay in LN susceptibility and highpoint. However, these collective insights highlight the intricate genetic interplay in LN susceptibility and highlight the importance of considering diverse populations to unravel the complexities of SLE genetics.

CONCLUSION

This study highlights (for the first time) the link between large panels of cytokine SNPs. Our finding showed that IL-10 (-1082) G allele variants, IL-10 (-1082) GG genotype, homozygous (GCC/GCC) genotype, and IL-10 haplotype of GCC are linked as risk factors for the vulnerability to LN. Therefore, these SNPs might be used as potential predictive markers for early LN risk assessment. It would be simpler for those susceptible to LN to take the required steps to stall the onset of the illness. Additionally, knowing how these cytokines work may help in the development of more potent strategies for treating LN and its related complications. It is also essential to understand the origin and course of LN to diagnose, prevent, intervene with, and manage these conditions. However, additional genetic markers that are considered as potential risk genes for developing LN in Jordanian patients should be examined to uncover all possible risk genes influencing LN pathogenesis.

Declaration of competing interest

The authors confirmed that none of their personal or financial ties could be interpreted as having an impact on the work presented in this paper.

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تعدد أشكال النوكليوتيدات المفردة للسيتوكاينز المختلفة بين المرضى الأردنيين المصابين بالتهاب الكلية الذئبي: دراسة تجريبية

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ملخص

يعد التهاب الكلية الذئبي (LN)أحد العلامات السريرية الأكثر شيوعًا في مرض الذئبة الحمراء (SLE) وله تأثير ملحوظ على معدلات الامراض والوفيات المرتبطة بمرض الذئبة الحمراء. تشارك العوامل البيئية والوراثية في تطور مرض الذئبة الحمامية. ومع ذلك، هناك اهتمام متزايد بتحديد العلامات الجينية، وخاصة السيتوكينات، التي قد تشير إلى القابلية للإصابة بمرض الذئبة الحمراء، والتنبؤ بدور الأعضاء الوشيكة للإصابة بالمرض، وتتبع التغيرات في نشاط المرض. الهدف من هذه الدراسة هو فحص مجموعة واسعة من المتغيرات الوراثية للسيتوكاينز وارتباطها بالتهاب الكلية الذئبي LN في إجمالي 83 عينة من الأردن. تم إجراء النمط الجيني للسيتوكاينز باستخدام تقنية .(PCR/SSP) تشير نتائجنا إلى وجود زيادة في الشكل الجيني G في النمط الجيني −1082 (A/G لـ IL-10) في عينات مرضى LN (P = 0.001) مقارنة بالأشخاص الأصحاء في حين كان الشكل الجيني A هو السائد (P = 0.001) عند الأشخاص الأصحاء. وعلى مستوى النمط الجيني لمنطقة (−1082 A/G لـ IL-10، ارتبط النمط الجيني GG بـ (P <0.001) LN، بينما النمط الجيني G/A كان هو السائد عند الأشخاص الأصحاء (P = 0.027). علاوة على ذلك، لم تتم ملاحظة أي ارتباطات مهمة في تعدد الأشكال الجينية السيتوكينية الأخرى (IFN-γ، IL-6، TNF-α LN) لدى مرضى التهاب الكلية الذئبي في الأردن. بشكل عام، تشير دراستنا التجريبية إلى أن الشكل الجينيG والنمط الجيني GG في المنطقة (−1082 A/G لـ IL-10 تزيد من خطر الإصابة بالتهاب الكلية الذئبي لدى المرضى الأردنيين المصابين بمرض الذئبة الحمامية. بينما تسود المتغيرات A والنمط الوراثي GA عند الأشخاص الأصحاء.

الكلمات الدالة: التهاب الكلية الذئبي، السيتوكاينز، الأردن.

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